The mechanism of microfibrillar orientation is not completely understood. Microtubules have been proposed as an orienting force for microfibrils (*14*), but in this study, microtubules have not been observed. Perhaps the partial orientation of microfibrils could occur as a result of intermittent binding between the surfaces of microfibrils. This is consistent with the evidence presented in Fig. 2.

Although alkali insoluble β-1,4 glucans can be synthesized in vitro, the apparent necessity for the presence of the intact plasma membrane has made it thus far impossible to demonstrate in vitro synthesis of microfibrillar cellulose (*15*). It is interesting that a chitin-synthesizing system has been shown to make microfibrils in vitro (*16*). A globular enzyme complex is associated with the tip of a growing chitin microfibril, similar to what has been observed in this study.

The significance of this report is that for the first time in higher plants, the cellulosic microfibril and its presumptive synthesizing complex have been demonstrated at the ultrastructural level. Furthermore, we believe that the morphological evidence represents a true picture of the assembly process free from artifactual distortion.

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Revertants of Human Cells Transformed by Murine Sarcoma Virus

Abstract. *Revertants of nonproducer human osteosarcoma (NP/KHOS) cells induced by Kirsten murine sarcoma virus were isolated after incubating at high temperature (40.5°C) overnight and subcloning at 36°C. The morphologic variants, from which murine sarcoma virus could no longer be rescued, had growth properties similar to those of the nontransformed, parent human osteosarcoma cells and did not release RNA-dependent DNA polymerase activity. These revertants were nontumorigenic in nude mice. The revertants supported leukemia virus growth and showed an enhanced sensitivity to murine sarcoma virus superinfection. Thus, the revertants were from human cells transformed by an oncogenic RNA virus.*

Cells transformed by murine sarcoma virus (MSV) can revert to variant forms in which their morphology and function resemble that of normal cells (*1, 2*). It has been reported that MSV transformed mouse cells termed S^+L^- (sarcoma-positive, leukemia-negative) (*3*) spontaneously gave rise to morphologic variants from which MSV could no longer be rescued (*1*). However, no such spontaneous reversion was found in human S^+L^- cells (*4*). Another type of morphological revertant (*2*), which contained rescuable MSV genome, has been observed at a low frequency from the MSV-induced rat nonproducer (NP) cells by single cloning procedures. Frequency of reversion has sometimes been increased by first treating the cells with sublethal doses of halogenated pyrimidines (*5*) or by adapting them to high temperature (*6*). Nonproducer, human osteosarcoma (KHOS) cells isolated from transformed foci induced by Kirsten murine sarcoma virus (Ki-MSV) (*7*) produce neither infectious virus nor murine leukemia virus (MuLV) antigens, but they contain the MSV genome which can be rescued by superinfection with MuLV. The NP cells produced tumors when transplanted subcutaneously into athymic (nude) mice (*8*). We now report the isolation and characterization of revertants from Ki-MSV transformed human NP cells. The revertants were isolated after incubation at high temperature (40.5°C) overnight and subcloning at 36°C. The morphologic variants, from which MSV could no longer be rescued, had growth properties similar to those of the nontransformed, parent human osteosarcoma (HOS) cells and did not release RNA-dependent DNA polymerase activity. These revertants were nontumorigenic in nude mice. To our knowledge, this is the first observation of revertants from human cells transformed by an oncogenic RNA virus.

The human NP (KHOS) cells cloned from a transformed focus (*7*) were maintained under liquid medium (*8*) for 30 passages to "fix" the premanently transformed state before use in our study. One-day-old cultures of KHOS were incubated overnight at 40.5°C and maintained at 36°C for two passages. During this period, transformed cells were detached and decanted from the cultures.

Table 1. Properties of revertants from human nonproducer cells (KHOS).

Properties	HOS cells	KHOS cells	Revertants from KHOS	
			240S	312H
Morphology	Flat	Transformed	Flat	Flat
Saturation density* ($\times 10^5$/cm^2)	1.7	4.8	0.85	0.75
Cell aggregates†				
Size	Small	Large	Small	Small
Viability of cells ($\times 10^5$)	1.3	6.2	0.5	0.4
Plating efficiency (%) in soft agar	1.5	9.4	1.1	1.1
Type C virus particles	Negative	Negative	Negative	Negative
CF titers of MuLV gs antigen	< 2	< 2	< 2	< 2
Reverse transcriptase‡	Negative	Negative	Negative	Negative
Tumorigenicity in nude mice§	Negative	Positive	Negative	Negative
MSV rescued by type C viruses‖	None	Present	None	None

*Maximum number of cells obtained after initial planting with 5×10^3 cell/cm^2 and then incubating at 36°C under conditions where growth medium was changed every 3 days. †Cell aggregates formed after 3 days with an agar static system. Viability of cell aggregates determined by 4 days after planting 2×10^5 cells per plate initially. ‡RNA dependent DNA polymerase activity was measured by incorporation of [^{3}H]thymidine triphosphate into acid-precipitable materials in 100× concentrated supernatant of cultures (*8*). §Five million cells inoculated into each nude mouse. ‖Supernatants from type C virus infected cultures were taken at 14 days, were passed through an 0.4-μm HA filter, inoculated into horse skin cells and human embryonic skin and muscle cells and examined for foci.

The remaining attached cells were then plated at low density and allowed to form colonies. Flat-looking areas were cloned by the cylinder technique. A total of 172 clones was selected; the majority were morphologically unstable, with foci of transformed cells (Fig. 1A). However, two clones (240S and 312H) appeared stable after 10 months of continuous culture in their "flat" fibroepithelial-like morphology (Fig. 1, E and G) resembling untransformed HOS cells (Fig. 1C).

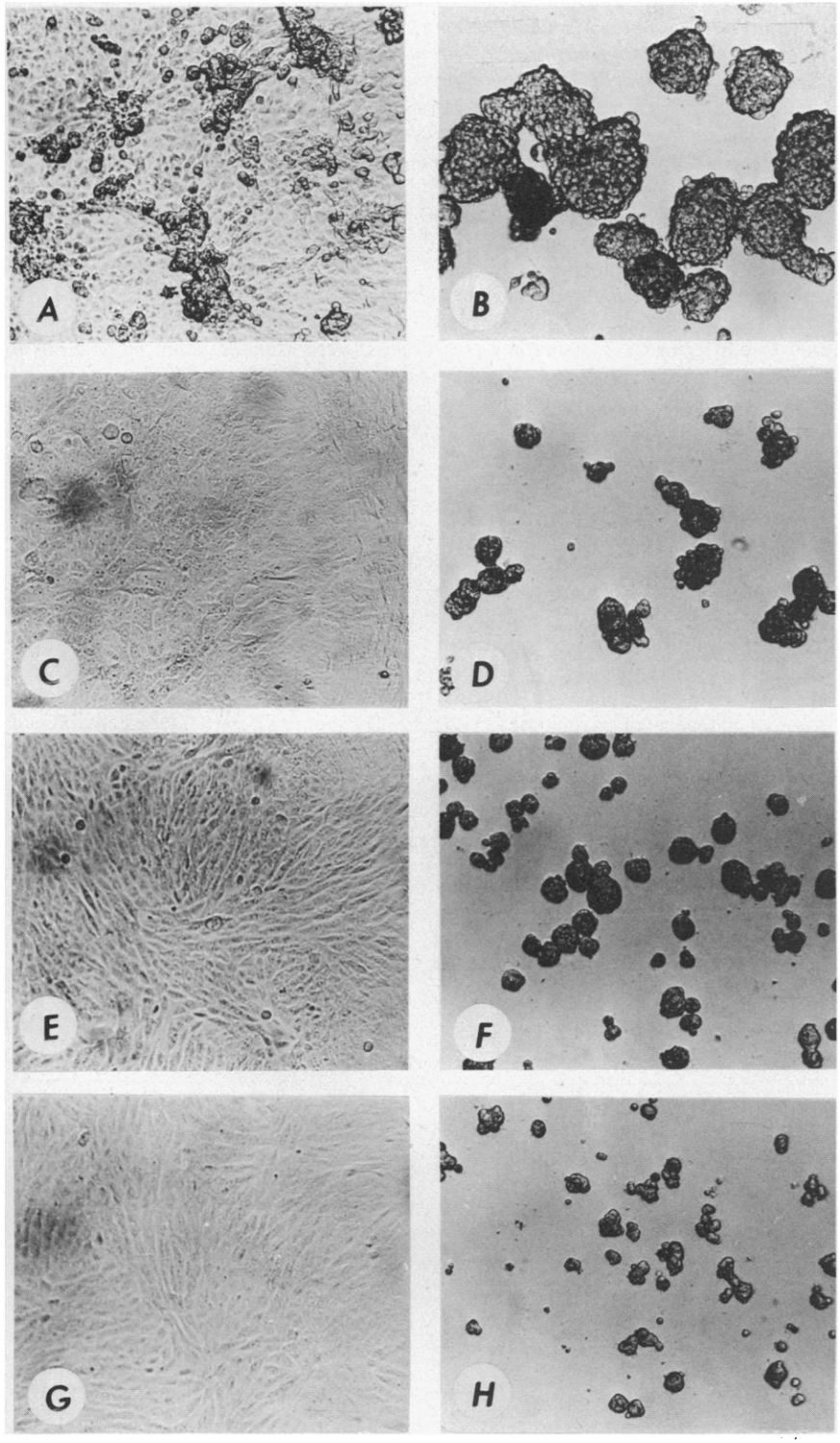

Fig. 1. Morphology and cell aggregates of revertants of human osteosarcoma (HOS) cells transformed by Ki-MSV, nonproducer cells (KHOS). Aggregates formed after 3 days with the use of an agar static system (cells were allowed to aggregate undisturbed in liquid growth medium above an agar base layer) (A) Human nonproducer cells (KHOS); (B) aggregates of KHOS cells; (C) HOS cells; (D) aggregates of HOS cells; (E) revertant 240S cells from KHOS cells; (F) aggregates of flat revertant 240S; (G) revertant of 312H cells from KHOS cells; and (H) aggregates of revertant 312H cells (× 45).

Study of several cell characteristics showed that the revertants 240S and 312H were more similar to HOS than to the parent KHOS line (Table 1). Saturation densities of 240S and 312H were lower than both HOS and KHOS. The revertants (Fig. 1, F and H) and HOS (Fig. 1D) formed smaller aggregates than KHOS (Fig. 1B) when suspended in liquid growth medium above an agar base (*9*) (Table 1). Viable cell counts performed on trypsinized aggregates on four consecutive days indicated that revertants (240S and 312H) and HOS cells underwent a significant decline in the number of viable cells; whereas the Ki-MSV transformed nonproducer (KHOS) cells exhibited growth in the aggregate form. Plating efficiency in soft agar was reduced as compared to that of KHOS cells. The revertants were nontumorigenic in nude mice. MuLV P-30 antigen, type C virus particles, and reverse transcriptase activity could not be demonstrated either in the revertants or in KHOS cells (Table 1).

To determine whether the morphologic variants still harbor the MSV genome, revertants were infected with helper type C viruses (Ki-MuLV, Woolly monkey leukemia virus, and RD-114 virus). Supernatants from infected revertant cells were taken at 14 days, filtered, inoculated into cells from human embryonic skin and muscle and from horse skin and examined for foci. No transformation was observed in the revertant cells directly superinfected with the type C viruses. Likewise, no foci were observed in the human and horse cells after inoculation with supernatant from the revertant cells infected with type C viruses. Revertants were tested repeatedly without any evidence of MSV rescue.

Susceptibility of revertants to MSV and MuLV infection was examined (Table 2). Tenfold dilutions of Ki-MSV stock from the Ki-MSV releasing HOS line (*8*) were inoculated into revertants and HOS cells that had been treated with DEAE-dextran (25 μg/ml, 30 minutes), and examined for the presence of foci. These cultures were harvested at 20 days. Supernatants from cultures were examined for polymerase activity and focus-forming activity. Revertants produced two- to fourfold more foci than did HOS cells (Table 2). The foci observed in revertants were morphologically distinguishable from those of HOS. The maximum number of foci was observed in morphologic variants between 14 and 17 days after infection, compared to 10 days in HOS cells. An enhancement of susceptibility to MuLV over that of HOS

cells was also observed in one of the revertants.

The relationship between the revertants, KHOS, and HOS cells was established by chromosome analysis (Table 3). HOS and KHOS cells had remarkable marker chromosomes by conventional staining (*8, 10*); the same markers were noted in the revertant cells (W. Nelson-Rees, personal communication). Trypsin-Giemsa banding of cells revealed banded markers previously reported in the parental HOS cells (TE-85, clone F-5) (*10*). The revertants had no increased chromosome numbers when compared to their progenitor cells.

Our experiment demonstrated that, after exposure to high temperature, morphologic variants of KHOS cells were obtained by cloning at 36°C. Prior to exposure of this line to high temperature, revertants could not be obtained by cloning at 36°C (*1*) or by prior treatment with sublethal dose of bromodeoxyuridine (*5*). The revertants had growth properties similar to those of the nontransformed, parent HOS cells and did not retain a rescuable MSV genome. These revertants like HOS cells were nontumorigenic in nude mice, whereas KHOS cells were tumorigenic in nude mice. Thus, the morphologic variant lines showed good correlation between the morphology, growth properties, and tumorigenicity. The revertants supported leukemia virus growth and showed an enhanced sensitivity to MSV superinfection. These human revertants may be a useful in vitro tool in assaying for putative human tumor viruses and chemical carcinogens in human cell systems. In addition, these revertants should provide an opportunity for examination of the control mechanism of cell transformation in the human cell system. Such studies have obvious implications for viral and chemical carcinogenesis.

It has been reported that HOS (TE-85, clone F-5) cells can be transformed by chemical carcinogens [*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 7,-12-dimethylbenz[*a*]anthracene (DMBA)] (*11*). We found also that treatment with DMBA and 3-methylcholanthrene induced rapid transformation in 312H revertant cells and that these chemically transformed 312H cells produced tumors when injected into nude mice (unpublished data).

Aggregation properties (size, viability, and proliferation of cell aggregates) of revertants had a proportionate correlation with growth in soft agar and tumorigenicity as we observed previously in the aggregation study of HOS, MNNG-transformed HOS, and virus-transformed KHOS cells (*9*). These criteria based on aggregation properties can be utilized to evaluate the tumorigenic potential of these cell types.

Table 2. Relative susceptibilities of HOS and revertants cell lines to MSV and MuLV. Each plate was inoculated with 2×10^5 cells.

Cell lines	MSV* (FFU/0.2 ml)	MuLV† ($TCID_{50}$/0.2 ml)
HOS	1.0×10^4	$10^{4.0}$
240S	4.0×10^4	$10^{4.5}$
312H	2.1×10^4	$10^{4.0}$

*Ki-MSV was titrated on cells first treated with DEAE-dextran (25 μg/ml for 30 minutes) before infection. Results are expressed as focus forming units (FFU) per 0.2 ml of inoculum. †The Ki-MuLV was titrated by the reverse transcriptase assay (*15*). The supernatants were collected on day 20 after infection and assayed for enzyme activity, which is expressed in terms of 10^8 cells. The susceptibility of each cell line to Ki-MuLV, was determined by the tissue culture infective dose, $TCID_{50}$, which was calculated by the Reed-Muench method.

The mechanism by which high-temperature exposure of KHOS cells induces morphologic variants is unknown, but may be due to preferential adhesion of revertants at 40.5°C. It has been reported that polyoma-transformed hamster cells (*12*) multiplied at 41.5°C, while normal and variant cells did not. Thus, high-temperature selection of morphologic variants may be species-, cell-, or virus-dependent. Although the mechanism remains unknown, there is the obvious fact that each cell in KHOS has the intrinsic control mechanisms potentially capable of yielding morphologic variants since they were the progeny of a single clonal cell line. The failure to demonstrate the presence of the Ki-MSV genome in revertants could be due either to elimination or to repression of the genome; subsequent experiments showed no evidence of the presence of the Ki-MSV genome in the revertants by nucleic acid hybridization techniques (unpublished). Major changes in genomic content have been reported to accompany reversion and retransformation (*13*). An increase in chromosome number generally accompanied reversion of transformation and a decrease accompanied retransformation. In contrast, Nelson-Rees and Scher (*14*) reported that there was no correlation within various classes of transformants in regard to total chromosome number or number of metacentrics and the extent of cellular growth. Nor do the data reported here support any major change in the chromosome number of the revertant cells. In our revertant cells, the modal chromosome numbers are similar to those of their progenitor cells.

Table 3. The karyology of revertants from human nonproducer cells (KHOS). Giemsa conventional markers for all cells consisted of a peculiar submetacentric chromosome (*10*).

Cell lines	Morphology	Range of number of chromosomes
		Modal No.
HOS	Flat	43–47
		45
KHOS (P-18)	Transformed	51–53
		52
240S (P-9)	Flat	46–47
		47
312H (P-5)	Flat	46–50
		48

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