Variant Lines of Mouse Kidney Cells Transformed by an SV40tsA Mutant with Growth Properties of Wild-Type Transformed Cells at Nonpermissive Temperature

By H. OTSUKA, D. R. DUBBS AND S. KIT

Division of Biochemical Virology, Baylor College of Medicine, Houston, Texas 77030, U.S.A.

(Accepted 25 September 1978)

SUMMARY

mKSA207 cells, a BALB/c mouse kidney line transformed by a tsA mutant of SV40, are temperature-dependent for the expression of the 'standard transformed phenotype'. At the permissive temperature (33.5 °C), the mKSA207 cells resembled wild-type (wt) SV40 transformants; they contained the intranuclear SV40 T antigen, grew to high saturation density in monolayer culture in either 10% or 0.5% serum, and also in methylcellulose suspension culture and became multinucleate in cytochalasin B. At the nonpermissive temperature (39.8 °C), the mKSA207 cells lost some of their transformed properties; they grew only to low density in 10% serum, hardly grew at all in 0.5% serum or in methylcellulose suspension culture, and remained mono- or binucleate in cytochalasin B. At 40 °C in low serum, mKSA207 cells lost the intranuclear T antigen and when fed 10% serum at 39.8 °C, accumulated large amounts of T antigen in the cytoplasm. Derivatives of mKSA207 have been selected at 39.8 °C in liquid medium and methylcellulose suspension culture. The heat-adapted lines, like wt SV40 transformants, exhibited the standard transformed phenotype at both 33.5 and 39.8 °C. It is unlikely that acquisition of temperature-independence for the transformed phenotype was due to reversion of the tsA gene to wild-type because the heat-adapted cell lines displayed the cytoplasmic T antigen at 39.8 °C, characteristic of the parental mKSA207 cells and SV40 rescued from one of the heat-adapted lines was temperature sensitive for growth. The T antigen levels (complement fixation units per 10^6 cells) of heat-adapted lines grown at 39.8 °C were comparable to those of mKSA207 cells grown at 33.5 or 39.8 °C.

INTRODUCTION

Cells transformed by wild-type SV40 usually exhibit many growth characteristics which are different from normal cells. Among these are the ability to grow to high saturation density in monolayer culture in either low or high serum, the ability to grow in soft agar or methylcellulose suspension culture, and the tendency to become multinucleate in the presence of cytochalasin B, a drug which inhibits cytokinesis (Dulbecco, 1969; Kelly & Sambrook, 1973; Hirano & Kurimura, 1974). In contrast, cells transformed by SV40tsA mutants lose some of the transformed properties at nonpermissive temperatures, although they behave like standard transformants at permissive temperatures (Butel et al. 1974; Brugge & Butel, 1975; Martin & Chou, 1975; Osborn & Weber, 1975; Tegtmeyer, 1975; Martin et al. 1977).
Not all cell lines transformed by wild-type SV40 display the 'standard transformed phenotype'; some 3T3 lines transformed by wild-type SV40 have growth properties similar to normal 3T3 cells or intermediate between those of normal 3T3 and 'standard transformants' (Risser & Pollack, 1974; Pollack et al., 1975; Vogel & Pollack, 1975). In addition, certain 3T3 lines transformed by wild-type SV40 show ts growth or temperature dependent serum requirements (Renger & Basilico, 1972; Toniolo & Basilico, 1975). The preceding observations suggested that SV40tsA transformants might also be selected which exhibit the 'standard transformed phenotype' at the nonpermissive temperature.

To test this hypothesis, experiments were initiated with a mouse kidney line (mKSA207) which had been transformed at the permissive temperature by SV40 mutant, tsA207 (Dubbs et al. 1978). As expected, the mKSA207 cells behaved like standard transformants at the permissive temperature (33.5 °C), but lost some of the transformed properties at the non-permissive temperature (39.8 °C). Furthermore, mKSA207 cells lost T antigen when incubated in low serum at 40 °C and accumulated it in the cytoplasm when they were fed 10% serum and incubated at 39.8 °C. From the mKSA207 transformed cells, derivative cell lines have been selected which express transformed properties at both permissive and non-permissive temperatures yet have T antigen in the cytoplasm at the nonpermissive temperature. The properties of these heat-adapted cell lines are described in this communication.

METHODS

Cell culture. mKSA207 cells are primary BALB/c mouse kidney cells transformed at 33.5 °C by SV40tsA207 (Dubbs et al. 1978). mKS(wt) cells are primary BALB/c mouse kidney cells transformed at 36.5 °C by wild-type SV40 virus. Both lines were grown at 33.5 °C and subcultured at 4 to 7 day intervals in Eagle's minimal essential medium (APMEM; Auto Pow, Flow Laboratories Inc., Rockville, Maryland) supplemented with 30% calf serum. CV-1 cells, an established line of African green monkey kidney cells (Jensen et al. 1964), were propagated at 36.5 °C in APMEM supplemented with 10% foetal calf serum.

Isolation of derivatives of mKSA207 and mKS(wt). After approx. 80 passages in culture, mKSA207 cells were recloned in monolayer culture at 33.5 °C and one of the clones (mKSA207-C) was used in this study. mKSA207 or mKSA207-C cells were inoculated at a density of 3 × 10⁴/cm² in 10 ml APMEM supplemented with 10% calf serum and incubated at 39.8 °C. Medium was changed every second day. When cultures reached confluency, they were trypsinized, subcultured and maintained in the same way. After several passages, cells became heat-adapted and were subcultured at 3 to 4 day intervals without intermediate medium changes. The heat-adapted lines, mKSA207-H and mKSA207-CH, were derived from mKSA207 and mKSA207-C, respectively.

mKSA207-S4 and mKSA207-S6 were isolated from methylcellulose suspension cultures of mKSA207-H. mKSA207-H cells were suspended at 10⁴ cells/ml in APMEM containing 1.3% methylcellulose (Matheson, Coleman and Bell, 4000 cP at 2%), and 10% foetal calf serum. Twenty g of the suspension were weighed into a bacterial plastic Petri dish (Falcon, 100 x 10 mm) and incubated at 39.8 °C for 2 weeks with addition of fresh medium on day 7. Colonies were picked and initially propagated in monolayer culture at 33.5 °C until several million cells were obtained; afterwards they were incubated at 39.8 °C and subcultured at 3 to 4 day intervals. Heat-adapted cells were used for these experiments after 10 to 29 passages at the high temperature.

mKS(wt)-H was isolated as a clone of mKS(wt) incubated at 39.8 °C in APMEM supplemented with 10% foetal calf serum.
Variant lines of SV40tsA-transformed cells

Growth in monolayer culture. Trypsinized cells were suspended in APMEM supplemented with 10% calf serum at 2 x 10^4 cells/ml and plated in 1 ml samples into multiwell tissue culture plates containing twenty-four 16 mm wells (Falcon). After 24 h incubation at 33.5 °C, the medium was removed, monolayers washed once with the medium, and 1 ml medium was added to each well. The medium was either APMEM supplemented with 10% calf serum or Eagle's basal medium (BME) supplemented with 0.5% calf serum. The plates were incubated at 33.5 or 39.8 °C and after various time intervals, cells per well were counted using a Coulter counter.

Growth in methylcellulose suspension culture. Trypsinized cells were suspended at 10^5 cells/ml in APMEM containing 1.3% methylcellulose and 10% foetal calf serum. Twenty g samples were weighed into 100 mm bacterial plastic dishes. The cultures were incubated at 33.5 or 39.8 °C. At various times, cultures were diluted fivefold with cold phosphate buffered saline (PBS) and cells were collected by centrifugation at 600 g for 10 min, washed twice with PBS, and stored at -20 °C. When all the samples of an experiment had been collected, the frozen cells were thawed and washed three times with cold 0.5 N-perchloric acid (PCA). Cold PCA insoluble materials were dissolved in 2 ml of 0.3 N-KOH, incubated at 37 °C for 16 h, and 0.32 ml of 5 N-PCA was added. The precipitates were collected at 4 °C by centrifugation, washed once with cold 0.5 N-PCA, suspended in 2 ml of 0.5 N-PCA and hydrolysed at 70 °C for 30 min. The amount of hydrolysed DNA was estimated by the method of Burton (1956).

Plating efficiency in monolayer culture. Two day old cultures of mKSA207, mKSA207-C or mKS(wt), growing exponentially at 33.5 °C, or of the heat-adapted lines growing at 39.8 °C, were used. The cells were trypsinized, washed twice in APMEM + 10% foetal calf serum, suspended in the same medium and 5 ml samples containing 200, 1000, 2500, 5000 or 10000 cells were plated into 50 mm plastic Petri dishes. The dishes inoculated with 200 cells were incubated at 33.5 °C for 10 days with a medium change on the fifth day. The dishes containing more than 1000 cells were incubated at 33.5 °C for 1 day and then incubated at 39.8 °C for 10 days with medium changes on the fourth and seventh days. After incubation, cultures were fixed with ethanol, stained with Giemsa, and the visible colonies were counted in dishes containing less than 200 colonies.

Multinucleation by cytochalasin B. Cells were attached to coverslips at 33.5 °C for 24 h and treated with cytochalasin B [2.5 µg/ml for mKS(wt) and 1.5 µg/ml for the other cells] at 33.5 or 39.8 °C for 4 days with a change of medium on the second day. Coverslips were fixed with ethanol, stained with Giemsa and the number of nuclei per cell was determined microscopically.

T antigen determination. SV40 T antigen was detected by indirect immunofluorescence using sera from hamsters with SV40 tumours and anti-hamster gamma globulin prepared in goats and conjugated with fluorescein isothiocyanate (Antibodies, Inc., Davis, California). Prior to the immunofluorescence tests, cells grown on coverslips were fixed at -20 °C in ethanol, placed on dry ice for 15 min, and stored at -70 °C (Kaplan et al. 1975). SV40 T antigen was also demonstrated by complement fixation (CF) in a semi-micro test employing 4 units of antibody and 2 full units of complement. T antigen was expressed as CFU/10^6 cells.

Rescue of virus from transformed cells. mKSA207-S4 and mKSA207-S6 cells were fused with CV-1 cells using u.v.-irradiated Sendai virus. Fusion mixtures were plated into 50 mm plastic Petri dishes and incubated at 33.5 °C overnight to permit attachment of cells. Medium containing 0.9% agar was added and incubation was continued at 33.5 °C. On the seventh and fourteenth days, the cultures were fed with 3 ml agar medium and, on the twenty-first day, agar medium containing neutral red was added. Well isolated plaques were picked.
Fig. 1. Growth of (a) mKSA207, (b) mKSA207-C and (c) mKS(wt) in monolayer culture in 10% serum at 33.5 °C (○--○) and 39.8 °C (●●●). Cells (3 × 10⁴/ml) were seeded in multiwell tissue culture dishes (2 cm²/well) in APMEM + 10% calf serum and incubated at 33.5 °C for 24 h to permit attachment of cells. At time 0, the cultures were washed, new medium (APMEM + 10% calf serum) was added, and cultures were incubated at 33.5 °C or 39.8 °C. Medium was changed again on day 2 and day 4.

and virus propagated in monolayer cultures of CV-1 cells at 33.5 °C for 2 weeks. SV40 virus was assayed on confluent monolayers of CV-1 cells at 33.5 °C.

Alternatively, the fusion mixtures were inoculated into 8 oz prescription bottles and incubated for 14 days at 33.5 °C. The cells and supernatant fluids were harvested, the cells disrupted by freeze-thawing and sonication, and the lysates used for blind passage in CV-1 cultures and assayed for SV40 on CV-1 monolayers.

One step growth curve of SV40 virus. Confluent monolayer cultures of CV-1 cells in 2 oz bottles were infected with virus from mKSA207-S4, at 2 p.f.u./cell and incubated at 33.5 or 39.8 °C. At various time intervals, cells were scraped into medium, dispersed by sonication and assayed for virus at 33.5 °C.

RESULTS

Parameters of growth

mKSA207 cells transformed by a tsA mutant of SV40 resemble mKS(wt) at 33.5 °C but lose some of their transformed properties when incubated at 39.8 °C (Dubbs et al. 1978). Heat-adapted lines of mKSA207 and mKSA207-C were selected at 39.8 °C. Growth properties of the heat-adapted lines were studied at 33.5 and 39.8 °C and compared to properties of parental lines. Five parameters of growth were studied for temperature sensitivity: (i) ability to grow in monolayer culture in 10% serum; (ii) ability to grow in monolayer culture in 0.5% serum; (iii) ability to grow in methylcellulose suspension culture; (iv) multinucleation in cytochalasin B; and (v) plating efficiency in 10% serum on plastic.
Variant lines of \textit{SV}40\textit{tsA}-transformed cells

Temperature-dependent properties of \textit{mKSA}207 and \textit{mKSA}207-C

Growth of \textit{mKSA}207, \textit{mKSA}207-C and \textit{mKS}(wt) in monolayer culture in 0.5% serum is shown in Fig. 1. All three cell lines grew well at 33.5 °C, but at 39.8 °C \textit{mKSA}207 and \textit{mKSA}207-C grew at a much slower rate than at 33.5 °C or than \textit{mKS}(wt) at 39.8 °C. The maximum cell population attained at 39.8 °C with the \textit{tsA} transformants was only about one third that at 33.5 °C and about one half that of \textit{mKS}(wt) at 39.8 °C.

Temperature-dependent growth of the \textit{tsA} transformant was particularly striking in low serum. In 0.5% serum, \textit{mKSA}207 and \textit{mKSA}207-C grew as well at 33.5 °C as did \textit{mKS}(wt) (Fig. 2). However, at 39.8 °C, where \textit{mKS}(wt) grew almost as well as at 33.5 °C, the \textit{tsA} transformants underwent only one or two cell divisions and then deteriorated in spite of frequent changes of medium.

The \textit{tsA} transformants also differed markedly from \textit{mKS}(wt) in their ability to grow in methylcellulose suspension culture (Fig. 3). (Growth in methylcellulose suspension cultures was assessed by measuring the amount of DNA because cells grew as aggregates and it was difficult to make accurate cell counts in a haemocytometer or Coulter counter.) As shown in Fig. 3(a, b) \textit{mKSA}207 and \textit{mKSA}207-C grew slowly in methylcellulose suspension medium even at 33.5 °C. At 39.8 °C, \textit{mKSA}207 showed only a two- to threefold increase in DNA, while with \textit{mKSA}207-C, DNA content declined rapidly after the first day. On the other hand, \textit{mKS}(wt) grew equally well at 33.5 and 39.8 °C in methylcellulose (Fig. 3c).

In the presence of cytochalasin B, normal mouse kidney (mk) cells remain either mononucleate or become binucleate (Fig. 4a, b). In contrast, at either 33.5 or 39.8 °C, \textit{mKS}(wt) become multinucleate; nearly all the cells contained three or more nuclei (Fig. 4c, d). At
37.5 °C, mKSA207-C responded to cytochalasin B in a manner similar to mKS(wt); more than 80% of cells contained three or more nuclei (Fig. 4e). On the other hand, at 39.8 °C the mKSA207-C behaved like normal mouse kidney cells when incubated with cytochalasin B. Eighty per cent of cells remained mono- or binucleate (Fig. 4f), with large, abnormal nuclear morphology. Similar results were obtained with mKSA207 (data not shown).

The tsA transformants also differed from mKS(wt) in their ability to form colonies at 39.8 °C. The efficiency of plating of mKS(wt) was reduced 8- to 20-fold at 39.8 °C, as compared to 33.5 °C. However, the efficiency of plating of the tsA transformants was reduced 80- to 100-fold (Table 1).

*Isolation of temperature-resistant derivatives of mKSA207 cells*

In an earlier study, mKSA207 did not grow at temperatures above 39.5 °C in 10% calf serum medium (Dubbs et al. 1978). However, it was observed that mKSA207 cells responded to the addition of medium with 10% serum by initiating DNA synthesis. When the inoculum was four times higher and medium containing 10% calf serum was replenished every second day, the tsA transformants grew slowly at 39.8 °C (Fig. 1a, b). Therefore, we adapted mKSA207 and mKSA207-C to grow at 39.8 °C for prolonged periods by using high inoculum and frequent medium changes. Initially, the cells grew slowly at 39.8 °C, requiring 6 to 7 days to reach confluency. After several passages, they grew faster with fewer medium changes. The heat-adapted lines were called mKSA207-H and mKSA207-CH.

The heat-adapted line mKSA207-H was further adapted by growing in methylcellulose suspension culture at 39.8 °C. Many colonies developed after 2 weeks incubation. Several
Fig. 4. The effect of cytochalasin B on (a, b) mouse kidney (e, d) mKSA(wt), (e, f) mKSA207-C, and (g, h) mKSA207-S6 at 33.5 °C (a, c, e, g) and 39.8 °C (b, d, f, h). Cells were allowed to attach to coverslips by incubation for 24 h at 33.5 °C. Cytochalasin B [2.5 µg/ml for mKSA(wt) and 1.5 µg/ml for the other lines] was added and the cultures were incubated at 33.5 and 39.8 °C. The medium was changed on day 2. On day 4, the coverslips were fixed and stained.

Table 1. Plating efficiency (e.o.p.)* of various cell lines at 33.5 °C and 39.8 °C

<table>
<thead>
<tr>
<th>Cell line</th>
<th>E.o.p. (%) At 33.5 °C</th>
<th>E.o.p. (%) At 39.8 °C</th>
<th>Relative e.o.p. (39.8/33.5 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mKSA207</td>
<td>57†</td>
<td>0.5‡</td>
<td>0.008</td>
</tr>
<tr>
<td>mKSA207-C</td>
<td>25</td>
<td>0.3</td>
<td>0.012</td>
</tr>
<tr>
<td>mKSA207-S4</td>
<td>75</td>
<td>10.1</td>
<td>0.13</td>
</tr>
<tr>
<td>mKSA207-S6</td>
<td>69</td>
<td>9.4</td>
<td>0.14</td>
</tr>
<tr>
<td>mKSA207-CH</td>
<td>51</td>
<td>3.3</td>
<td>0.065</td>
</tr>
<tr>
<td>mKSA(wt)</td>
<td>52</td>
<td>2.7</td>
<td>0.052</td>
</tr>
<tr>
<td>mKSA(wt)-H</td>
<td>76</td>
<td>9.7</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* Per cent of cells plated yielding colonies.
† 200 cells were plated in 50 mm plastic dishes in 10% serum and incubated at 33.5 °C; medium was changed on the fifth day.
‡ Samples of cell suspension containing 1000 to 10,000 cells were plated as above, incubated at 33.5 °C for 24 h and then shifted to 39.8 °C with medium changes on fourth and seventh days. Visible colonies were counted in plates with less than 200 colonies.
Table 2. \( T \) antigen in \( m\text{KSA207} \) and heat-adapted \( m\text{KSA207-CH} \), \( m\text{KSA207-S4} \) and \( m\text{KSA207-S6} \) cells

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Cell line*</th>
<th>Temperature (°C)</th>
<th>Days</th>
<th>CF titre</th>
<th>CFU/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( m\text{KSA207} )</td>
<td>33.5</td>
<td>4</td>
<td>32</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>( m\text{KSA207-CH} )</td>
<td>39.8</td>
<td>2</td>
<td>16</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>( m\text{KSA207-S4} )</td>
<td>39.8</td>
<td>3</td>
<td>16</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>( m\text{KSA207-S6} )</td>
<td>39.8</td>
<td>3</td>
<td>32</td>
<td>7.8</td>
</tr>
<tr>
<td>2</td>
<td>( m\text{KSA207} )</td>
<td>39.8</td>
<td>2</td>
<td>32</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>( m\text{KSA207-S4} )</td>
<td>39.8</td>
<td>3</td>
<td>32</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>( m\text{KSA207-S6} )</td>
<td>39.8</td>
<td>3</td>
<td>16</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* \( m\text{KSA207} \) cells were seeded at \( 1.8 \times 10^4 \) cells/cm²; the heat-adapted cells at \( 3.6 \times 10^4 \) cells/cm². APMEM + 10% serum was replaced on day 2.

† \( m\text{KSA207} \) cells were seeded at \( 1.8 \times 10^4 \) cells/cm² in APMEM + 10% serum and incubated at 33.5 °C for 2 days. For this group only, \( m\text{KSA207} \) cells were then depleted of \( T \) antigen by incubation for 3 days at 40 °C in BME + 0.5% serum. Medium was replaced with APMEM and cultures were shifted to 39.8 °C for one more day before \( T \) antigen was measured.

Large colonies were picked, propagated in monolayer cultures and two clones, \( m\text{KSA207-S4} \) and \( m\text{KSA207-S6} \), were selected for further study.

After eight passages at 39.8 °C, the heat-adapted cells, when grown at 33.5 °C, displayed an intranuclear \( T \) antigen by immunofluorescence. At 39.8 °C, only a faint nuclear fluorescence was observed; most of the \( T \) antigen was cytoplasmic as in \( m\text{KSA207} \) or \( m\text{KSA207-C} \). In \( S6 \) cells, the cytoplasmic \( T \) antigen appeared as small, brightly fluorescent globules, but in \( S4 \) the cytoplasmic \( T \) antigen was more diffuse.

\( T \) antigen in the transformed cells was measured by CF and expressed as CFU/10^6 cells. \( m\text{KSA207-S4} \) and \( m\text{KSA207-S6} \) cells, which had been growing continuously at 39.8 °C, were seeded at \( 3.6 \times 10^4 \) cells/cm²; \( m\text{KSA207} \) cells, growing at 33.5 °C, were seeded at \( 1.8 \times 10^4 \) cells/cm². \( T \) antigen measurements were made on \( m\text{KSA207-CH} \), \( m\text{KSA207-S4} \) and \( m\text{KSA207-S6} \) at 2 days after seeding, when the cells were growing exponentially and on day 3 as they approached the stationary phase. The level of \( T \) antigen in \( m\text{KSA207-S4} \) at 39.8 °C on day 2 was comparable to that of \( m\text{KSA207} \) at 33.5 °C on day 4 (Table 2). This was also the level of \( T \) antigen obtained when \( m\text{KSA207} \) cells, which had been depleted of \( T \) antigen by incubation at 40 °C for 3 days in low serum, were incubated 24 h longer at 39.8 °C in 10% serum (Table 2, Expt. 2). The CFU \( T \) antigen/10^6 exponentially growing \( m\text{KSA207-S6} \) cells was slightly higher than in \( m\text{KSA207} \) (Table 2) at 2 days, while that in \( m\text{KSA207-CH} \) was about 50% less. \( T \) antigen levels decreased in all three heat-adapted lines after 3 days at 39.8 °C as the cells approached saturation density. These results indicate that abnormally high levels of \( T \) antigen do not accumulate in the heat-adapted lines at 39.8 °C.

Temperature-independent growth of heat-adapted lines

The three heat-adapted lines, \( m\text{KSA207-CH} \), \( m\text{KSA207-S4} \) and \( m\text{KSA207-S6} \), were studied for temperature sensitivity using the five parameters of growth described earlier. All three lines grew equally well at 33.5 and 39.8 °C in monolayer cultures in 10% serum (Fig. 5). In low serum (0.5%), the cells could also grow at both 33.5 and 39.8 °C (Fig. 6).
Variant lines of SV40tsA-transformed cells

Fig. 5. Growth of (a) mKSA207-S4, (b) mKSA207-S6 and (c) mKSA207-CH in monolayer culture in 10% serum at 33.5 °C (○—○) and 39.8 °C (●—●). See Fig. 1 for culture details.

Fig. 6. Growth of (a) mKSA207-S4, (b) mKSA207-S6 and (c) mKSA207-CH in monolayer culture in 0.5% serum at 33.5 °C (○—○) and 39.8 °C (●—●). See Fig. 2 for culture details.
Fig. 7. Growth of (a) mKSA207-S4, (b) mKSA207-S6 and (c) mKSA207-CH in methylcellulose suspension culture at 33.5 °C (O--O) and 39.8 °C (●--●). See Fig. 3 for culture details.

The cells grew at the same rate at the two temperatures for 2 days, but then began to slow at 39.8 °C, in a manner analogous to mKS(wt) (Fig. 2c).

Heat-adapted cells also gained the capacity to grow in methylcellulose suspension at 39.8 °C (Fig. 7). The DNA content of the cultures increased four- to eightfold at 39.8 °C, but at a slower rate than at 33.5 °C. With cultures of mKSA207-S4 and -S6, the maximum DNA content attained at 39.8 °C was only 30% and 50% of that attained at 33.5 °C. Surprisingly, mKSA207-CH, which was adapted in monolayer culture, grew nearly as well in methylcellulose suspension medium at 39.8 as at 33.5 °C (Fig. 7c). None of the heat-adapted lines, however, grew as well in suspension culture as did mKS(wt) (Fig. 3c).

The relative plating efficiencies for heat-adapted lines on plastic in 10% serum were also much higher than for the original tsA transformants and comparable to those of mKS(wt) and mKS(wt)-H (Table I).

When the heat-adapted cell lines were exposed to cytochalasin B, they became multinucleate at both 33.5 and 39.8 °C (Fig. 4g, h). With mKSA207-S6, approx. 85% of cells had three or more nuclei at 33.5 °C, and 75% at 39.8 °C. Similar results were obtained with mKSA207-S4 (data not shown).

Rescue of SV40 from heat-adapted lines

The resident virus can easily be rescued from mKSA207 cells by fusing with permissive CV-1 cells using u.v.-irradiated Sendai virus. Approximately one in 10^6 or 10^7 mKSA207 cells formed infectious centres. Virus was also rescued from mKSA207-S4 and, like that rescued from mKSA207, was shown to be temperature sensitive for growth. However, in five independent trials no virus was rescued from mKSA207-S6.
DISCUSSION

The mKSA207 cells described in this study have temperature-dependent growth properties similar to those of SV40tsA-transformed rodent cells described by others (Brugge & Butel, 1975; Osborn & Weber, 1975; Tegtmeyer, 1975; Martin et al. 1977). In contrast, the heat-adapted lines derived from mKSA207 exhibit at both nonpermissive (39.8 °C) and permissive temperatures (33.5 °C) the phenotype of 'standard wild-type SV40 transformants'. The heat-adapted cells grow to high saturation densities at both temperatures in 10% serum or in low serum, grow in methylcellulose suspension culture and become multinucleate in the presence of cytochalasin B.

Besides the heat-adapted cell lines described here, cell lines transformed by analogous gene A mutants of polyoma virus, which exhibit the transformed phenotype at high temperature, have been isolated (Fried, 1965; DiMayorca et al. 1969; Eckhart, 1969; Seif & Cuzin, 1977). One of sixteen SV40tsA-transformed cell lines isolated by Brockman (1978) showed temperature-resistant growth properties. Graf & Beug (1976) also isolated transformed variants of rat (NRK) cells infected by temperature-sensitive avian sarcoma virus mutants that grew at nonpermissive temperatures.

Seif & Cuzin (1977) have emphasized that the phenotype of tsA transformants may depend on the method of clone selection. They found that clones (N) of rat embryo cells transformed by polyoma tsA mutants selected in liquid medium in monolayer were temperature-dependent for growth characteristics, whereas those clones (A) selected in soft agar were temperature-independent. The mKSA207 is a clonal line selected in liquid medium, as is subclone mKSA207-C. These lines are temperature-dependent, like most other SV40tsA transformants isolated in a similar way, and correspond to the N clones of Seif & Cuzin (1977). TsA transformants of polyoma virus have usually been selected in soft agar and were hence of the A type (Eckhart, 1969; Fried, 1965). The heat-adapted lines reported here originated from clonal lines of temperature-dependent cells. Variants of the A variety arose spontaneously and were selected during growth at high temperature or in methylcellulose. It is interesting that mKSA207-CH cells, selected for the ability to grow in 10% serum at 39.8 °C, also gained the capacity to grow in low serum or methylcellulose culture at 39.8 °C.

The many studies showing that cells transformed by tsA mutants of SV40 have temperature-dependent growth properties but that cells transformed by wild-type SV40 do not, have generally been interpreted to signify that a functional SV40 gene A product is essential for the maintenance of the transformed phenotype (Brugge & Butel, 1975; Osborn & Weber, 1975; Tegtmeyer, 1975; Martin et al. 1977). This interpretation receives strong support from the recent experiments of Steinberg et al. (1978) on revertants isolated from a transformed rat line (14B), which contained only one insertion of the wild-type SV40 genome. The revertants fell into three classes with regard to virus DNA sequences: (i) the first class in which the SV40 DNA was retained; (ii) the second class in which some of the SV40 DNA sequences mapping in gene A were deleted; (iii) the third class in which the SV40 DNA was totally lost and cured cells were generated. All three classes of revertants were T antigen-negative, density-sensitive, more serum-sensitive than line 14B and anchorage-dependent.

Unlike the revertant cell lines described by Steinberg et al. (1978), the parental mKSA207 cells and the heat-adapted cell lines contained multiple copies of the SV40 DNA and they expressed T antigen activity. The T antigen levels, measured by complement-fixation tests, were not grossly elevated in the mKSA207-S4, mKSA207-S6 and mKSA207-CH lines. This is in contrast to one exceptional SV40tsA transformant with temperature-resistant growth properties described by Brockman (1978) which did overproduce T antigen. It should be
noted, however, that SV40 gene A codes for at least three polypeptides, large-T, small-t and TSTA (Crawford et al. 1978). The function of one or more of these may have been regained in the heat-adapted cells, even though the T antigen activity remained cytoplasmic. The distributions of these three gene A products in mKSA207 and heat-adapted cells will be compared in another study. However, it should be noted that the site of the original tsA mutation in SV40tsA207 DNA is in Hind II + III fragment I, distal to the terminator codon of small-t (Lai & Nathans, 1975; Crawford et al. 1978) and in the same DNA fragment as some of the tsA mutants studied by Brockman (1978).

It is unlikely that the altered growth properties of heat-adapted mKSA207 lines were caused by reversion of the tsA gene to wild-type. Unlike SV40 wt transformants which contain intranuclear T antigen at both 39.8 and 33.5 °C, the heat-adapted sublines contain a cytoplasmic T antigen at 39.8 °C, indicating that the abnormal character of the gene A product of the parental mKSA207 line was retained (Dubbs et al. 1978). Furthermore, the virus rescued from mKSA207-S4 was ts for growth.

The hypotheses under consideration which might help to explain the acquisition of a temperature resistant expression of the transformed phenotype by heat-adapted mKSA207 lines are that either spontaneous or induced hereditary alterations in cellular DNA or changes in the state of the integrated SV40 DNA have occurred. With regard to the first possibility, spontaneous or induced cell mutations might bypass the need for products of SV40 gene A or reduce the requirements for these proteins. At present, there is no direct evidence that the heat-adapted variants of mKSA207 cells were derived by selection of cells that had undergone somatic cell mutations affecting growth at 39.8 °C. However, ‘spontaneously’ transformed mouse lines are known and it has been shown that somatic cell mutations can regulate the expression of the transformed phenotype (Miyashita & Kakunaga, 1975; Bouck & DiMayorca, 1976; Leavitt et al. 1977). Also, Marshak and collaborators (1975) and Theile & Strauss (1977) have demonstrated that SV40 infection can induce gene mutations in cultured cells, perhaps by insertion of virus DNA sequences into cellular DNA.

With respect to the state of the SV40 DNA in transformed cells, it is known from genetic and biochemical experiments that: (i) double transformants can readily be isolated (Dubbs & Kit, 1970; Prasad et al. 1975); (ii) multiple integration sites exist and the sites of integration of SV40 DNA into host DNA differ in different transformed cell lines; (iii) transformed cell lines contain virus DNA sequences in fragments of many different sizes; (iv) different regions of the SV40 genome are represented at different frequencies; and (v) the junctions between virus and cellular DNA sequences map at different places on the virus genome (Botchan et al. 1974, 1976; Ketner & Kelly, 1976). It might be anticipated that SV40 transcripts and their translation products would be altered if rearrangements in the integrated SV40 DNA occurred, such as deletions, duplications or transpositions to different integration sites. Rearrangements of integrated SV40 DNA sequences might also have an impact on the expression of contiguous cellular genes.

Several observations suggest that alterations in SV40 DNA sequences have in fact occurred in the heat-adapted mKSA207 cell lines. Although ts SV40 was rescued in every trial from parental mKSA207 cells, five attempts to rescue virus from mKSA207-S6 cells were negative. Preliminary experiments utilizing restriction endonuclease EcoR1 and SAl digestion of transformed cell DNAs, the Southern technique for transfer of DNA fragments to nitrocellulose filters, and hybridization with nick-translated 32P-SV40 DNA have also been carried out (unpublished experiments). These studies have revealed that there are a minimum of three SV40 integration sites in the DNA of heat-adapted mKSA207-S4 and mKSA207-S6 variants and that the restriction fragments containing SV40 DNA sequences from the heat-
Variant lines of SV40tsA-transformed cells

adapted lines differ electrophoretically from those of the temperature-sensitive parental mKSA207 cells. Analyses with additional restriction endonucleases are being carried out and may serve to elucidate some of the differences between the heat-adapted variants and the parental mKSA207 cells.

This research was aided by Robert A. Welch Foundation grant no. Q-163 and by USPHS grants no. CA-06656-15 and no. 1-K6-AI2352-15 from the National Cancer Institute and the National Institute of Allergy and Infectious Diseases.

A preliminary report of this work was presented at the Sixty-Ninth Annual Meeting of the American Association for Cancer Research, 5 to 8 April 1978.

REFERENCES


H. OTSUKE, D. R. DUBBS AND S. KIT


(Received 4 July 1978)