Production of Recombinant Rat Viruses as a Method of Oncogene Isolation in Coculture Medium

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ABSTRACT

A simple oncogene isolation was proved using the SD1-T rat embryonic cell line. The SD1-T cell line, which releases endogenous rat leukemia virus, was cocultured with (a) normal rat kidney cells transformed by cloned v-mos DNA, (b) the rat mammary tumor cell line (63SP), or (c) normal rat kidney cells transformed by 63SP DNA. Within 1 mo, oncogenic viruses were recovered from all three coculture supernatants. During this period, increase of oncogenic transcripts was observed in the cocultured cells. The oncogenic viruses appeared to contain the mos gene in cocultures (a) and ras-related sequences in cocultures (b) and (c). The emergence of virus containing mos from mos DNA-mediated normal rat kidney transformants demonstrated "rescue" of the active cellular oncogene by the rat leukemia virus. This coculture system seems to facilitate "rescue" of oncogenes functioning in the tumor and transformed cells.

INTRODUCTION

Methods for isolation of cellular oncogenes from tumor cells have recently been developed. These methods mainly depend on focus formation of fibroblast cells after transfection of tumor cell DNA. Many ras-related cellular oncogenes [Ha-ras (1-4), Ki-ras (5-8), N-ras (8-11)] and several sequences other than ras (12, 13) have been isolated by NIH/3T3 transformation. However, only a few kinds of cellular oncogenes have been isolated by DNA transfection, compared with the variety of viral oncogenes isolated. So, viral systems for oncogene isolation must be reconsidered. Ki-MSV2 and Ha-MSV were obtained from sarcomas by the infection of rats with MuLV (14, 15). Moreover, AF-1 virus was isolated from sarcomas that were induced after several passages of F-MuLV in mice (16). These sarcoma viruses were considered to be due to the recombination between MuLV and endogenous ras oncogenes. A similar recombinational event between a cellular oncogene and nonpathogenic RSV has been reported (17). Rasheed et al. (18) reported production of RaSV by cocultivation of rat SD1-T cells (19) with 4NQ cells (a chemically induced carcinoma cell line). The RaSV was found to be a recombinant virus between RaLV and the ras oncogene in 4NQ DNA (20), but the precise mechanism of this recombination has not been elucidated. In this work, we obtained new recombinant viruses by the same coculture method (SD1-T and a tumor cell line) and observed the increase of oncogenic mRNA during the coculture period.

MATERIALS AND METHODS

Cell Lines. The cell lines used in this study were the Osborn-Mendel rat kidney cell line (NRK), Sprague-Dawley rat mammary tumor cell line (63SP), and Sprague-Dawley rat embryonic cell line (SD1-T), releasing RaLV. The cell lines used and developed in this study were maintained in Dulbecco's minimal essential medium supplemented with 10% FBS (GIBCO) under 5% CO2 in air at 37°C.

Transfusion of DNA into NRK Cells. Plasmid pLM-1 (cf. Fig. 1) and 63SP DNA were transfected into NRK cells essentially as described by Peruchó et al. (21). Inocula of 1 x 106 cells in 10-cm culture dishes were cultured for 16 h, refed with 9 ml of fresh medium, and cultured for an additional 8 h. A DNA calcium phosphate-coprecipitated suspension was prepared by introducing a solution of 240 mM CaCl2 containing each DNA into an equal volume of HEPE-S-buffered saline (42 mM HEPE-S-276 mM NaCl-14 mM NaHPO4) with bubbling. The mixture was then allowed to stand for 30 min at room temperature. Samples of 100 µg of 63SP DNA or 0.1 µg of pLM-1 plasmid DNA plus 20 µg of NRK cellular DNA were applied per dish. The cells were transferred to fresh medium 16 h after transformation. After additional culture for 24 h, the cells in each dish were distributed into 3 dishes and cultured in medium containing 5% FBS for 2 wk. The transformed cells, which became visible as actively growing foci, were cloned on soft agar, and cell lines were established. Five independently transfected cell lines each from 63SP DNA-transfected and v-mos DNA-transfected NRK foci were established and named TNRK-63D-1-5 and TNRK-mos-1-5, respectively.

Cocultivation and Virus Infection. SD1-T cells (1 x 106) were seeded with the same number of TNRK-63D-1, TNRK-mos-1, or 63SP cells into 10-cm dishes. The supernatants were collected on Days 3, 7, 14, 28, and 35 of cocultivation. The medium was changed every 3 days and on the day before each collection of the supernatant. The medium collected was centrifuged at 5000 rpm for 30 min, and the clear supernatant was used for infection experiments. No viable cells were observed in the supernatant (data not shown). NRK cells were inoculated at 3 x 104 cells per 6-cm dish, cultured for 24 h, and treated with the above supernatant diluted 10-fold in the presence of polybrene (8 µg/ml) at 37°C for 2 h with shaking every 15 min. Then the medium was replaced by fresh medium containing 5% FBS, and after culture for 10 days, the foci that formed were counted. Cocultivated cell lines from the individual foci were isolated as described above. Five transformed cell lines, each obtained by infection with the coculture supernatants of SD1-T with TNRK-63D-1, TNRK-mos-1, and with 63SP, were cloned and named TNRK-63DCI-1-5, TNRK-mosCl-1-5, and TNRK-Cl-1-5, respectively. The transformed cell lines obtained were tested for tumor-forming ability in nude mice. The coculture supernatants on Day 28 were also tested for ability to induce transformation after one of the following three treatments: 0.45-µm filtration; freeze-thawing; and heat inactivation at 65°C for 2 h.

Neutralization of the Coculture Supernatant by Viral Antiserum. The supernatants of NIH-Cl-57 (releasing F-MuLV) and SD1-T were centrifuged at 25,000 rpm in a Beckman SW28 centrifuge, and the pellets of F-MuLV and RaLV viruses were collected. These viruses were suspended in phosphate-buffered saline and injected into DDS mice 3 times with an interval of 1 wk between injections. Then the sera from immunized and nonimmunized mice were incubated with the coculture supernatants of 63SP, TNRK-63D-1, and TNRK-mos-1 with SD1-T for 2 h. The coculture supernatants treated with mouse sera were inoculated onto NRK cells, and virus infection was detected by counting the foci.
cells per 15-cm dish were cultured for 2 days before medium collection. From Day 3 of cultivation, the culture supernatant was collected at 6-h intervals for 3 days. Volumes of 30 liters of the supernatants from each virus-infected transformant, TNRK-C1-1, TNRK-mos-1, and TNRK-63DC1-1, were treated with polyethylene glycol- NaCl, and viruses were precipitated by centrifugation at 3000 rpm for 30 min. The viruses were dissolved in 100 mM Tris-100 mM NaCl-10 mM EDTA and centrifuged in a stepwise sucrase gradient (60, 35, and 20%) at 25,000 rpm for 3 h in a Beckman SW 28 rotor and then in a continuous sucrase gradient (60 to 20%) at 50,000 rpm for 4 h in a Beckman SW 50.1 rotor. The resulting virus band was withdrawn and centrifuged at 45,000 rpm in a 50 Ti rotor for 45 min, and then its RNA was extracted with a phenol/chloroform mixture. The RNA was purified by repeated precipitation with ethanol and stored at −80°C. Before use, the RNA was dissolved in 1 M glyoxal-50% dimethyl sulfate-10 mM sodium phosphate (pH 7.0) at 50°C for 1 h and separated by electrophoresis on 1.2% agarose gel in a sodium-phosphate buffer at pH 7.0. The gels were then blotted onto nitrocellulose filters. The filters were dried and hybridized with P-labeled Ra-ras, v-mos, or Rashed RaLV DNA probe, washed, and autoradiographed.

Cloning of the “Rescued” Oncogene. A sample of 200 µg of TNRK-C1-1 DNA was digested with EcoRI and subjected to electrophoresis on agarose gel. Part of the gel was then used for Southern hybridization analysis. With the Ra-ras probe, a “novel” ras-related sequence was detected as an additional autoradiographic band. The area of the agarose gel corresponding to the novel ras-related band was cut out and frozen at −20°C. The frozen gels were homogenized in 10 mM Tris-1 mM EDTA and stored for 6 h at room temperature. The supernatant of the homogenate was extracted twice with phenol, and the DNA was precipitated with ethyl alcohol. The DNA was redigested with BamHI and again probed with ras DNA, and the 4.8-kilobase fragment containing ras was reextracted. This fragment was inserted into the EcoRI-BamHI sites of pBR322 and transferred to Escherichia coli HB101. The ras-positive transformants were picked up individually by the colony hybridization procedure, and the cloned DNA fragment was analyzed (22).

RESULTS

Focus Formation of NRK Cells after Transfection of v-mos and 63SP DNA. The pLM-1 plasmid constructed (cf. Fig. 14), which contains v-mos and LTR of SFFV (23, 24) and 63SP DNA, was transfected into NRK cells. After 2 wk, transformed foci appeared (483/1 µg pLM1 DNA; 7100 µg 63SP DNA). Cells from these foci formed colonies on soft agar, while normal NRK cells did not. Cell lines were cloned from these isolated colonies and designated as TNRK-mos-1-5 and TNRK-63D-1-5, respectively. Southern hybridization analysis showed that newly appearing v-mos and Ra-ras sequences were present in these transformants and that the DNA integration sites differed in the different transformants. All the transformed cells formed tumors in nude mice (data not shown). The Southern blot hybridization patterns of TNRK-mos-1 and TNRK-63D-1 are presented in Fig. 1, B and C. These results showed that 63SP DNA contains an oncogene related to Ra-ras DNA and that NRK cells, like NIH/3T3 cells, are good recipient cells for use in oncogene research.

Appearance of Oncogenic Virus during Cocultivation. SD1-T cells were cocultured with TNRK-mos-1, TNRK-63D-1, or 63SP cells. The medium of these cocultures and cultures of each of the four cell lines singly was collected at intervals and inoculated into NRK cells. After culture for 12 days, the foci that had appeared were counted. No foci were obtained with medium of single cell lines, but after culture for over 2 wk, the media of all three cocultures showed focus-forming activity on NRK cells (Table 1). The focus-forming activity increased with the period of coculture, reaching a plateau after coculture for...
Table 1  Number of foci obtained after inoculation of coculture supernatant.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Coculture period (days)</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
<th>42</th>
<th>28*</th>
<th>28*</th>
<th>28f</th>
</tr>
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<tbody>
<tr>
<td>SD1-T</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>63SP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TNRK-63D-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TNRK-mos-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>63SP SD1-1</td>
<td>0</td>
<td>2.8 ± 1.2</td>
<td>7.1 ± 1.3</td>
<td>21.0 ± 3.3</td>
<td>24.6 ± 4.5</td>
<td>20.3 ± 4.9</td>
<td>16.9 ± 5.1</td>
<td>21.8 ± 3.1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNRK-63D-1 SD1-1</td>
<td>0</td>
<td>5.1 ± 3.0</td>
<td>6.9 ± 0.8</td>
<td>24.8 ± 3.9</td>
<td>23.9 ± 5.4</td>
<td>21.6 ± 3.8</td>
<td>19.1 ± 4.5</td>
<td>21.6 ± 4.2</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNRK-mos-1 SD1-1</td>
<td>0</td>
<td>3.2 ± 0.1</td>
<td>9.0 ± 3.9</td>
<td>35.1 ± 2.1</td>
<td>28.3 ± 4.5</td>
<td>29.3 ± 4.9</td>
<td>24.3 ± 3.5</td>
<td>23.6 ± 5.1</td>
<td>0</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Footnotes:

a Millipore filtration (0.45 μm).
* Freeze thawing.
' Heat treatment.
" Mean ± SE.

Table 2  Effect of treatment with viral antiserum on focus-forming activity.

<table>
<thead>
<tr>
<th>Treated supernatant</th>
<th>No. of foci</th>
<th>Anti-RaLV</th>
<th>Anti-F-MuLV, Normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>×1000*</td>
<td>×100</td>
<td>×10</td>
</tr>
<tr>
<td>63SP + SD1-T</td>
<td>3.3 ± 0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TNRK-63D-1 + SD1-T</td>
<td>3.6 ± 4.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TNRK-mos-1 + SD1-T</td>
<td>6.5 ± 3.7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Footnotes:

a Dilution for treatment.
* Mean ± SE.

about 1 mo. Transformed NRK cells were cloned on soft agar and named TNRK-mosCl-1-5, TNRK-63DCI-1-5, and TNRK-Cl-1-5, corresponding to the infected supernatants of cocultures. On Southern blot hybridization analysis, the DNAs of all the transformants gave bands of newly integrated v-mos- or Ra-ras-related sequences (some of the results are shown in Fig. 1C). All these cell lines also formed tumors in nude mice. The focus-forming activity of the culture medium was lost on heat treatment, but not filtration or freeze thawing (Table 1). The transforming activity of the coculture medium was inhibited by anti-RaLV serum prepared in DDD mice, but not by anti-F-MuLV serum (Table 2). These results indicate that the transformation activity that appeared in the coculture medium was due to oncogenic viruses and that RaLV served as a helper virus.

Increase of Oncogenic mRNA during Coculture. RNA was extracted from SD1-T, NRK, 63SP, TNRK-mos-1, TNRK-63D-1, TNRK-mosCl-1, TNRK-63DCI-1, and TNRK-Cl-1 and from the cocultured mixtures of cells and spotted on a nitrocellulose filter. Fig. 2B shows the hybridization of spots with the v-mos DNA probe. The results show that, during coculture of SD1-T with TNRK-mos-1, the mos RNA content per cell increased gradually for a month. TNRK-mos-1 also produced the same mos RNA, but SD1-T and NRK cells had no mos RNA. The pattern of increase was the same as that of the transforming activity in the coculture medium (cf. Table 2). TNRK-mosCl-1, a transformed NRK clone obtained by infection with the supernatant of a coculture of SD1-T and TNRK-mos-1, appeared to have a large amount of mos RNA, which corresponded to that in a 1-mo coculture of SD1-T with TNRK-mos-1 cells.

Spot hybridization analysis with the Ra-ras DNA probe gave similar results to those with the mos probe. On coculture of SD1-T with 63SP or with TNRK-63D-1 cells, the amount of Ra-ras RNA increased steadily for 1 mo, corresponding to the increase of the transcripts in these cocultures, and was the same as that of the transforming activity of the coculture medium on NRK cells (Fig. 2A; Table 2). The ras RNA content per cell was higher in 63SP and TNRK-63D-1 than in SD1-T or NRK cells. TNRK-63DCI-1 and TNRK-Cl-1 produced similar amounts of Ra-ras RNA to those in 1-mo cocultures of SD1-T with 63SP and with TNRK-63D-1, respectively. These increases of mos and Ra-ras transcripts during coculture indicate the active production of new oncogenic viruses.

Fig. 2. Spot hybridization of RNA. RNA was extracted from cocultured cells or cultures of single cell lines. In A, Ra-ras DNA was used as a probe. In B, mos DNA was used as a probe. +, cocultivated cells.
This cloned fragment could transform NRK cells by DNA transfection (data not shown).

**DISCUSSION**

On coculture of SD1-T with tumor cells we obtained new recombinant oncoviruses within 1 mo. The emergence of RaLV in only 1 mo suggested a high recombination efficiency of RaLV. The main advantage of this coculture method over the DNA transfection method is its simplicity: we could recover a ras-related oncovirus directly from the coculture of SD1-T with 63SP cells. In genomic DNA transfection it is thought to be difficult to isolate oncogenes with long interspersed intron sequences, and this may be one reason why only a few kinds of genes have been isolated by DNA-mediated cell transformation. Shimotono and Temin (26) showed that intron sequences in the retrovirus genome were eliminated within a few generations. Then it may be possible to "rescue" larger oncogenes using the coculture method. In addition, viral LTR should promote the expression of rescued oncoviruses, making it easier to obtain foci in recipient cells. We are now trying to rescue oncogenes by coculture of SD1-T with many kinds of tumor cell lines established from rat tumors in which unknown oncogenes operate.

**ACKNOWLEDGMENTS**

We thank Dr. Rasheed, professor, for gifts of SD1-T cells, Ra-ras clones, and RaLV DNA clones and for helpful discussions. We also thank Dr. Matsugi for injecting transformed cells into nude mice and Dr. Soma for construction of the pLM-1 plasmid.

**REFERENCES**

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