Establishment and Characterization of Indian Muntjak Cell
Lines Transformed with Simian Virus 40

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SUMMARY

Kidney cells of an Indian muntjak were transformed with simian virus 40 (SV40). The transformation efficiency of the tertiary cultures was very high when estimated by the agar suspension culture method. The efficiency was about 0.015% when infected at an input multiplicity of 0.4 p.f.u./cell. Clonal cell lines were established from the colonies in soft agar medium. Most of the cell lines and their subclones produced a small amount of infectious SV40. The SV40 virion antigen-positive cells in a clone increased from 0.2% to about 40% by the treatment with mitomycin C. More than 70% of the cells in two cell lines were normal in G- and C-banded karyotypes, indicating that chromosomal change is not a necessary step in the process of transformation of the Indian muntjak cells with SV40.

INTRODUCTION

Risser & Pollack (1974) observed wide variation in transformed properties among the clones of simian virus 40 (SV40)-transformed cells. This observation suggests that the process of cellular transformation with SV40 is the result of several complicated interactions of cellular and virus genes, not simply of a single virus gene acting directly to transform a cell. These interactions can be studied by the cytogenetic analysis of the stages of transformation. Numerical chromosome variations and karyotype abnormalities have been observed in papova virus-transformed cells. However, it remains unsolved whether the presence of certain chromosomes plays a role in the expression or suppression of the transformed phenotype. Recently, Yamamoto et al. (1973) reported specific chromosome loci correlated with the expression or suppression of the malignancy in Syrian hamster cells transformed with polyoma virus.

The Indian muntjak (Muntiacus muntjak vaginalis) cells possess the lowest chromosome number of any mammalian cells yet reported, with six in the female and seven in the male (Wurster & Benirschke, 1970) and are ideal for cytogenetic studies. The present communication describes the establishment of several SV40-transformed muntjak cell lines and their virological and cytogenetical characterization in order to evaluate their usefulness for cytogenetic analysis of transformation with SV40.

METHODS

Cell cultures. A secondary culture of newborn male Indian muntjak kidney cells was kindly provided by Dr Hajim Katsuta. The kidney cells were designated as Mm-2K (Takaoka

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Hamster cells were prepared by the method previously described (Yamaguchi & Kuchino, 1975) from brain tissue of newborn Syrian hamsters. The GC7 monkey cell line (Yamaguchi & Kuchino, 1975) and W-3Y-23, a clonal line of SV40-transformed rat 3Y1 cell (Segawa et al. 1977), were previously described. The cells were cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% foetal bovine serum.

Virus. A virus stock of SV40 strain 777 was prepared in GC7 cells as described (Yamaguchi & Kuchino, 1975).

Detection of infectious virus. Medium and cells (about $5 \times 10^6$ cells/ml) were frozen and thawed (five cycles) and tested for SV40 infectivity on GC7 cells. Aliquots (0.2 ml) of the samples were added to confluent GC7 cell cultures in roller tubes. After a 2 h adsorption period, 1 ml of maintenance medium (Eagle's MEM supplemented with 2% foetal bovine serum) was added. The cultures were incubated at 37°C and the medium was changed on days 5 and 10. The development of a cytopathic effect within 2 weeks was interpreted as an indicator of the presence of infectious SV40.

Plaque assay. Plaque assay was performed on GC7 cells as described previously (Yamaguchi & Kuchino, 1975).

Transformation assay. Transformation assay was performed by the agar suspension culture method (Macpherson & Montagnier, 1964). Briefly, confluent monolayer cultures were infected with SV40 for 2 h and then incubated overnight. After trypsinization, $4 \times 10^6$ cells were planted in growth medium containing 0.4% agarose (Seaplaque agarose: Marine Colloids Inc., Rockland, U.S.A.) and 20% foetal calf serum over a layer of 0.7% agarose medium. An additional agarose medium was overlaid on day 10. Colonies larger than 0.2 mm in diam. were counted on day 21.

Immunofluorescence staining. Cells were stained for the SV40 T antigen with the fluorescein-conjugated antibody as described (Kuchino & Yamaguchi, 1975). The antisera against SV40 virion (V) antigen were prepared in rabbits as described (Kuchino & Yamaguchi, 1975). Cells were stained for the SV40 V antigen as described for the SV40 T antigen. Percentages of fluorescence-positive cells were calculated from the results of counting either 200 or total positive cells on two coverslips (18 mm).

Induction by mitomycin C. Confluent cultures in 35 mm plastic dishes were exposed to mitomycin C (0.1 µg/ml, P-L Biochemicals, Inc.) at 37°C for 24 h in the dark. After washing and feeding the cultures with fresh growth medium, they were incubated at 37°C for 72 h and fixed for immunofluorescent staining or harvested for assay of infectious SV40.

Induction by cell fusion. A stock of Sendai virus strain Z was kindly provided by Dr. R. Hirai. SV40-transformed cells were fused to permissive GC7 cells using u.v.-irradiated Sendai virus as described (Yamaguchi et al. 1969). Cultures were incubated at 37°C for 7 days after the cell fusion reaction and tested for infectious SV40.

Chromosome analysis. Metaphase chromosome preparations for conventional Giemsa staining were made by the method previously described (Huh et al. 1977). Briefly, cells were treated for 4 h with $5 \times 10^{-7}$ M-calcine and specimens for chromosome analysis were prepared by the dropping and flaming method (Yoshida, 1966). More than 20 metaphase spreads per clone were photographed and used for chromosome analysis.

Chromosome preparations for banding were processed according to the air drying method. Chromosomes were G-banded by the method of Seabright (1971) and C-banded by the method of Sumner (1972).
SV40-transformed Indian muntjak cell

Table 1. Transformation of Indian muntjak and hamster cells by SV40*

<table>
<thead>
<tr>
<th>Cells†</th>
<th>M.o.i.‡</th>
<th>No. of colonies/plate§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muntjak (p3)</td>
<td>40</td>
<td>TMMC</td>
</tr>
<tr>
<td>0.4</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hamster (p2)</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

* Transformation assay was performed by the agar suspension culture method. Colonies larger than 0.2 mm in diam. were counted three weeks after the virus infection.
† Tertiary cultures of Indian muntjak cells and secondary cultures of hamster cells prepared from kidney and brain tissue, respectively.
‡ Input multiplicity of infection (p.f.u./cell).
§ Average of two plates.
|| Too many to count.

Table 2. Production of infectious SV40 from clonal lines of transformed cells

<table>
<thead>
<tr>
<th>First cloning</th>
<th>Second cloning*</th>
</tr>
</thead>
<tbody>
<tr>
<td>14/15†</td>
<td>10/11, 11/11, 12/12, 12/12, 12/12, 12/12</td>
</tr>
</tbody>
</table>

* Six virus producing clones were used for recloning.
† No. virus producing clones

RESULTS

Transformation of muntjak kidney cells with SV40

When confluent tertiary cultures of the muntjak cells were infected with SV40 and transferred into soft agar medium, colonies became visible to the naked eye 7 to 10 days after infection. The diameter of the colonies in infected cultures was 0.5 to 0.9 mm on day 21. Most of the cells in uninfected cultures divided several times in soft agar medium and the diameter of the colonies became approx. 0.05 mm on day 21. The efficiency of transformation was about 0.015% when infected at an input multiplicity of 0.4 p.f.u./cell (Table 1). Secondary cultures of hamster cells were infected as control for comparison at an input multiplicity of 40 p.f.u./cell. Only a few colonies were formed in soft agar medium and the size of colonies was smaller (0.2 to 0.4 mm) than that in the infected muntjak cell cultures. The growth rate of the uninfected muntjak cell cultures became slower upon repeated passages. The transformation efficiency of the muntjak cells at the 8th passage decreased to 0.003% even at an increased input multiplicity of 5000 p.f.u./cell.

Isolation and characterization of SV40-transformed muntjak cell lines

From the colonies developed in soft agar medium, 15 colonies were isolated. Cells derived from the colonies grew well and formed multilayers, in contrast to the uninfected muntjak cell cultures in which few cells were growing at this stage (2 months of cultivation). The SV40 T antigen was detected in the nuclei of nearly all the cells of all clones. Infectious SV40 was detected in all the clones but one (SV-Mm-21; Table 2). A possible carrier state of infectious SV40 was examined by re-cloning in soft agar medium. Seventy colonies were isolated from six virus-producing clones. All the clones except one (SV-Mm-12A) were positive for infectious virus (Table 2). Several virus-producing subclones in exponential growth phase were assayed for infectious SV40 and tested for SV40 V antigen. The titre of
Table 3. Effect of anti-SV40 serum on production of V antigen and infectious virus in SV40-transformed muntjak cells

<table>
<thead>
<tr>
<th>Treatment of antiserum</th>
<th>V antigen-positive nuclei (%)</th>
<th>Yield (p.f.u./10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0.022</td>
<td>6.7 x 10⁴</td>
</tr>
<tr>
<td>+</td>
<td>0.025</td>
<td>9.2 x 10⁴</td>
</tr>
</tbody>
</table>

* SV40-transformed muntjak kidney cells (Mm-zK-12B) were cultured in the presence of anti-SV40 serum for 5 passages (18 days). The cells were washed and cultured for 3 days either in the presence (for assay of V antigen) or in the absence (for assay of infectious SV40) of the antiserum. Control cultures were passed in parallel in the absence of the antiserum.

Table 4. Effect of mitomycin C on induction of V antigen and infectious virus in SV40-transformed muntjak cells

<table>
<thead>
<tr>
<th>Mitomycin C treatment</th>
<th>No. of cells per culture†</th>
<th>V antigen-positive cells (%)</th>
<th>Yield (p.f.u./culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1.5 x 10⁶</td>
<td>0.24</td>
<td>7.5 x 10⁵</td>
</tr>
<tr>
<td>+</td>
<td>2.6 x 10⁶</td>
<td>38</td>
<td>2.0 x 10⁷</td>
</tr>
</tbody>
</table>

* Confluent cultures of SV40-transformed muntjak kidney cells (SV-Mm-12B) in 35 mm plastic dishes were exposed to mitomycin C (0.1 µg/ml) for 24 h in the dark. After washing and feeding the cultures with fresh growth medium, they were further incubated at 37 °C for 72 h and fixed for immunofluorescent staining or harvested for assay of infectious SV40.
† Number of cells attached to a plastic dish at the time of harvest.

SV40 was in the range of 1 x 10⁵ to 4 x 10⁵ p.f.u./10⁶ cells per 3 days. Only 0.002 to 0.03 % of the nuclei were positive for V antigen. The percentage of V antigen-positive cells and the production of infectious SV40 were found to be unchanged whether or not the cells were cultured in the presence of 1 % rabbit anti-SV40 serum (neutralization titre, 1:16000) for five passages over a period of 18 days (Table 3). The result also excluded a carrier state of infectious SV40. No infectious SV40 was rescued from either of the two nonproducing clones (SV-Mm-21, SV-Mm-12A) by cell fusion with permissive monkey cells using u.v.-inactivated Sendai virus, but it was rescued from the SV40-transformed rat cells (W-3Y-23) which were used as positive control.

Mitomycin C has been used to induce infectious virus from SV40-transformed semipermissive cells (Gerber, 1964; Burns & Black, 1968; Margalith et al. 1970; Kaplan et al. 1975b). In order to compare the inducibility of the transformed muntjak cells with that of the semipermissive cells, one of the clones (SV-Mm-12B) was treated with mitomycin C. The yield of infectious SV40 was enhanced about 30-fold and the percentage of V antigen-positive nuclei was increased from 0.2 to about 40 % by mitomycin C treatment (Table 4).

Karyotype analysis of SV40-transformed clones of muntjak kidney cells

The histogram of chromosome number distribution for the uninfected muntjak kidney cells at the 9th passage (P9) is shown in Fig. 1(a). The cells exhibited a diploid modal number of 7 (91 %). Karyotypes of 13 SV40-transformed clones at the 4th to 8th passages after cloning (P4 to P8) were similarly analysed. The clones could be classified roughly into three groups. Clones in the first group showed a diploid modal chromosome number of 7 (7 clones; e.g. Fig. 1b, e, f). Clones in the second group showed a tetraploid modal chromosome number (4 clones), but more than 60 % of the cells in each clone showed a diploid chromosome number. The Y chromosome was lost from most of the cells in the third group (2 clones; e.g. Fig. 1h).
The results suggest that the chromosomal change is not a necessary step in the process of transformation of the Indian muntjak cells with SV40. In order to identify each chromosome precisely, G- and C-bands of the chromosomes of the clones were studied (Fig. 2). Each homologous pair of the chromosomes of uninfected muntjak kidney cells (Mm-2K) showed an identical banding pattern (Fig. 2Aa, Ba) which was similar to that reported previously (Kato et al. 1974). The banding patterns of three clones showing a diploid chromosome number in more than 70%, were compared with those of uninfected cells. The patterns of SV-Mm-11 and SV-Mm-12 were substantially the same as those of uninfected cells (Fig. 2Ab, Ac, Bb). SV-Mm-13 showed a modal chromosome number of 7 (Fig. 1e), but a portion of distal end of one of the No. 1 chromosome pair, the largest metacentric, was translocated on the distal end of the long arm of one of the No. 2 chromosomes (Fig. 2Ae). No common chromosomal changes among the transformed clones could be detected in their karyotypes.

Karyotypes of two clones were analysed after further passages to examine their stability. SV-Mm-12A was derived from a colony of SV-Mm-12 (Fig. 1b) in soft agar. It still showed a diploid mode at the 6th passage after the re-cloning (Fig. 1c, 2Ad), but after another nine passages the chromosome numbers were rather widely distributed between 6 to over 20 (Fig. 1d). SV-Mm-21 (p8) showed two main cell populations, one (45%) with a diploid, the other (17%) with a tetraploid chromosome number (Fig. 1f). After six passages, the diploid population decreased to 34% and that of the tetraploid increased to 29%. The range of the chromosome number distribution became wider after the passages (Fig. 1g).

**DISCUSSION**

Indian muntjak cells which possess the lowest chromosome number of mammalian cells were transformed with SV40. The results revealed two interesting properties of the muntjak cells; high transforming efficiency and semipermissiveness for SV40 replication. The trans
formation efficiency of the tertiary cultures estimated by the agar suspension culture method was comparable to or even better than that of mouse 3T3 line (Black, 1966). A reason for the high transformation efficiency of the muntjak cells may be their ability to grow in soft agar medium although the growth was far poorer than that of SV40-transformed cells. This peculiar ability is gradually decreased by passage. Transformed clonal cell lines positive for SV40 T antigen were easily established from the colonies developed in soft agar medium. Infectious SV40 was recovered from most of the transformed cell lines. Only one clone (SV-Mm-21), among 15 established, did not induce infectious SV40 even after cell fusion with permissive monkey cells. This clone might have arisen by transformation with defective SV40 (Uchida et al. 1968). A non-producing subclone (SV-Mm-12A) was isolated from a virus-producing clone. Since the original virus-producing colony developed in soft agar medium was surrounded by untransformed cells, the colony could have been a mixture of transformed and a few untransformed cells. The untransformed cell could be transformed with newly produced defective SV40 during colony isolation procedures. Therefore, the virus non-producing subclone might be derived from such a cell. All the remaining clones and subclones derived from the producer clones were producing small amounts of infectious SV40 spontaneously. The fact that most of the transformed clones were producing infectious virus and the failure to isolate nonproducer transformed clones containing a resuable virus genome suggest that all the muntjak kidney cells susceptible to transforming infection with SV40 are potentially permissive for this virus. The semipermissive character

Fig. 2. G-banded (A) and C-banded (B) sample karyotypes of untransformed muntjak kidney cells (Mm-2K) and their SV40-transformed clones.
of the transformed cells resembles that of Chinese hamster cells (Martin & Chou, 1975; Lavialle et al. 1976), though the efficiency of spontaneous virus induction seems to be significantly higher in the transformed muntjak cells. The mechanism of infectious SV40-induction has been studied using transformed Syrian hamster cell lines (Burns & Black, 1969; Kaplan et al. 1975a, Rakusanova et al. 1976; Zamansky et al. 1976). The high efficiency of V antigen induction in the transformed muntjak cells (about 40% of the cell population) by the mitomycin C treatment (Table 4) may provide a useful system to analyse the mechanism.

Recently, Zuna & Lehman (1977) observed a marked tendency to hypotetraploidy in SV40-transformed Chinese hamster embryo cells at an early passage after their establishment and indicated a possible involvement of a tetraploid intermediate as a necessary step in the transformation process. Cytogenetic analysis of the transformed muntjak cells revealed the following: (1) More than half of the transformed clones showed a diploid modal chromosome number and, in two of these clones, more than 70% of the cells were diploid; (2) no specific chromosome abnormalities were observed in common among the transformed clones; (3) about 30% of the clones had a tetraploid modal chromosome number, but diploid cells also accounted for more than 20% of the cell population; (4) the cultures showed a tendency to shift towards polyploidy upon repeated passages. The results indicate that the chromosomal change is not a necessary step in the process of transformation of Indian muntjak cells with SV40 and suggest that heteroploidy is a secondary event occurring as a result of cell transformation. It has also been suggested by Weinstein & Moorhead (1967) in human cells and Lavialle et al. (1975) in Chinese hamster cells that a chromosomal aberration is not a prerequisite for transformation with SV40.

The isolation of the clones of various transformed phenotypes will be required for the cytogenetic analysis of transformed cells to study the chromosomal location of the cellular gene(s) responsible for the expression or suppression of transformed phenotype. The genetic instability of SV40-transformed muntjak cells, as stated above, made it essential to establish a karyologically stable stem line for such analysis.

The diploid transformed muntjak cell lines may be useful for cytogenetic studies in various fields. Though they were karyologically unstable, the diploidy can be maintained by re-cloning (Fig. 1b, c). Cell lines transformed with avian and murine sarcoma virus have also been established from muntjak kidney cells (Yuasa et al. 1978; M. Hatanaka, personal communication).

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REFERENCES


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