LIMITED AND UNLIMITED GROWTH OF SV40-
TRANSFORMED CELLS FROM HUMAN DIPLOID
MRC-5 FIBROBLASTS

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SUMMARY

Human foetal lung strain, MRC-5, was treated with simian virus 40 and cultures were obtained that had many of the properties of transformed populations. In 10 experiments, only two produced permanent lines, designated MRC-5V1 and MRC-5V2, which have grown to passage 750 and 650, respectively. In all cases, the SV40-treated cultures acquired many of the features of transformation, including production of T-antigen, loss of contact-inhibition, and ability to grow in low concentrations of serum. The presence or absence of other transformed characteristics, such as altered morphology, abnormal karyotype or ability to grow in soft agar, can be used to distinguish between individual newly infected cultures. However, the cells invariably entered a period of slow growth, or crisis, and in eight experiments the cultures subsequently died without the emergence of a permanent line. The report that late-passage diploid cultures are more easily transformed to permanent lines than young cultures has not been confirmed. MRC-5V1 initially had a sub-diploid chromosome number, but during serial passaging this gradually increased. MRC-5V2, which has a more extreme transformed phenotype than MRC-5V1, had a hyper-diploid chromosome number, which also increased during long-term growth. MRC-5V1 became polymorphic for glucose-6-phosphate dehydrogenase, as judged by the heat-lability and electrophoretic mobility of the enzyme. Fusions between MRC-5V1 and Lesch-Nyhan fibroblasts yielded hybrids with a limited lifespan, and certain sub-lines of MRC-5V1 also slowed down, exhibited characteristic signs of senescence and ceased to grow.

INTRODUCTION

Human diploid fibroblasts have a finite lifespan when cultured in vitro and do not produce permanent lines spontaneously. These can, however, be obtained following infection with simian virus 40 (SV40) (for a review, see Ponten, 1971). SV40-transformed permanent lines acquire new phenotypic features, including altered morphology, ability to grow in low concentrations of serum and soft agar, production of T'-antigen and an unstable karyotype (for a review, see Sack, 1980). Many properties of transformation can be expressed in newly SV40-infected cultures of human fibroblasts before they enter a period of cellular deterioration, generally known as crisis (Sack & Obie, 1981; Oshima, Pellett, Robb & Schneider, 1977; Moyer, Wallace & Cox, 1964), during which growth eventually ceases. At this time the cells resemble senescent diploid cells, since they are characteristically large, granular, irregular in shape and, in addition, they are continually shed into the medium (Girardi, Jensen & Koprowski, 1965). The crisis period has been shown to coincide roughly with the general senescence of normal cultures (Jensen, Koprowski & Ponten, 1963), but a
direct relationship between crisis and senescence still remains to be established. Transformed permanent lines can emerge from crisis, forming foci of rapidly dividing cells. A general confusion exists concerning human transformation experiments, since authors often make no distinction between transformation before crisis and the emergence of permanently transformed lines after crisis (Koprowski et al. 1962; Nishida, 1970; Greiner, Evans & Di Paolo, 1981). For this reason, newly infected pre-crisis cultures in this study will be referred to as pre-transformed.

The ease with which permanent lines could be obtained after SV40 infection differed greatly between laboratories. Girardi et al. (1965) and Todaro, Wolman & Green (1963) isolated permanent lines with high frequency, whilst others could produce none at all (Shein, Enders, Palmer & Grogan, 1964; Ruben & Rafferty, 1978; Rafferty, Ruben & Young, 1978; Gotoh, Gleb & Schlessinger, 1979; Sack & Obie, 1981). We have confirmed the results of Moyer et al. (1964), who showed that permanent transformation is a rare event. In one study, susceptibility to transformation was reported to increase with passage level of the diploid culture (Jensen et al. 1963), but we have been unable to substantiate this observation.

MRC-5 has been widely used in biochemical and cellular studies of in vitro ageing (e.g. see Holliday & Tarrant, 1972; Thompson & Holliday, 1973; Petes, Farber, Tarrant & Holliday, 1974; Linn, Kairis & Holliday, 1976; Shakespeare & Buchanan, 1978; Fulder, 1979; Murray & Holliday, 1981). In addition, experiments have been carried out specifically to test the commitment theory of ageing, which provides a possible basis for the difference in growth potential between diploid populations and transformed permanent lines (Kirkwood & Holliday, 1975; Holliday, Huschtscha, Tarrant & Kirkwood, 1977). In this study we have isolated and characterized two permanent lines from MRC-5, which will be used for comparative studies between transformed and diploid cell populations.

MATERIALS AND METHODS

Cells

The male foetal lung fibroblast strain, MRC-5, was obtained by courtesy of J. P. Jacobs, National Institute for Biological Standards and Control, Hampstead, London, where the strain was originally isolated and characterized (Jacobs, Jones & Baillie, 1970).

Lesch-Nyhan skin fibroblasts from a 9-year-old male patient (A.C.) were provided by Dr Elizabeth Spellacy from the Clinical Research Centre, Northwick Park Hospital. The monkey kidney cell line (CV1) was obtained from Dr W. C. Russell, Virology Division, this Institute.

Media and cell growth

In the first experiments (Table 1, expt 1) human diploid cells were routinely subcultured in 150 ml bow bottles in Eagle's BME medium (Gibco-Biocult Ltd, U.K.) containing 10% foetal calf serum (FCS), 10% tryptose-phosphate broth. This and all other media used contained 100 units of penicillin and 100 μg streptomycin per ml. The cells were incubated at 37°C, with their bottle tops screwed on tightly. For the remaining experiments in Table 1, the cells were subcultured in 25 cm² flasks (Nunc Ltd, U.K.) and incubated, with loose bottle tops, in a humidified atmosphere of 5% CO₂. A richer medium was used in later experiments for routine cell growth (Table 1, expts 6–10). This was F-15 Eagle's MEM (Gibco-Biocult) supplemented with 10% FCS, 2 mM-glutamine. Cells were subcultured when the culture had attained confluence. They were first washed in
SV40-transformed human fibroblasts

phosphate-buffered saline (Dulbecco-PBS 'A') before trypsinizing with 0.5 ml of trypsin-verseine (0.125% trypsin, 0.01% EDTA). The cell suspension was diluted by the addition of 9.5 ml medium before subculture at 1:4 or 1:8 split ratios for younger cultures, or 1:2 split for slower growing cultures. The growth medium of non-confluent cultures was changed every 3-4 days. The cumulative number of population doublings (p.d.) was calculated by counting the number of cells harvested from each confluent bottle with a Coulter Counter (model ZB1, Coulter Electronics Ltd, Harpenden, U.K.). (To retain equivalence of p.d. with passages, we score a 1:4 split as 2 passages and a 1:8 split as 3 passages.) All cells were routinely screened for mycoplasmal contamination by the method of Chen (1977). They were all found to be negative. Stocks of cells were maintained by storing them in growth medium containing 10% glycerol in liquid nitrogen.

Pre-transformed cells were cultured in a manner similar to that for normal cells, but the growth medium was supplemented with horse anti-SV40 serum (Flow Laboratories, U.K.) at TCD50 for the first two experiments in Table 1. Anti-SV40 was not used in subsequent experiments, since its presence did not affect the growth of pre-transformed cultures. The transformed lines were grown in MEM supplemented with 2% FCS, 1 mg/ml glucose and 2 mM-glutamine. The medium was replaced every 2-3 days.

Methionine-free medium consisted of F-11 (Gibco-Biocult) supplemented with 10% dialysed FCS, 0.1 mM-folic acid, 1.5 mM-hydroxy-cobalamine, 0.1 mM-homocysteine thiocarbonate (all from Sigma Chemical Co., U.K.). For methionine-supplemented medium 0.1 mM-L-methionine replaced homocysteine. The selective medium for the fusion experiment was methionine-free with the addition of 10-4 M-hypoxanthine, 4 X 10-7 M-aminopterin, 1.6 X 10-5 M-thymidine (HAT medium).

The doubling time of transformed cells was estimated by seeding 104 and 2 X 104 cells, respectively, into each well of two Linbro trays (24 wells of 1.75 cm2 from Linbro Flow Laboratories). Six hours after seeding and every 24 h thereafter, two wells from each dilution were trypsinized and counted. Cells grown on coverslips were examined for morphological changes after fixation with methanol/acetone in the ratio of 3:2 and then stained with Giemsa stain. The cells were also examined by phase-contrast microscopy.

For growth in soft agar, 106 and 2 X 105 cells were suspended separately in growth medium containing 0.3% Bacto-agar (Difco Laboratories, Detroit, Michigan, U.S.A.) and layered onto a base of 0.45% Bacto-agar (Macpherson & Montagnier, 1964). Colonies were scored 10-14 days later.

SV40 virus and infection

Three strains of virus were used to infect cultures of MRC-5. These were DM, 45-54 and WT80, kindly supplied by Dr D. Metz and Dr G. Stark, both from Virology Division of this Institute, and Dr K. Rafferty of the Department of Anatomy, University of Illinois, Chicago, respectively.

Log-phase cultures of MRC-5 at various population doubling levels were infected with one of the strains of SV40 at the specified level of plaque-forming units (p.f.u.)/ml (see Table 1). The virus was left to adsorb for 2 h at 37 °C with gentle shaking at frequent intervals. The cultures were then washed three times in warm PBS to wash off any unadsorbed virus before adding fresh growth medium.

Isolation of transformed lines

Transformed cell lines were isolated according to the following procedures. (1) In mass culture (MC), the newly infected cells were subcultured when they reached confluence and maintained as a normal cell culture in its pre-transformed state until they entered crisis. (2) In the focal isolation method (FI), the cells were subcultured only once after SV40 infection and then left until foci appeared. The nutrient medium was replaced every week. A selected focus was then individually isolated by washing the cells with warm PBS, followed by the addition of 1 ml of trypsin. Before the cells detached, a long Pasteur pipette with a bent end was used to suck up the cells and redistribute them into a 5.5 cm² Leighton tube. When this was confluent, all the cells were transferred to a 25 cm² flask and grown as a pre-transformed culture. A cloning ring was not used, since cross-contamination did not occur between foci, as each pre-transformed culture isolated in this manner had its own transformed phenotype (see Results).

T-antigen test

Cells were examined for the presence of SV40 T-antigen by the indirect immunofluorescence test
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(Pope & Rowe, 1964), using hamster anti-SV40 T serum (kindly supplied by Dr D. Lane, Imperial College, London) and fluorescein-conjugated goat anti-hamster immunoglobulin G (IgG) (Flow Laboratories, U.K.).

**Determination of free virus**

Samples of 0.1 ml of undiluted, and 1:2 and 1:10 dilutions of nutrient medium taken from a flask of growing transformed cells, were inoculated in pairs into Linbro trays (Linbro, Flow Laboratories) previously seeded with CV1 (monkey kidney) cells in log-phase. After incubating for 1 h at 37°C with CO₂, the medium was removed and 0.5 ml of overlay (20% 5 X MEM, 0.75% agarose, 0.04% glutamine, 2% FCS, 0.21% sodium bicarbonate) at 37°C was added. Five days later another 0.5 ml of fresh overlay was overlaid. When plaques appeared, the wells were stained with 0.1% neutral red (Gurr Ltd) in saline and scored.

**Chromosome counts**

Metaphases were examined routinely in pre-transformed and transformed cultures. Cells were arrested in division by addition of 0.08 µg/ml colchicine for 3 h, harvested and swollen for 20 min at 37°C in 0.003 M-potassium chloride and 0.01 M-sodium citrate. The cells were fixed in methanol/acetate acid (3:2), spread on slides and the chromosomes were then stained in 2% Giemsa solution (Gurr Ltd) in phosphate buffer (pH 6.8) for 1 h. At least 100 metaphases were counted from each population. Karyotype analysis, without banding, was done on selected samples of metaphases of transformed lines.

**Glucose-6-phosphate dehydrogenase (Glc-6-P dehydrogenase)**

Cell-free extracts were prepared from cultures before, during and after crisis, and the proportion of heat-labile Glc-6-P dehydrogenase was measured according to the methods described by Holliday & Tarrant (1972), except that 1 mM-mercaptoethanol was excluded from the extraction buffer.

**Cell hybridization**

Equal numbers of transformed MRC-5V1, passage 675, were fused to normal Lesch-Nyhan skin fibroblasts at passage 21, according to the method described by Bunn & Tarrant (1980). The hybrids were selected in medium appropriate for the HAT selection regime and lacking methionine; 7.5 x 10⁵ and 10⁴ fused cells were inoculated into 1.75 cm² wells (Linbro, Flow Laboratories, U.K.) and scored for hybrid colony growth. These were transferred into larger vessels only in those cases in which one clone appeared per well. The chromosomes were counted in each isolated hybrid clone and these were scored for morphological appearance, and for finite and infinite growth.

**RESULTS**

**SV40 infection and pre-transformed cultures**

Cultures of MRC-5 at various population doubling levels (p.d.l.) were infected with SV40 and the results of 10 such experiments are summarized in Table 1. In all cases cells that had some of the properties of transformed cells appeared after infection and we refer to these as pre-transformed cells. These properties include changes in morphology, presence of T-antigen, mitotic activity in confluent cultures (indicating loss of contact-inhibition) and growth in soft agar, although in some cases the latter test was not done. During the growth of pre-transformed cultures large numbers of cells were continually being shed into the medium, indicating that the final lifespan is an underestimate.

Although these pre-transformed cultures were all positive for T-antigen, they were not identical in other properties. For instance, in expt 8 the pre-transformed culture
Table 1. **SV40 transformation of MRC-5**

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Time of infection (p.d.l.)</th>
<th>Strain of virus (p.f.u./cell)</th>
<th>Method of selection</th>
<th>No. of foci per 10^6 cells</th>
<th>Death of untreated control (p.d.l.)</th>
<th>Time of entry into crisis (p.d.l.)</th>
<th>Length of crisis (weeks)</th>
<th>Transformed line</th>
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<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>DM (1000)</td>
<td>MC*</td>
<td>-</td>
<td>52</td>
<td>54</td>
<td>8</td>
<td>MRC-5V1</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>DM (1000)</td>
<td>MC</td>
<td>-</td>
<td>42</td>
<td>52</td>
<td>~14</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>DM (10000)</td>
<td>MCC</td>
<td>(2% FCS)</td>
<td>54</td>
<td>58</td>
<td>&gt;=5</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>45-54 (660)</td>
<td>MC</td>
<td>46</td>
<td>48</td>
<td>46-68</td>
<td>2-12</td>
<td>MRC-5V2</td>
</tr>
<tr>
<td>5</td>
<td>51</td>
<td>45-54 (750)</td>
<td>FI† (9)</td>
<td>25</td>
<td>ND†</td>
<td>53</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>W830 (500)</td>
<td>MC</td>
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<td>53</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>52</td>
<td>W830 (500)</td>
<td>FI (0)</td>
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<td>ND</td>
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<td>-</td>
</tr>
<tr>
<td>8</td>
<td>21</td>
<td>45-54 (200)</td>
<td>FI (20)</td>
<td>11</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>53</td>
<td>45-54 (130)</td>
<td>MC</td>
<td>-</td>
<td>54</td>
<td>58</td>
<td>&gt;=4</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
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<td>45-54 (80)</td>
<td>FI (11)</td>
<td>14</td>
<td>ND</td>
<td>66-81</td>
<td>~1-12</td>
<td>-</td>
</tr>
</tbody>
</table>

* Mass culture method.
† Focal isolation method, and number of foci isolated.
‡ ND, not determined.
§ All foci died before growing into large populations.
exhibited fewer properties of transformation; such as, normal fibroblast morphology and little mitotic activity at confluence. In expt 2, the cells exhibited an intermediate morphology and high mitotic activity at confluence, which indicated a faster growth rate than the uninfected control. All pre-transformed cultures entered crisis later than their respective controls became senescent. During the crisis period, many more cells were shed into the medium and those remaining attached were very granular in appearance, irregular in shape, often large and multinucleate. Cultures in crisis were discarded when almost all cells had detached, which varied from 2 to 14 weeks. In many cases, clones of freshly dividing cells appeared against a background of non-growing cells during crisis, but these eventually died before the culture became confluent. Only one permanent line emerged during crisis (Table 1), and this was designated MRC-5V1.

In the focal isolation (FI) experiments (Table 1, expts 4–10), the differences between the individual focal isolates were more obvious. For example, in expt 10, one of the newly isolated pre-transformed cultures had an epithelial morphology, high mitotic activity and increased growth rate, while another from the same experiment retained all the properties of a normal uninfected cell line, except high mitotic activity. All these cultures entered crisis, which varied in length from 2 to 12 weeks. In this series of experiments only one culture survived crisis to form a permanent line, which was designated MRC-5V2.

There seems to be no particular feature of pre-transformed cells that makes it possible to determine whether or not a cell line would eventually develop into a permanent line. For instance, the age at infection, the multiplicity of infection, the number of foci appearing shortly after SV40 infection, the method of selection or increasing the size of the population of cells entering the crisis period, did not increase the probability of cells surviving crisis. Growth in reduced serum (Table 1, expt 2) did not predispose the cells to survive crisis. We have no evidence that the viral strain is important in determining immortalization. We have also tried, with no success, to increase the probability of transformation by treating pre-transformed cultures just prior to or during crisis with 1 μg/ml of 4-nitroquinoline-oxide, which is a potent carcinogen (Kakunaga, 1978), and bromodeoxyuridine, which is known to alter gene expression in many systems (for a review, see Rutter, Pictet & Morris, 1973).

Emergence of permanent transformed lines

In this section we describe in more detail the origins of the two permanent lines, MRC-5V1 and MRC-5V2.

MRC-5V1. In expt 1 (Table 1) nine foci appeared 13 p.d. after mass culture infection. These foci were dispersed by treatment with trypsin. From this time onwards the pre-transformed culture exhibited increased mitotic activity, was positive for T-antigen, but still maintained a normal fibroblast-like morphology. The growth rate of this pre-transformed culture was similar to that of the control, uninfected culture (Fig. 1a). However, growth rapidly decreased as the culture entered crisis at p.d.l. 53. At the beginning of crisis nearly 2 × 10^5 cells were accumulated and these were always maintained at a reasonably high density by pooling the cells into smaller
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culture vessels approximately every 2–3 weeks. After 60 days, dividing cells could be seen again and a permanent line emerged (MRC-5V1).

MRC-5V2. In expt 4 (Table 1), the first foci appeared approximately 21 days after SV40 infection. Nine of these were selected for further study using the focal isolation method. All the pre-transformed lines that could be sub-cultured were positive for T-antigen. Five of these could not be sub-cultured further, while three eventually died in crisis between p.d.l. 46 and 55. The properties of these three pre-transformed cultures differed from each other: one had a distinct epithelial morphology, the second a fibroblast-like morphology, while the third was epithelial-like in appearance. The latter two lines did not produce colonies of growth in soft agar, while the former was lost due to fungal contamination. These differences made it unlikely that there was any cross-contamination between foci.

The ninth focal isolate exhibited many of the properties of fully transformed cells. The growth rate was greatly increased, the cells exhibited high mitotic activity, grew in reduced serum and soft agar (8–9% plating efficiency), and were distinctly epithelial in morphology. When the ninth focal isolate attained full population size, the culture was divided into four sub-lines: two were grown in small flasks (25 cm²) in 2% FCS and 10% FCS, while the other two were grown in larger flasks (81 cm²) in 2% FCS and 10% FCS. The two sub-lines in the smaller vessels grew more rapidly than their counterparts in the larger vessels (Fig. 1B), yet they failed to survive crisis. They died at p.d.l. 67 and 68, respectively. The large population in 10% FCS was the first to enter crisis at p.d.l. 62. At this time many cells were being shed into the medium, but crisis itself was not as severe as for MRC-5V1 (i.e. there were less abnormal cells). Two weeks later many dividing clones appeared (170 were counted in one flask) and the dispersal of these with trypsin resulted in a line, which was grown for 10 p.d. and then frozen. The other large population growing in reduced serum entered crisis later, at p.d.l. 68. A similar pattern of behaviour in crisis was also observed for this line but its time in crisis was longer, approximately 3 weeks. This permanent line, designated MRC-5V2, was chosen for further study.

Transformed properties of MRC-5V1 and MRC-5V2

To demonstrate that MRC-5V1 and MRC-5V2 are derived from MRC-5 and were not contaminants, the electrophoretic properties of certain enzymes were checked, in collaboration with Dr S. Povey, Galton Laboratory. MRC-5 is heterozygous for phosphoglucomutase and 6-phosphogluconate dehydrogenase loci (Jacobs, Garrett & Merton, 1979). MRC-5V1 and MRC-5V2 were shown to have electrophoretic profiles similar to MRC-5 for these enzymes. Both MRC-5V1 and MRC-5V2 have the B form of Glc-6-P dehydrogenase, indicating that they could not be HeLa contaminants, since this line has the A form of the enzyme. The Y chromosome in MRC-5 was still present in both transformed lines, as detected by bright fluorescence, using the technique of Q banding, which shows it is of human origin (Miller et al. 1971).

Table 2 summarizes the transformed properties of MRC-5V1 and MRC-5V2. MRC-5V2 always exhibited a distinct epithelial morphology (Fig. 2E), even in its
Fig. 1. The growth of transformed MRC-5 following infection with SV40. A. MRC-5V1: infected by the mass culture method (●—●), and the uninfected control (○—○), which died out at the same time. B. MRC-5V2: infected by the focal isolation method. Sub-lines grown in small Falcon flasks in 10% FCS (▲—▲) and 2% FCS (△—△) and then in large Falcon flasks in 10% FCS (●—●) and 2% FCS (○—○) (MRC-5V2). The arrow in B indicates that the culture was frozen in liquid nitrogen. Crosses indicate death of culture.
pre-transformed state. On the other hand, MRC-5V1 maintained a morphology between epithelial and fibroblast-like when the culture was sparse (Fig. 2b), but appeared more epithelial as the culture became confluent (Fig. 2c). There was less uniformity in cell size and shape for this line compared to MRC-5V2. For instance, large cells with up to four nuclei were present (Fig. 2c). Both MRC-5V1 and MRC-5V2 grew equally in reduced serum but MRC-5V1 needed twice the normal concentration of glucose (2mg/ml) for maximum growth, whereas MRC-5V2 did not have this requirement. Both transformed lines had lost contact-inhibition, since they continued to grow even when the cultures were confluent. However, this was only a partial effect with MRC-5V1, since its growth rate was reduced at confluence (results not shown). In growth experiments over 7 days, the doubling times for MRC-5V1 and
MRC-5V2 were always slower than MRC-5; they did not significantly change with p.d.l., even when the cumulative growth rate increased (Fig. 3). For example, the doubling time of MRC-5V1 at passage 84 was 37 h and at passage 416 it was 38 h. The doubling time for MRC-5V2 remained in the range 42–56, compared with 18–21 h for early-passage MRC-5 cells (Holliday & Kirkwood, 1981). Since the doubling time of the transformed strains was lower than MRC-5, it might be thought that any spontaneously occurring transformed cells would be selected against in young or mid-term cultures and this might explain the failure to observe transformed foci in MRC-5 cultures. However, we have shown, by mixing a small proportion of MRC-5V1 cells (0.1–10%) with MRC-5, that the transformed cells invariably take over the culture during approximately 10–20 p.d. We assume that this is due to loss of contact-inhibition, which enables the transformed cell to grow even when the culture is confluent.

Both MRC-5V1 and MRC-5V2 produced colonies in soft agar; however, no growth was seen below a certain cell density. For MRC-5V1 this threshold was about 10⁵ cells per 55 mm plate and for MRC-5V2 about 10⁶ cells per plate. After about 200 p.d. these thresholds were reduced 10-fold. Estimates of the proportion of cells that form colonies at or above these thresholds were in the range 8–10% for MRC-5V2, but only 0.6% for MRC-5V1 at passage 200, rising to 7% at passage 386. Both transformed lines have an extremely low plating efficiency in normal medium (<0.01%), whereas, in our hands, the parental strain, MRC-5, has a plating efficiency of 2–10%.

Both MRC-5V1 and MRC-5V2 had T-antigen in the nuclei of all their cells, but the nuclei exhibited a variable intensity of fluorescence, which did not seem to be related to the size of the nucleus. Free virus was still found in the medium shortly after crisis, from both MRC-5V1 and MRC-5V2, but this ability to shed virus was lost with
prolonged growth in culture (Table 2). During a 3-month period no tumours were produced in nude mice when between $5 \times 10^6$ and $2 \times 10^7$ cells of MRC-5V1 and MRC-5V2 were injected subcutaneously into the left hind-foot. The same negative result had been previously reported with SV40-transformed human cells (Stiles, Desmond, Sato & Saier, 1975). On the other hand, Kahn et al. (1980) reported that their transformed lines were tumorogenic.

SV40-transformed human fibroblasts have been shown to have a growth requirement for methionine, whereas normal fibroblasts do not (Hoffman & Erbe, 1976; Kamely, Weissbach & Kerwar, 1977). Both MRC-5V1 and MRC-5V2 were grown in B12- and folate-supplemented media in which methionine was present or replaced by
homocysteine. MRC-5V1 divided two to three times in methionine-free medium and then growth ceased, whereas MRC-5V2 grew equally well with or without methionine.

The summary of results in Table 2 shows that MRC-5V1 and MRC-5V2 have their own individual characteristics but, in general, MRC-5V2 was judged to possess a more extreme transformed phenotype.

**Heteroploidy of permanent lines**

Metaphase preparations were examined at intervals throughout the growth of MRC-5V1 and MRC-5V2. Chromosome counts were made but no detailed karyotype analysis was done. These results are shown in Fig. 4. MRC-5V1 has a modal number between 38 and 42, with only a small proportion of the metaphases in each population having a hyper-diploid number of chromosomes – in the range of 60–70. Few other transformed human lines have previously been found to be sub-diploid (Weinstein & Moorhead, 1965). We do not know if the predominantly low chromosome number in most MRC-5V1 cells is compensated by rearrangements that might form larger
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Fig. 4 also shows that there is a gradual increase in the proportion of hyper-diploid for MRC-5V1 with time in culture. Only 15% of the metaphases were hyper-diploid shortly after crisis, but the proportion had increased to 45% approximately 600 p.d. later. This suggests that the MRC-5V1 population is continually changing; either there is selection for cells with higher chromosome numbers, or hyper-diploid cells are continually being derived from sub-diploid ones. On the other hand, MRC-5V2 is more stable, with no noticeable change in chromosome number during growth. Most of the metaphases are hyper-diploid, with a modal number between 60 and 80 chromosomes.

In both strains abnormal chromosomes were common, including breaks, translocations, dicentrics and occasionally ring chromosomes. In some cases, all these abnormalities could be seen in one metaphase. However, no particular marker

![Graph showing the percentages of hypo- and hyper-diploid metaphases in MRC-5V1 and MRC-5V2 at increasing passage levels. The asterisk indicates that the analysis was done before crisis.](image-url)
chromosome could be seen consistently in MRC-5V1, but in MRC-5V2 a long-armed acrocentric chromosome was detected in all metaphase karyotypes at passage 101, and again at passage 286. It is interesting to note from Fig. 4 that the extent of heteroploidy in the pre-transformed state is probably related to the transformed state, since MRC-5V2 was hyper-diploid both before and after crisis.

**Growth potential**

MRC-5V2 seems to possess infinite growth potential, since it has shown no signs of slowing down during 5 years of continuous sub-culture (approx. 650 p.d.). The growth rate in terms of cumulative p.d., has increased slightly in later passages, but there has been no discernible change in the doubling time, morphology or chromosome number.

On the other hand, MRC-5V1 did not grow as a continuous cell line (Fig. 3). The primary line was lost due to bacterial contamination at p.d.l. 300, and then several ampoules of frozen stocks had to be defrosted sequentially in order to recover this cell line. The first ampoule (I) was defrosted at p.d.l. 274 and as soon as cells were available they were re-frozen (ampoule II). The remaining cells were lost almost immediately, probably due to an unknown environmental cause. Ampoule II was then defrosted and the cells achieved 30 p.d. before the culture slowed down and died out (Fig. 5). Some of these cells had been frozen at p.d.l. 287 (ampoule III) and, on defrosting, six sub-lines were established. All these slowed down and died out after 20–30 p.d. The cells became more granular, larger and more fibroblast-like, looking very much like senescent fibroblasts – a conclusion that was confirmed by two independent observers. Finally, the cells were again removed from a frozen ampoule (IV) at p.d.l. 294. Two sub-lines died out, but two survived to maintain the cell line. During the course of 6 years of continuous sub-culture (~750 p.d.) its growth rate has gradually increased (Fig. 3). However, the doubling time in 6-day growth experiments has been fairly stable throughout, so the faster cumulative growth could be attributed to a greater release from contact-inhibition. Further evidence for this is shown by the increased efficiency of growth in soft agar and the appearance of a more epithelial-like morphology in the latter passages.

**Effects of bromodeoxyuridine (BrdUrd) and temperature**

It has been shown that the growth potential of MRC-5 is severely reduced by 0.3 µg/ml of BrdUrd, but there is little effect at lower concentrations (unpublished results). It was then found that MRC-5V1 is much more sensitive to BrdUrd than the parent diploid strain. BrdUrd at 0.1 µg/ml prevented more than 1–2 p.d., but growth of MRC-5V2 continued at a concentration of 1.0 µg/ml.

Transformed lines also showed differences from MRC-5 in their tolerance of temperatures higher or lower than 37°C. It was previously shown that MRC-5 can grow at 40°C and achieves roughly half the lifespan of control cultures at 37°C (Thompson & Holliday, 1973). MRC-5V1 and MRC-5V2 had a very limited growth at 40°C. In several experiments with MRC-5V1, the cells divided very slowly and achieved only 1–5 p.d. However, the effect did not appear to be analogous to the premature
Fig. 5. The terminal growth of a subline of MRC-5V1, from defrosted ampoule II (see Fig. 3).

senescence of MRC-5 at 40 °C. At 39°C MRC-5V1 died within 3–10 p.d., but MRC-5V2 continued to grow. MRC-5V1 was also unable to grow continuously at 33 °C or 34 °C, whereas MRC-5 achieved a considerable number of population doublings at these temperatures. The results show the transformed lines are heat-sensitive and MRC-5V1 is cold-sensitive.

Glucose-6-phosphate dehydrogenase

It has been shown that senescent cultures of MRC-5 and skin fibroblasts produce a significant fraction of heat-labile Glc-6-P dehydrogenase (Holliday & Tarrant, 1972; Holliday & Thompson, 1982). If the crisis of pre-transformed strains is the same as
senescence, it should be possible to detect altered enzyme during or before this period. However, the results obtained were not consistent. MRC-5V1 contained 20% heat-labile dehydrogenase during crisis, and when the permanent line emerged the proportion of heat-labile enzyme fell to control levels (0–10%). In the case of MRC-5V2, no significant proportion of heat-labile dehydrogenase was detected to 10 p.d. before crisis, and the results were variable when the enzyme was examined from other populations during crisis.

In other experiments on Glc-6-P dehydrogenase during the growth of MRC-5V1, it was noticed that the heat-lability of the enzyme had altered dramatically after many p.d.s. In cell-free extracts at least 50% of the enzyme was extremely sensitive to heat inactivation at 60°C (Fig. 6, curve B). This inactivation curve is completely different from those obtained with extracts from senescent cells (Fig. 6, curve A). It led us to examine the enzyme by starch-gel electrophoresis. A new band was seen that migrates more quickly towards the cathode than either the parental B form of the enzyme of the A form. This band disappeared entirely when the gel was incubated at 60°C for 1–2 min prior to staining, whereas the normal band (approximately 50% of

![Fig. 6. The heat inactivation of Glc-6-P dehydrogenase from senescent MRC-5 and MRC-5V1. Curve A, MRC-5, passage 56 (60°C); B, MRC-5V1, passage 270 (59°C); C, MRC-5V1, passage 509 (59.7°C).](image-url)
activity) was not inactivated (Fig. 7). Further experiments were carried out on MRC-5V1 at various passage levels (see Fig. 3) and it became evident that the population had become polymorphic for the enzyme at approximately passage 100 and this persisted for about 200 further passages. Subsequently, the heat-labile form of the enzyme was no longer detectable on gels, although the heat-inactivation profile showed that the enzyme was still quite unstable (Fig. 6, curve C).

**Hybridization**

To study the expression of the transformed phenotype in hybrids between normal and transformed cell lines, a new method of selection was devised. This entailed a HAT regime to select against the normal Lesch-Nyhan cells and medium lacking methionine to inhibit growth of MRC-5V1. Fifteen hybrid clones appeared 2 weeks after fusion, which represents a frequency of hybrid formation of 0.05%. These were transferred into two wells (3.7 cm²) and then into two Leighton tubes (11 cm²) when there were sufficient cells. Their morphology was essentially epithelial, although cells in sparser areas exhibited a more fibroblast-like form. All the clones eventually died.

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**Fig. 7.** Horizontal starch gel electrophoresis of Glc-6-P dehydrogenase. Lanes 1, MRC-5, passage 25; 2, MRC-5V1, passage 96; 3, MRC-5V1, passage 306. A. Extracts at room temperature; B, extracts heated for 1 min at 60°C prior to electrophoresis.
between 34 and 40 p.d. A normal population of Lesch-Nyhan died after 37 p.d. Chromosomes were counted in five hybrid clones. Their modal number was 50–60 chromosomes, which is more than that of either parent, but less than would be expected from the sum of the two parental cells. The variation in chromosome number was much less than in the MRC-5V1 parent. These observations are similar to those of Bunn & Tarrant (1980) on hybrids between Lesch-Nyhan and HeLa cells.

**DISCUSSION**

In the experiments reported here we have reinvestigated the sequence of events involved in the transformation of cultured human fibroblasts by SV40, and characterized two permanent lines that were obtained. One of the aims of the investigation was to find out whether the crisis period that terminates the first stage in transformation is equivalent to the senescence of normal diploid fibroblasts, and another was to try to gain some insight into the basic difference between diploid and heteroploid transformed cells, with regard to their limited and unlimited growth potential.

We found that many properties of transformation were acquired soon after viral infection, such as high mitotic activity at confluence, presence of T-antigen, anchorage independence and growth in low concentrations of serum. We refer to these cultures as pre-transformed, because it was invariably observed that growth eventually ceased and the cells entered a degenerative phase, commonly referred to as 'crisis'. The phenotypes of pre-transformed cultures were often quite distinct, but no particular phenotype predisposed the culture to survive crisis and form a permanent line. In a similar study, Sack & Obie (1981) noted individual phenotypes in their cloned pre-transformed cultures. Oshima et al. (1977) were also unable to correlate any pre-transformed feature, such as virion production or T- and V-antigen expression, with the ability to become fully transformed.

Our results demonstrate that the emergence of permanent lines is a very uncommon event, since in 10 experiments with MRC-5 only two yielded transformed lines, indicating that the frequency of transformation at the cellular level is probably \(< 10^{-7}\), and there was also no indication that late-passage diploid cultures were more susceptible to transformation. These observations confirm those of several other investigators, who either had similar difficulties in obtaining established cell lines after SV40 infection of human cells (Moyer et al. 1964), or did not obtain any at all (Shein et al. 1964; Ruben & Rafferty, 1978; Rafferty et al. 1978; Gotoh et al. 1979; Sack & Obie, 1981). They do not support the results of Jensen et al. (1963), Todaro et al. (1963) and Girardi et al. (1965), who reported a high rate of success in producing permanent cell lines, especially with senescent populations. The variable length of the crisis period (i.e. from 2–14 weeks) suggests that the rare events involved in immortalization occur during rather than before crisis, as selection of an immortal clone would not take more than about 4 weeks (assuming a doubling time of approximately 35 h). Moreover, within the crisis period foci may appear that have limited capacity for growth. In other experiments with SV40-treated human skin fibroblasts, the crisis period sometimes lasted up to 200 days before permanent lines emerged (unpublished results).
The individual nature of the pre-transformed cultures was further exemplified by comparing the histories of our two transformed lines, MRC-5V1 and MRC-5V2. Pre-transformed MRC-5V1 had fairly minor changes in growth properties, while the phenotype of pre-transformed MRC-5V2 was more extreme. Both permanent lines retained similar features of their pre-transformed state, including morphological appearance and chromosome number. The major phenotypic differences between MRC-5V1 and MRC-5V2 are their morphological appearance, modal chromosome number, glucose and methionine requirements, sensitivity to BrdUrd and elevated incubation temperature. Long-term growth has shown that both lines have lost their tendency to shed free virus into the medium, and rates of cumulative population doublings have increased in later passages, without a detectable change in growth rate in short-term experiments. The gradual increase in anchorage independence probably indicates that there is also a greater release from contact-inhibition. In both lines, the chromosome number gradually increased during sub-culture, but this change was more marked in MRC-5V1. Karyotype instability has also been reported for other SV40-transformed lines (Begovich & Francke, 1979).

MRC-5V1 showed two further interesting examples of instability. The growth of some sub-lines slowed over a period of many population doublings and the culture died out, in a manner that was very reminiscent of the senescence and death of diploid fibroblasts. In several cases, cells recovered from liquid nitrogen also gave rise to populations with finite growth. However, it has been possible to keep the line growing and it has now reached p.d.l. 750. The question arises as to whether this line contains an immortal sub-population that constitutes only a small minority of the cells. If this sub-population is lost (perhaps by drift or stochastic fluctuation in cell numbers), then the remaining cells may continue to divide for a while before eventually dying out. We previously proposed that very early passage diploid fibroblasts contain a sub-population of immortal cells that are inevitably lost, because during growth they change with a given frequency to cells with finite growth potential (Kirkwood & Holliday, 1975; Holliday et al. 1977). In transformed cultures, a similar sub-population would normally be retained, but it might occasionally be lost, especially after a reduction in population size during freezing and thawing. Unfortunately, the difficulty of growing individual clones from MRC-5V1 has not made it possible to test this hypothesis directly. It is, however, known that many clones isolated from HeLa cell populations have limited growth potential (Martinez, Norwood, Prothero & Martin, 1978; K. A. Rafferty, personal communication).

The instability of MRC-5V1 was also shown at the biochemical level. The proportion of heat-labile Glc-6-P dehydrogenase was studied at intervals to see whether the populations maintained the low levels observed in young cultures of diploid human fibroblasts. Whereas in MRC-5V2 there was no significant change in the enzyme over 600 p.d., MRC-5V1 acquired a new form of the enzyme, which was extremely sensitive to heat and was also distinguishable from the normal enzyme by its faster migration in starch gels. However, this new form of Glc-6-P dehydrogenase disappeared after a further 200 p.d. The heat-inactivation experiments also suggested that other allelic forms of the dehydrogenase had been produced during the growth of
MRC-5V1. There have been two other reports of newly acquired isoenzymes of Glc-6-P dehydrogenase in mouse and human tumours (Hilf, Rector, Abraham & Lyon, 1973; Khan, Shin & Steinberg, 1978). In the former, a faster migrating band of the enzyme in polyacrylamide gels was detected in pre-neoplastic and neoplastic mammary adenocarcinomas from Balb/c mice, while in the latter, a new modified form of the enzyme was observed by isoelectric focusing in various human leukaemic cells. Extracts of leukaemic cells, when incubated with normal Glc-6-P dehydrogenase, led to a modification of this enzyme, identical to that seen in the leukaemic cells. We were not able to show this to be the case when our normal and transformed cell-free extracts were mixed.

We do not know whether the new forms of Glc-6-P dehydrogenase are due to post-synthetic modifications, or to changes in the structural gene. It may be that the well-known karyotypic instability of transformed cells is also accompanied by instability at the gene level. For instance, the amplification of genes in response to antimetabolite inhibitors has been demonstrated, so far as we are aware, only in permanent lines (e.g. see Schimke, 1982). In any event, the widely held view that the electrophoretic profile of any enzyme is an unchanging diagnostic characteristic of any particular cell strain or line, may need reassessment. In the case of MRC-5V1, it seemed that an initial homogeneous population, possibly a clone, had itself become polymorphic for Glc-6-P dehydrogenase. We presume that the changes seen in the properties of the enzyme are at least in part due to continuous selection of fast-growing sub-populations of cells, and their subsequent replacement by other populations.

One of the aims of this study was to try to determine whether the crisis of pre-transformed cells is the same as senescence (phase III) of diploid populations. Cells in crisis are invariably large, often multinucleate, granular and with variable morphology. Large numbers of cells detach and form debris in the medium. In these respects they resemble phase III diploid cells. We tried to exploit the observation that senescent MRC-5 cultures have elevated levels of heat-labile Glc-6-P dehydrogenase (Holliday & Tarrant, 1972). The results obtained were ambiguous, in that some cultures near to or within crisis contained 15–25% heat-labile enzyme, which is characteristic of senescent MRC-5, whereas others had a heat-labile fraction equivalent to young populations. (Note that any changes in a minor sub-fraction of enzyme molecules should be clearly distinguished from the major alterations in the enzyme that we observed during the long-term growth of MRC-5V1.) It is possible that in some cases there was a loss of unstable enzyme molecules in the slowly dividing or non-growing cells, before the heat stability of Glc-6-P dehydrogenase was examined. Better biochemical methods are needed to compare pre-transformed cells in crisis with senescent diploid fibroblasts.

The events that allow occasional cells to survive crisis and form a permanent line remain obscure. These cells are, in a sense, rejuvenated. For instance, it was recently shown that they contain a low level of autofluorescence (AF), which is a specific characteristic of early-passage MRC-5 cells (Rattan, Keeler, Buchanan & Holliday, 1982). It is probable that the escape from senescence is due to the loss of one or more cellular functions, since hybrids between diploid and transformed cells usually have
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a finite lifespan (Bunn & Tarrant, 1980; Muggleton-Harris & DeSimone, 1980; Periera-Smith & Smith, 1981). We exploited the methionine requirement of MRC-5V1 to select hybrids between this line and Lesch-Nyhan fibroblasts and also found that these eventually died out.

The early events in transformation clearly involve the frequent integration of SV40 and the expression of the T-antigens, but the later event or events during the crisis period, which give rise to permanent lines, are unknown. Since immortalization is very rare in human cells, it is reasonable to suppose that it depends on mutation, rearrangement or transposition of the SV40 genome in individual cells, although epigenetic switches in gene activity are also possible (Holliday, 1979; Harris, 1982). In rodent cells, immortalization occurs spontaneously and at high frequency after SV40 infection (Ponten, 1971). A comparative study of in vitro transformation in different species may throw light on the mechanisms whereby cells escape from senescence.

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