Selective Replication of Transformation-defective Avian Sarcoma Virus Mutants in Duck Embryo Fibroblasts

By MISUZU I. SHIMAKAGE, TOSHIO KAMAHORA, AKIRA HAKURA AND KUMAO TOYOSHIMA

Department of Tumor Viruses, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita City, Osaka, Japan

(Accepted 30 March 1979)

SUMMARY

When an avian sarcoma virus (ASV), subgroup C Bratislava 77 (B77-C) was inoculated into duck embryo fibroblast cultures (DEF) at a m.o.i. of 0.02, its replication was retarded by about 3 days compared with that in chick embryo fibroblast cultures (CEF). A transformation-defective (td) mutant was isolated during this period of retardation. Unlike the sarcoma virus, this td mutant replicated in both DEF and CEF with no retardation, even at a low m.o.i. The subgroup C Prague strain of Rous sarcoma virus (PR-C), which can infect DEF, also replicated in DEF slower than its td mutant, tdPR-C, at a m.o.i. of 0.02.

INTRODUCTION

We have studied the genetics of ASV, but our system was complicated by the admixture of a recombinant between sarcoma virus and endogenous retrovirus (Ando & Toyoshima, 1976; Toyoshima et al. 1976). DEF, in which no endogenous retrovirus genome was found by hybridization with avian leukemia virus (Varmus et al. 1973; Varmus et al. 1974; Shoyab & Baluda, 1975; Tereba et al. 1975; Stavnezer et al. 1976); would therefore be useful for genetic studies on ASV. Accordingly we have started genetic studies on ASV using DEF as host cells and ASV strains of B77-C and PR-C, which are infectious for both CEF and DEF (Duff & Vogt, 1969). These systems have been studied by several groups and there are reports that most transformed clones on DEF produced virus in which only the smaller b subunit of genomic RNA could be detected (Stone et al. 1975) and that B77 showed spontaneous mutations in plating efficiency on DEF (Zarling & Temin, 1976; Zarling et al. 1977). Little has been reported, however, about virus replication in DEF.

We intended to obtain virus clones with high plating efficiency on DEF for genetic studies on ASV but during this work we found that the growth of sarcoma viruses in DEF was retarded compared with their growth in CEF, whereas their td mutants grew in DEF as efficiently as in CEF. This paper describes differences in the replicating capacity of sarcoma viruses and their td mutants.

METHODS

Cell culture. Fertile chicken eggs were obtained from the Research Foundation for Microbial Diseases of Osaka University and primary cultures of fibroblasts were prepared from 11-day-old embryos by the standard technique (Vogt, 1969). The chicken embryos used were C/BE or C/O and were negative for avian leukemia virus, chick helper factor and group specific antigens of avian leukemia virus. Some fertile eggs of the Osaka duck were produced in our laboratory and others were obtained from commercial farms. Primary cultures of
Table 1. Efficiency of transformation of B77-C viruses re-cloned on DEF

<table>
<thead>
<tr>
<th>Virus clone</th>
<th>f.f.u./ml on DEF</th>
<th>f.f.u./ml on CEF</th>
<th>Ratio of DEF/CEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-1</td>
<td>$3.3 \times 10^6$</td>
<td>$2.4 \times 10^6$</td>
<td>1.38</td>
</tr>
<tr>
<td>2</td>
<td>$5.5 \times 10^6$</td>
<td>$7.0 \times 10^5$</td>
<td>0.79</td>
</tr>
<tr>
<td>3</td>
<td>$4.2 \times 10^6$</td>
<td>$2.0 \times 10^5$</td>
<td>2.10</td>
</tr>
<tr>
<td>4</td>
<td>$4.6 \times 10^6$</td>
<td>$3.9 \times 10^5$</td>
<td>1.18</td>
</tr>
<tr>
<td>5</td>
<td>$3.4 \times 10^5$</td>
<td>$4.2 \times 10^5$</td>
<td>0.81</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>1.27</td>
</tr>
</tbody>
</table>

DEF were prepared from 12- to 14-day old embryos and were D/ABDE and D/ABE. All cultures were incubated at 39 °C.

**Viruses and cloning.** B77-C virus, obtained from Dr P. K. Vogt, was originally isolated from a virus-producing rat tumour cell line. B77-C was cloned in CEF or DEF by the standard procedure (Vogt, 1969), except that foci were picked up 8 to 10 days p.i. Standard *tdB77-C* (*tdB77*), originally isolated by Toyoshima et al. (1970), has been passaged only in CEF. The original PR-C was obtained from Dr P. K. Vogt and has been passaged many times in CEF in our laboratory as regular PR-C stock. Another stock was prepared from a lower passage level of this PR-C. Transforming viruses were assayed by focus formation and the titres were expressed as focus-forming units (f.f.u.)/ml.

**Assay for *td* mutants.** Interfering capacity was determined on CEF by the standard technique after three successive transfers (Rubin & Vogt, 1962) and titres were expressed as interfering units (IU)/ml.

**Gel electrophoresis.** The size of virus RNA was determined by the standard technique (Duesberg & Vogt, 1973) with some modifications.

**Results**

**Isolation of B77-C clones which transform DEF as efficiently as CEF**

The efficiency of transformation (e.o.t.) of the original B77-C on DEF was one-third of that on CEF and production of sarcoma virus was about ten times less in DEF than in CEF. But since B77-C clones isolated on DEF generally showed higher e.o.t. on DEF than the original stock, we repeated cloning on DEF to select clones showing e.o.t. ratios on DEF and CEF of about 1 (Table 1). One of these clones, D1, showed similar e.o.t. on DEF from eight different embryos.

**Growth rates of B77-C clones isolated on DEF**

The growth rates of clone D1 on DEF and CEF were tested (Fig. 1). When CEF and DEF were infected with D1 at a m.o.i. of 2, the growth curves of the transforming virus were indistinguishable (Fig. 1a). However, at a m.o.i. of 0.02, virus production in DEF was retarded about 3 days compared with that in CEF (Fig. 1b). Since the e.o.t. values of the virus on CEF and DEF were nearly equal, the growth curves assayed on CEF and DEF were directly comparable.

Other clones isolated on DEF were also tested at small inoculum size in DEF. The initial parts of the growth curves were somewhat different in each experiment and decrease in the growth curves at 3 to 4 days after infection was observed in some experiments. However, retardation of virus production was observed in all experiments without exception.

The amount of cell associated virus in DEF of Fig. 1(b) did not exceed that of the culture fluid (data not shown), excluding the possibility of increase of cell associated virus by trapping of virions during the period of retardation.
Selective replication of ASV td mutants

Fig. 1. Growth of D1 in CEF and DEF at m.o.i. of 2(a) and 0.02(b). Virus was inoculated on to 1 x 10^6 cells/6 cm plastic plate. After 16 h of adsorption, the cultures were washed and re-fed with medium containing 1% DMSO. Medium was changed every 2 days. DEF, ○—○; CEF, △—△.

Isolation of a td mutant

No td mutant could be isolated from the stock of D1 by the end-point dilution method in CEF. Therefore, viruses grown in CEF and DEF were labelled with 3H-uridine and their RNA size was examined. Most virions in both preparations contained the a subunit as their major component. These results suggested that this stock did not contain a higher level of td than transforming virus.

Next we examined the possibility that the td mutant was replicated selectively in earlier stages of replication in DEF when growth of sarcoma virus was restricted. In DEF infected with D1 at a m.o.i. of 0.02 (Fig. 1 b), no excess td mutant was detected on the first or second day by interference test but on the third and fourth days, the production of td mutants was 10 to 100 times greater than that of sarcoma virus. After the fifth day, sarcoma virus production increased rapidly and exceeded production of the td mutant. Then we isolated td mutant from the virus stock of the third day by the end-point dilution method.

Growth of the td mutant

Growth of the td mutant isolated from clone D1 in the above experiment was tested on both DEF and CEF (Fig. 2). Virus production of the td mutant at a m.o.i. of 0.02 increased rapidly as early as 2 days p.i. and continued to increase without retardation. Replication of the td mutant, unlike that of the sarcoma virus, was the same in DEF as in CEF.

The growth rate of tdB77, which had been isolated and passaged only in CEF, was tested in CEF and DEF to see the effect of passage history on growth. In addition, three dilutions were used to test the dependency of growth on the inoculum size (Fig. 3). TdB77 started to grow in DEF after a short incubation period and its titre increased rapidly at all inoculum sizes, although the titre at a m.o.i. of 0.001 remained 1 log below the titres shown at higher multiplicities. This pattern of virus growth in DEF was similar to that in CEF as observed in the case of the td mutant isolated on DEF. Therefore, the passage history did not seem to have any noticeable influence on the growth pattern of td mutant.

As shown in Fig. 1(a), replication of sarcoma virus in DEF was much better at the higher
Fig. 2. Growth of the td mutant of DI isolated on DEF. The clone D1 was inoculated at a m.o.i. of about 0.02 on to DEF (○—○) or CEF (△—△). Medium was changed as for Fig. 1.

Fig. 3. Growth of CEF-passaged tdB77 at various multiplicities in DEF. TdB77 was inoculated at a m.o.i. of about 0.1 (□—□), 0.02 (●—●), 0.001 (■—■), respectively. Growth of this tdB77 at a m.o.i. of about 0.02 in CEF is shown in the same figure (△—△). Conditions of growth were as for Figs. 1 and 2.

m.o.i. of 2, although only some of the cells were doubly infected under these conditions. To investigate whether co-infection with the td mutant facilitated growth of the sarcoma virus, DEF were inoculated with B77-C at a m.o.i. of 0.02 and after 100 min cells were superinfected with tdB77 at a m.o.i. of 0.001, 0.1 or 1.0. No evidence of facilitation of growth of sarcoma virus was detected at any infectivity of tdB77 tested.

**Isolation of tdPR-C**

When B77-C was inoculated into DEF at a low m.o.i., production of sarcoma virus was slower than in CEF, but when td mutants were inoculated into DEF at the same m.o.i., they replicated as rapidly as in CEF. To examine whether this phenomenon was common to other avian sarcoma viruses that could infect DEF, we examined the growths of PR-C and its td mutant in CEF and DEF.

The e.o.t. of the regular PR-C stock on DEF was less than one hundredth of that on CEF. The CEF and DEF cultures, which did not have any foci, were transferred three times and then challenged with PR-C to test for the presence of interfering virus. Td mutants were isolated from DEF cultures, but no interfering virus was isolated from CEF cultures. This tdPR-C was so called 'long td' (Yoshida & Ikawa, 1977) in RNA size, measured by polyacrylamide gel electrophoresis (Fig. 4). The e.o.t. on DEF of this PR-C stock was considerably lower than those reported previously (Friis, 1973; Fujita *et al.* 1974; Popović *et al.* 1977) and those used in the next section. Although the reason for the low e.o.t. of this one stock is unclear, it may be due in part to long undiluted passages in CEF.
Selective replication of ASV td mutants

Fig. 4. Polyacrylamide gel electrophoresis of heat-dissociated 60 to 70S RNA of tdPR-C. (a) Heat-dissociated RNAs of $^{32}$P-tdPR-C (●) and $^3$H-TY9 (○) as a long td marker (Yoshida & Ikawa, 1977); (b) heat-dissociated RNAs of $^{32}$P-tdPR-C (●) and $^3$H-tdB77 (○) as a standard td marker, were subjected to electrophoresis in 2% polyacrylamide gel for 6 h at 100 V (Duesberg & Vogt, 1973).

Fig. 5. (a) Growth of PR-C and (b) growth of tdPR-C at a m.o.i. of 0.02 in DEF (○—○), and CEF (△—△). Conditions were as for Figs. 1 and 2.

Growth of PR-C and tdPR-C

In this experiment a sarcoma virus clone with a short history of CEF passage was used; it had been isolated on CEF from the second stock of PR-C and then kept in the frozen state. The e.o.t. ratio of this clone on DEF and CEF was about one third. The tdPR-C used had been isolated on DEF in the preceding experiment. DEF and CEF were infected with PR-C at a m.o.i. of 0.02, determined by assay on the respective cells; that is, the vol. inoculated into DEF was three times that inoculated into CEF (Fig. 5). Sarcoma virus production was somewhat slower in DEF than in CEF and the final yield on virus was also lower in DEF than in CEF. When DEF was infected with tdPR-C at a m.o.i. of 0.02
(Fig. 5b), progeny virus appeared after a short latent period, grew rapidly and reached a higher titre than that of the sarcoma virus (Fig. 5a).

DISCUSSION

With both B77-C strain and PR-C strain at low m.o.i., sarcoma virus production in DEF was lower the first few days p.i. than that in CEF, but td mutants isolated from these sarcoma virus strains grew rapidly in both DEF and CEF. When a clone of B77-C was inoculated into DEF at a m.o.i. of 2, virus production was similar to that of CEF. There are various possible explanations for this difference in growth at a m.o.i. of 2: the proportion of cells which received multiple infection of sarcoma virus at a m.o.i. of 2 would be 0.59 and that at a m.o.i. of 0.02 would be 0.0002. Therefore, if duck cells infected with two or more sarcoma virions at a m.o.i. of 2 produced more progeny virus at an early stage of infection, this might result in a significant difference in the growth curve from that at a m.o.i. of 0.02. A second possibility is that when cells are infected with sarcoma virus they may facilitate virus production in neighbouring infected cells and when the proportion of infected cells is high this influence may be appreciable. A third possibility is that although we failed to demonstrate helper function of td mutants on sarcoma virus production in DEF, some other defective particles may promote replication of the sarcoma virus.

Stone et al. (1975) reported that the proportion of a and b subunits of virus RNA of B77 did not change significantly during passage on DEF, but that most transformed clones on DEF produced virus in which only b subunits could be detected. We found that B77-C cloned on DEF contained a subunit RNA as the major component and we failed to isolate the td mutant by focus cloning on DEF. TdB77-C, which existed in cloned virus as a minor component, was isolated due to its faster replication in DEF than B77-C in the early stage of infection. TdPR-C was obtained by limiting dilution on DEF. Isolation of tdPR-C may have been due to the poor plating efficiency of the original PR-C on DEF, or to the good growth of tdPR-C on DEF or to both these factors. Selection in DEF will be applicable for isolation of td mutants of other ASV.

The difference in the growth patterns of sarcoma virus in DEF and CEF suggests that there may be some difference between the cellular mechanisms for control of production of sarcoma viruses and Td mutants. Recently Neiman et al. (1978) reported the difference between replicating capacities of sarcoma virus and td virus in a chicken lymphoid cell line MSB-1 and suggested that the difference might occur before integration of provirus DNA. We obtained a B77-C clone completely adapted to DEF that showed the same e.o.t. on CEF and DEF and grew as well in DEF as in CEF even at a low m.o.i., and from this clone we isolated a stable host range mutant that has different e.o.t. on CEF and DEF (M. I. Shimakage et al. unpublished data). More host range mutants are required, however, to study the difference in the cellular mechanisms of control of ASV and their td mutants.

We thank Dr Mitsuaki Yoshida for much helpful advice regarding polyacrylamide gel electrophoresis. We are also grateful to the members of the Research Foundation for Microbial Diseases of Osaka University for their generous help in obtaining fertile eggs of known genetic constitution free of avian leukemia virus. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES


Selective replication of ASV td mutants


(Received 29 December 1978)