Isolation and Characterization of Host Range Mutants of Avian Sarcoma Virus

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SUMMARY

Two host range mutants of avian sarcoma virus (ASV) were isolated from a clone of the Bratislava 77 strain, subgroup C (B77-C). An HR- mutant was obtained by adaptation to duck cells and showed almost the same efficiencies of transformation (e.o.t.) and growth in cultures of chick embryo fibroblasts (CEF) and duck embryo fibroblasts (DEF). An HR+ mutant showed lower e.o.t. and slower growth on DEF than on CEF. Characterization of these mutants and wild-type B77-C showed that (i) unlike the host range mutants reported previously, these mutants and wild-type B77-C have common antigenicity, (ii) the number of infective centres is almost the same for the two mutants and the wild-type, and so the adsorption and integration of these viruses are similar, and (iii) the content of viral RNA in DEF infected with HR- is increased and decreased in the case of HR+ compared with the wild-type.

INTRODUCTION

Duck embryo fibroblasts (DEF) seem useful for studying the genetics of avian sarcoma virus (ASV), since they contain no detectable endogenous viral genome that can hybridize with avian leukosis virus (Varmus et al., 1973, 1974; Tereba et al., 1975; Stavnezer et al., 1976). However, one problem found when using DEF is that the efficiency of infection by ASV is low (Duff & Vogt, 1969; Altaner & Temin, 1970; Shimakage et al., 1979). Mutants with higher efficiencies of infection on DEF have been reported, but these mutants are very unstable (Zarling & Temin, 1976; Zarling et al., 1977). Restriction of ASV replication in avian cell cultures other than chick embryo fibroblasts (CEF) (Neiman et al., 1978; Shimakage et al., 1979) and restricted expression of the ASV genome in mammalian cells (Graf & Friis, 1973; Graf & Beug, 1976; Kryzek et al., 1977, 1979; Quintrell et al., 1980) have also been reported. However, little is known about the mechanism of restriction of ASV replication in DEF. One approach to elucidation of the mechanism is isolation of host range mutants on duck cells.

In this paper, we report the isolation of two duck cell-dependent mutants and show that the level of viral RNA synthesis may determine these host range dependencies.

METHODS

Cells. 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/O or C/BE, which were negative for avian leukosis virus, chick helper factor and group-specific antigens of avian leukosis virus. Some fertile eggs of Osaka ducks were obtained in our laboratory and others were obtained from commercial farms. Primary cultures of DEF were prepared from 12-day-old embryos and were D/ABDE or D/ABE. All cultures were incubated at 39 °C in Medium 199 containing 10%, tryptose-phosphate broth, 5% calf serum, 0.5% chicken serum and 1 µg/ml aphomycin B.

Viruses. B77-C virus, obtained from Dr P. K. Vogt (University of Southern California, School of Medicine), 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 at 8 to 10 days after infection. Transforming viruses were assayed by focus formation (Vogt, 1969) and titres were expressed as focus-forming units (f.f.u.)/ml.

**Mutagenesis.** DEF cells in 9-cm dishes were infected with the cloned virus (HR⁻) and when production of virus became sufficiently high, the cultures were treated with 5-azacytidine (5-AC; 1 μg/ml) for 24 h. This treatment reduced virus production to 1/100th of that of the control.

**Antisera.** Chicken antisera to B77-C wild-type (wt) and mutants were prepared in 6-month-old White Leghorn chickens raised in our laboratory. The chickens had no detectable neutralizing antibodies to B77-C viruses before immunization. They were initially inoculated with 0.1 ml of virus material, partially inactivated with u.v. light (approx. 10¹ to 10² f.f.u./ml), and 9 weeks later were challenged with 0.1 ml of the original virus (approx. 10⁵ to 10⁶ f.f.u./ml). Three weeks after the last injection, the chickens were bled. Antisera and normal chicken sera were inactivated for 30 min at 56 °C before use. Neutralization tests were performed at 37 °C for 40 min.

**Infective centre assay.** Infective centre assays were carried out as described by Weiss et al. (1973) with some modifications. Chick and duck cells (1 x 10⁶ cells/5 cm dish) were infected with viruses at an m.o.i. of 0.005 and incubated at 39 °C for the indicated times. The cultures were treated with 10 μg/ml mitomycin C for 2 h at 37 °C. After washing twice with phosphate-buffered saline (PBS) cells were trypsinized, serially diluted and seeded onto the indicator monolayers (8 x 10⁵ cells/5 cm dish). The cultures were overlaid 2 h later with agar-containing medium.

**Preparation of cDNA.** cDNA were prepared with SR-RSV-D RNA as described previously (Stehelin et al., 1976) with some modifications. Concentrated virions were incubated at 40 °C for 12 h in 0.5 ml of reaction mixture (0.4 mM each of dGTP, dATP and dCTP, 10 μM-[3H]TTP (200 μCi), 8 mM-MgCl₂, 100 μg/ml actinomycin D, 10 mM-dithiothreitol, 0.02% Triton X-100, 8 mM-Tris-HCl pH 7.8). After addition of 0.5 ml TNE buffer (10 mM-Tris-HCl, 0.1 mM- NaCl, 1 mM-EDTA, pH 7.4) containing 20 mM-EDTA, 1% SDS, 100 μg yeast RNA and 200 μg/ml proteinase K, the reaction mixture was incubated at 37 °C for 1 h. The DNA product was extracted twice with phenol saturated with TNE buffer and once with chloroform–isoamyl alcohol (24:1, v/v), and precipitated with 2 vol. ethanol. The ethanol precipitate was treated with 3 m-NaOH at 37 °C for 2 h, neutralized and precipitated with 50 μg yeast RNA and 2 vol. ethanol.

**Preparation of cellular RNA.** Cells were infected with virus at a multiplicity of infection (m.o.i.) of 0.005 and 5 days later the cell monolayers were washed twice with TNE buffer. After addition of 1 ml TNE buffer to each 9 cm dish, cells were mixed with 200 μg/ml proteinase K and 0.5% SDS and passed three times through a 20-gauge needle. After incubation at 37 °C for 45 min, the mixtures were extracted twice with phenol and once with chloroform–isoamyl alcohol (24:1, v/v), and precipitated with 2 vol. ethanol (Hayward, 1977).

**RNA–DNA hybridization.** 3H-labelled cDNA (700 ct/min) was hybridized to the cellular RNA in 50 μl of reaction mixture (0.6 M-NaCl, 2 mM-EDTA, 50 mM-Tris-HCl pH 7.4, 0.2% SDS) at 68 °C for 4 to 145 h. Then, 0.5 ml of buffer (0.1 M-sodium acetate pH 4.5, 0.7 M-NaCl, 3.6 mM-ZnCl₂), 0.1 ml of S1 nuclease (2000 units/ml), 0.01 ml of denatured calf thymus DNA (1 mg/ml) and 0.34 ml H₂O were added and the sample solution was incubated at 37 °C for 2 h. The hybrids were precipitated with 10% trichloroacetic acid and collected on cellulose nitrate filter paper and radioactivity was counted.

**RESULTS**

**Isolation of B77-C clones that transformed DEF and produced virus on DEF as efficiently as on CEF**

Cloned wt B77-C can transform DEF, but its efficiency is lower than with CEF (Altaner & Temin, 1976; Shimakage et al., 1979) and virus production by DEF was also restricted (Fig. 1). To isolate a B77-C mutant that showed higher e.o.t. and better virus replication in DEF, we repeatedly cloned the virus on DEF and selected clones with similar e.o.t. on DEF and CEF. Even these selected clones grew less well in DEF, taking about 3 days longer at a low m.o.i. than in CEF (Shimakage et al., 1979). Accordingly, we repeated rapid passage of these clones in DEF to select a transforming virus that grew rapidly in DEF. After 11 passages and two further clonings, one clone, HR⁻, was found to grow faster in DEF than the original clone, even at low m.o.i. (Fig. 2).

We next examined the stability of the transforming ability of this clone on DEF. During three successive passages of HR⁻ on CEF, the ratios of e.o.t. on DEF and CEF were between 1.07 and 0.92. Thus, clone HR⁻ was stable, unlike the clones isolated by Zarling & Temin (1976). We used this clone as an HR⁻ mutant.
**Host range mutants of ASV**

**Isolation of a B77-C mutant that transformed DEF and produced virus less effectively in DEF than in CEF**

We tried to isolate a host range B77-C mutant that showed DEF-dependent inefficiency with regard to e.o.t. and virus production (HR⁺ mutant). Mutagenized HR⁻ virus stocks were prepared as described in Methods. Five-hundred clones were isolated from plates that showed 60 foci or less and their transforming ability was tested in DEF. Clones that showed a lower e.o.t. on DEF than on CEF were recloned and propagated on DEF. Their e.o.t. and virus production on CEF and DEF were reexamined. In this way an HR⁺ clone was selected as a restricted mutant. This mutant was tested with DEF and CEF prepared from 10 different embryos. The DEF/CEF ratio of e.o.t. of the HR⁺ mutant was $(2.76 \pm 1.93) \times 10^{-2}$, that of HR⁻ was $(7.23 \pm 0.31) \times 10^{-1}$ and that of the wild-type was $(2.50 \pm 0.77) \times 10^{-1}$. Multiplication of HR⁺ in DEF was much slower than that in CEF, and the maximum yield in DEF was 100 times less than in CEF (Fig. 3). In our experimental conditions, each growth curve (Fig. 1, 2 and 3) was reproducible when showing infection with these viruses at low m.o.i. (0.003 to 0.02).

Because DEF, infected with the wt B77-C which showed slow growth in DEF, produced more transformation-defective (td) mutants than sarcoma-inducing virus during the first few days of infection (Shimakage et al., 1979), the culture fluid of DEF infected with the HR⁺ clone was examined for the presence of excess td mutants during the first week of infection. However, no excess production of td mutants could be detected. The virus passaged twice in CEF showed the same host range specificity as HR⁺, suggesting that this host range specificity was stable.

**Common antigenicity of these mutants and B77-C**

These mutants belong to subgroup C, because the mutants HR⁻ and HR⁺ could infect C/BE CEF as well as C/O CEF, and interfered with subgroup C avian leukosis viruses. Since the host range of ASV is determined by its envelope glycoproteins, which give rise to subgroup-specific antigenicities, we prepared antisera to both mutants and the wt B77-C to detect minor antigenic differences. No differences in the antigenicities of these three viruses could be detected by neutralization tests (Table 1).
Table 1. Neutralization test

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Virus</th>
<th>wt</th>
<th>HR-</th>
<th>HR+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-wt</td>
<td>120*</td>
<td>160</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Anti-HR-</td>
<td>300</td>
<td>100</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Anti-HR+</td>
<td>80</td>
<td>84</td>
<td>160</td>
<td></td>
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</tbody>
</table>

* Dilutions of antisera that reduced the infectivity of each virus by 50%. Values are average dilutions in three independent tests.

Table 2. Infective centres of wt, HR- and HR**

<table>
<thead>
<tr>
<th>Treatment with mitomycin C after infection</th>
</tr>
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<tbody>
<tr>
<td>24 h</td>
</tr>
<tr>
<td>Virus</td>
</tr>
<tr>
<td>wt</td>
</tr>
<tr>
<td>HR-</td>
</tr>
<tr>
<td>HR+</td>
</tr>
</tbody>
</table>

* Viruses were inoculated as described in Methods. Infected cells were treated with mitomycin C for 2 h at 24 h and 5 days after infection. Infective centres are expressed as f.f.u./5 cm dish.

Infections with HR+ and HR-

We examined the e.o.t. and viral growth curves of these mutants in the presence of various concentrations (1 to 10 μg/ml) of polybrene and with u.v.-inactivated Sendai virus (1000 to 5000 haemagglutinating units/5 cm dish) to facilitate penetration of the HR+ mutant, but no enhancement was observed with HR+ on DEF (data not shown). These results suggest that the host dependencies of the HR+ and HR- mutants on DEF were determined subsequent to adsorption. We also examined whether the level of virus fixation varied in different mutants, and therefore carried out infective centre assays with these viruses using CEF as indicator cells (Table 2). The results of three independent experiments showed that the infective centres at 24 h after infection were somewhat less on DEF than on CEF but that this DEF/CEF ratio was similar for HR-, HR+ and the original B77-C. Thus, we conclude that the reverse transcription and integration at these times did not differ in these viruses.
Host range mutants of ASV

Viral RNA levels of mutants in infected cells

The levels of viral RNA in cells infected with HR−, HR+ and B77-C were estimated by hybridization with [3H]cDNA to SR-RSV-D RNA. CEF and DEF were infected with the virus stocks at an m.o.i. of 0.005 and incubated at 39 °C. After 5 days, the cellular RNA was extracted and hybridized as described in Methods and the culture fluids were assayed for virus titres on CEF. The results are shown in Fig. 4. The relative C,t (concentration of RNA × time of incubation) of DEF/CEF was about 1/2 with HR−, 10−1 with wt B77-C and 10−3 or less with HR+. These data are compatible with the results of virus titrations carried out at the same time.

In this experiment we used a low m.o.i.; thus, secondary infection may have occurred. Therefore, we estimated the number of virus-producing cells by infective centre assay 5 days after infection (Table 2). There was no significant difference in the proportion of virus-producing cells in DEF and CEF cultures. Therefore, at least part of the differences in virus production and efficiency of transformation was due to the difference in the content of viral RNA.

DISCUSSION

In this paper we report the isolation of two stable host range mutants, HR+ and HR−, from wt B77-C. Although DEF-dependent mutants of B77 ASV have been reported (Zarling & Temin, 1976), they were unstable spontaneous mutants and the mutations affecting host range involved changes of envelope antigenicity (Zarling et al., 1977). Our mutants differed from those of Zarling and Temin in that they were stable and showed common antigenicity to that of the parent B77-C. Analysis of these mutants indicated that different levels of viral RNA were responsible for differences in virus production and e.o.t.

There are several reports on cellular control of the process of ASV replication: Neiman et al. (1978) reported that ASV DNA could not be integrated normally into chicken lymphoid cells; Deng et al. (1974) and other authors (Deng et al., 1977; Quintrell et al., 1980; Chiswell et al., 1982) reported that mammalian cells modulated the expression of integrated ASV genomes by regulating the production of viral mRNA. We have also reported that CEF produced avian sarcoma viruses as efficiently as td viruses, but that DEF produced sarcoma viruses less efficiently than td viruses early after infection at low m.o.i. (Shimakage et al., 1979). These data suggest that there are some differences in the cellular controlling systems of different avian cells or of those of avian cells compared with mammalian cells, which affect the course of replication.
of BS7-C and other ASVs. The present results indicate that one difference might reside in the process of transcription. We can not, however, exclude the possibility of differential stability of viral RNA in CEF and DEF.

Further characterization of host range mutants and of the permissiveness of DEF for replication of ASV and its td mutants should be useful in elucidating cellular controlling mechanisms in virus replication.

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REFERENCES


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