Characteristics of a Retrovirus Associated with a Hamster Melanoma

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SUMMARY

The continuous culture of a hamster melanoma cell line has led to the spontaneous appearance of a retrovirus (HaRV) with typical type-C characteristics. The virus differs from all other known hamster viruses in its ability to transform murine as well as rat and hamster cells with apparent one-hit kinetics. Guinea pig, human and feline cells were not transformed although reverse transcriptase activity was detected in the supernatant from infected human cells. HaRV-transformed hamster embryo cells produced solid tumours (all non-pigmented) in 4 out of 35 animals when injected into hamsters while HaRV-transformed murine cells produced no tumours in mice. Injection of HaRV alone in hamsters, mice and rabbits did not induce tumours. HaRV possesses a 70S RNA which dissociates to 35S in DMSO and has a reverse transcriptase which utilizes the 70S virus RNA as a template. The size, morphology and density (1.15 g/ml) are similar to other known type-C viruses. Polyacrylamide gel electrophoresis indicates the presence of polypeptides analogous to those found in other type-C viruses.

INTRODUCTION

Retroviruses have been isolated from a wide variety of mammalian species (Aaronson & Stephenson, 1976) and many of these isolates are known to be oncogenic (Huebner et al. 1970). There has been considerable speculation as to the nature of the relationship between these viruses and their hosts.

Several reports of the isolation of type-C like viruses from hamster cells have appeared including the hamster leukaemia virus (HaLV), the hamster sarcoma virus (HaSV) and the D-9 virus (Bassin et al. 1968; Kelloff et al. 1970; Somers et al. 1973). These viruses have been reported to have little or no endogenous reverse transcriptase activity and to be unable to transform murine cells (Kelloff et al. 1970; Verma et al. 1974).

The hamster retrovirus (HaRV) which is the subject of this study, is produced spontaneously by pigmented cells (Y-22) established from a naturally occurring hamster melanoma. Since virtually all reports of hamster viruses are concerned with the HaLV-HaSV hamster–murine pseudotypes, there is very little data in the literature concerning native hamster type-C viruses. We present here certain characteristics of HaRV which show it to be distinct from other reported hamster type-C viruses although possessing properties in common with other mammalian type-C viruses. Additional properties of the HaRV polymerase which further distinguish HaRV from other hamster viruses are presented in the accompanying paper (Gregerson et al. 1979).

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Methods

Cells. HaRV appeared spontaneously after 104 passages of a line of pigmented cells (Y-22) derived from the Greene hamster melanoma found in a Syrian hamster. The Y-22 cells, which continue to produce virus, are grown as monolayers in RPMI 1640 with 5 to 10% foetal calf serum and antibiotics. Ten millilitres of culture supernatant produced total poly(rA).oligo (dT) reverse transcriptase activity of 2.5 nmol dTMP/h.

Virus preparation. Culture supernatants were removed, clarified by low-speed centrifugation and spun at high speed to pellet the virus which was resuspended in HNG [25 mM-HEPES, pH 7.4, 150 mM-NaCl, 5 mM-DTT, 50% (w/v) glycerol] and stored at −15 °C. This virus preparation retains >90% of its poly(rA).oligo (dT) reverse transcriptase activity for at least 6 months. Highly purified virus was obtained by isopycnic centrifugation in 30 to 80% glycerol gradients followed by passage through a porous glass bead column (Darling et al. 1977) and subsequent re-banding of the virus in another glycerol gradient.

Electron microscopy. Cultured cells were gently pelleted and fixed with either Dalton's chrome osmium for 1 h or 3% glutaraldehyde in phosphate buffer (pH 7.4) for 1 h followed by chrome osmium. The pellet material was dehydrated, embedded in an Epon–Araldite mixture (Luft, 1961), ultrathin-sectioned and stained in uranyl acetate followed by lead citrate (Reynolds, 1963).

Cellular transformation studies. To assess the ability of HaRV to transform cells, the focus formation assay of Aaronson & Weaver (1971) was performed on several cell lines including hamster embryo cells, guinea pig cells (primary culture), normal rat kidney cells (primary), NIH/ Swiss mouse 3T3, Balb mouse 3T3, rat embryo fibroblasts and human skin fibroblasts. After 1 week the cells were methanol-fixed, dried and stained. The number of foci per Petri dish was compared with the reverse transcriptase activity obtained in a polymerase assay of the virus stock used for infections. Virus for infections was passed through a 0.22 μm filter.

Oncogenicity studies. Hamster and murine cells were infected with HaRV as described earlier, cultured for 3 weeks and 5 × 10^6 cells injected subcutaneously into the shoulders of Syrian hamsters, or NIH/Swiss mice and Balb mice, respectively. Mock-infected cells were used as controls. The resultant tumours were examined by electron microscopy.

Several concentrations of HaRV alone were tested directly for tumorigenicity by subcutaneous injections in buffered saline into hamsters and mice, both new-born and young adult.

Polymerase assay procedure. Poly(rA).oligo (dT) reactions were performed in microculture plates in a total vol. of 130 μl. Samples of virus, 10 μl, were mixed with 20 μl of TNDN [50 mM-tris-HCl, pH 8.3; 60 mM-NaCl; 20 mM-DTT; 0.5% (v/v) NP-40] and incubated for 15 min at 4 °C. Fifty μl of assay solution B [0.6 mM-MnCl2 or 6.0 mM-MgCl2; 50 mM-tris-HCl, pH 8.3; 5 μg/ml oligo (dT)12–18; 1 mg/ml poly(rA)] was added followed by 50 μl of assay solution A [50 mM-tris-HCl, pH 8.3; 120 mM-NaCl; 40 mM-DTT; 1.0% (v/v) NP-40; 45 μM-rATP; 20 μM-dTTP; 1 μCi 3H-dTTP (sp. act. 50 Ci/mmol)]. The assay reactions were incubated for 90 min at 25 °C, terminated by the addition of an equal vol. of 10% TCA and the precipitate harvested on to glass fibre filters using a multiple sample harvester. These conditions were found to be optimal for the HaRV polymerase.

Sedimentation analysis of virus RNA. Purified virus pellets containing 3H-uridine labelled RNA were resuspended in 10 mM-tris-HCl, pH 7.5; 1.0% SDS and 0.1 M-LiCl, incubated for 20 min, layered on a 10 to 30% glycerol gradient, and spun for 3 h at 100000 g in an SW50-1 rotor. Lower mol. wt. RNA subunits were generated by mixing the virus pellet in 10 μl of 5 mM-tris-HCl, pH 7.0, 60 mM-NaCl, 20 mM-DTT and 0.5% NP-40 followed by
Fig. 1. Electron micrographs of HaRV. (a) Virus particles from 1.15 g/ml glycerol density gradient fraction; (b) negative staining of HaRV after fixation, surface projections can be seen; (c) virus particles budding in vitro from Y-22 cell; (d) virus budding from a pigmented cell in a Y-22-induced tumour; (e) virus budding from a cell in a tumour induced by HaRV-transformed hamster embryo cells.
150 μl of DMSO (Bader & Steck, 1969). After heating to 56 °C for 15 min, the sample was cooled on ice, SDS and LiCl were added to the above concentrations and the sample spun as above.

The simultaneous detection of 70S RNA and endogenous polymerase activity was performed using the procedure of Schom & Spiegelman (1971). Gradients were standardized using *Escherichia coli* ribosomal RNA and Rauscher MuLV-RNA.

**SDS-polyacrylamide gels.** Disc gel electrophoresis on 4 to 22% polyacrylamide gradients was carried out according to Laemmli (1970). The slab gels were stained with Coomassie blue R-250 and photographed through a red filter.

**RESULTS**

**Electron microscopy of HaRV**

The characteristic morphology and size (0.1 μm) of a type-C virus is expressed by HaRV under electron microscopic examination of isolated virions (Fig. 1a, b). HaRV can be seen budding from Y-22 cells grown *in vitro* (Fig. 1c), from a melanoma induced by the inoculation of a hamster with Y-22 cells (Fig. 1d) and from a tumour induced in hamsters by the injection of HaRV-transformed hamster embryo cells (Fig. 1e). As with most type-C virions, surface projections can be discerned. Fresh isolates of the virus banded at a density of 1.15 g/ml in glycerol density gradients (data not shown).

**Cellular transformation**

Fractions from virus centrifuged on glycerol gradients were passed through a 0.22 μm Millipore filter and incubated with normal cells. Focus formation occurred within 1 week on cells treated with the 1.15 g/ml density fraction which contained the virus. No foci resulted from fractions having other densities.

Guinea pig cells, hamster embryo cells (HEF), normal rat kidney (NRK), Swiss mouse 3T3 cells (NIH/3Swiss), Balb 3T3 mouse cells (Balb), rat embryo fibroblasts and human skin fibroblasts were checked for focus formation. No focus formation was observed on guinea pig, human and rat embryo cells. The log of the HaRV reverse transcriptase activity present in the inoculum was plotted as a function of the log of the titre estimate for the different cell lines (Fig. 2). The virus titre was determined by multiplying the number of foci per plate by the dilution of the virus for that plate. These values were then averaged to obtain the titre estimate for that particular virus preparation. Thus, each value of virus titre seen in Fig. 2 represents the average of foci from four to six plates.

For each of the four different cell lines shown in Fig. 2, the line drawn through the data points has the same slope as a theoretical Poisson curve for one-hit kinetics (Pincus et al. 1975). The inset in Fig. 2 illustrates the slopes of the theoretical lines for one-hit and two-hit kinetics. It can be seen that in each case, the data best fit a one-hit kinetics curve (see inset Fig. 2). Only a tenfold variation in the titres was found between the three different species. Representative foci on murine cells are shown in Fig. 3.

Cell medium was also harvested from the HaRV-infected cells and tested for the presence of reverse transcriptase activity (Table 1). No polymerase activity was present in newly-transformed murine, rat and guinea pig cells, although medium from the hamster embryo cells and human skin fibroblasts showed low, but significant RNA-dependent DNA polymerase activities using the poly(rA).oligo (dT) assay.
Fig. 2. Plot of HaRV polymerase activity versus titre estimate for several cell lines. The cells were infected with HaRV and stained after 1 week. Foci were counted and each value of the titre estimate was determined from 4 to 6 separate determinations. (a) ■ - ■, hamster embryo cells; (b) ○ — ○ Balb 3T3 cells; (c) ● - ●, NIH Swiss 3T3 cells; (d) □ — □, normal rat kidney cells. Inset: △ — △, theoretical curve for one-hit kinetics; ▲ — ▲, theoretical curve for two-hit kinetics.

Fig. 3. Phase contrast photomicrographs of foci produced on murine (Balb) cell, following infection with HaRV.
Table I. Release of reverse transcriptase activity by HaRV-infected cells*

<table>
<thead>
<tr>
<th>Test cells</th>
<th>ct/min x 10^{-3}</th>
<th>14 days post-infection</th>
<th>Presence of foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster embryo cells</td>
<td>1.3</td>
<td>4.1</td>
<td>+</td>
</tr>
<tr>
<td>Human skin fibroblasts</td>
<td>1.3</td>
<td>5.5</td>
<td>-</td>
</tr>
<tr>
<td>Balb mouse 3T3</td>
<td>1.3</td>
<td>2.2</td>
<td>+</td>
</tr>
<tr>
<td>C57Bl/6J embryo cells</td>
<td>1.2</td>
<td>1.4</td>
<td>N.D.†</td>
</tr>
<tr>
<td>NIH Swiss mouse 3T3</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>Guinea pig embryo cells</td>
<td>0.8</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Rat embryo cells</td>
<td>1.1</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>Normal rat kidney (primary)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>+</td>
</tr>
<tr>
<td>Y-22 cells</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.‡</td>
</tr>
</tbody>
</table>

* Approx. 2-5 ml of culture media were first spun at low speed to remove cellular debris and the supernatant spun at 100000 g for 60 min to pellet polymerase activity. The pellet was resuspended in 200 µl and 25 µl tested for poly(rA), oligo (dT) reverse transcriptase activity.

† N.D. = not determined.
‡ N.A. = not applicable.

Oncogenicity

HaRV-infected hamster embryo cells were able to induce solid tumours in 4 out of 35 inoculated hamsters. Mock-infected hamster embryo cells did not induce tumours in any animals. The tumours which appeared were palpable within two weeks and grew progressively to a large size. These tumours were transplantable to other Syrian hamsters and type-C viruses could be seen budding from the tumour cells by electron microscopy (Fig. 1e). A large number of R particles were also seen in some of these tumour cells. Although approx. 3% of the cells in these tumours contained pre-melanosomes (indicated by arrow in Fig. 1 e) and melanin, the tumours were not considered to be typical melanomas.

Numerous unsuccessful attempts were made to produce tumours by the injection of HaRV itself into hamsters of various ages.

No tumours were induced in mice injected with HaRV-infected murine cells although a small fatty deposit frequently appeared at the site of injection. This tissue was examined and did not appear to be a tumour. Animals kept up to 6 months did not show any visible effects.

Sedimentation of virus RNA

To determine the size and nature of the HaRV RNA, the virus RNA was labelled in culture by the addition of 3H-uridine, virus particles were purified and the RNA extracted and sedimented on SDS-LiCl glycerol gradients. The RNA was found to be 60 to 70S which decreased to 35S upon treatment with DMSO as found with other type-C viruses (Fig. 4a). Using the simultaneous assay of Schlim & Spiegelmann (1971), the radioactive fraction sedimented at 70S indicating endogenous reverse transcription on the 70S virus genome (Fig. 4b). Treatment of the RNA-DNA hybrid with RNase A eliminated the 70S peak.

Polyacrylamide gels

The above experiments strongly suggest that HaRV possesses the basic features of a type-C virus. It can be seen from the electrophoretic patterns in Fig. 5 that HaRV possesses polypeptides which are similar to those found in other mammalian type-C viruses. These are indicated to the right of lane (h) on Fig. 5 as p10, p12, p15 and p30. The profile obtained
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Fig. 4. Glycerol density gradient analysis of HaRV virus RNA. (a) Detergent disrupted virions were sedimented in glycerol gradients as described in the Methods section. \( \circ - \circ \), Without DMSO treatment; \( \bullet - \bullet \), with DMSO. (b) Simultaneous detection of 70S RNA and reverse transcriptase as described in the Methods section. \( \circ - \circ \), Intact RNA-DNA hybrid; \( \bullet - \bullet \), pre-treatment with RNase A. Markers are 60 to 70S virus RNA from Rauscher murine leukaemia virus and 16S and 23S *Escherichia coli* ribosomal RNA.

with Rickard FeLV has been included for comparison (lane k). The major core polypeptide of FeLV is known to be slightly smaller than the murine p30 and is often designated p27 (Strand & August, 1974). The HaRV p30 is apparently closer to murine p30 in size.
Fig. 5. SDS polyacrylamide gel electrophoresis of HaRV and several marker proteins on a 4 to 22% acrylamide gradient slab gel. (a) alkaline phosphatase subunit, 43,000; (b) arginase subunit, 60,000, (c) trypsin, 23,000; (d) rabbit immunoglobulin heavy chain, 55,000 and light chain, 25,000; (e) foetal bovine serum; (f) HaRV; (g) lysozyme, 14,000; (h) to (j) HaRV; and (k) Rickard FeLV.

DISCUSSION

The results presented here indicate that HaRV is type-C in nature and distinct from the other hamster type-C viruses in its transforming ability. RNA from virus particles banding at 1.15 g/ml was shown to be 60 to 70S and dissociated into 35S and smaller pieces upon treatment with DMSO. The simultaneous assay for the detection of 70S RNA and endogenous reverse transcriptase activity revealed that both the RNA and polymerase were associated with HaRV. The morphology of the virus observed on electron micrographs is that of a type C virus as are the proteins found on SDS-polyacrylamide gel electrophoresis.

The fact that HaRV was able to transform murine, rat and hamster cells is significant since HaRV is the only reported hamster retrovirus able to do so. The equal efficiency of transformation and apparent one-hit kinetics are difficult to interpret definitely because of the unknown contribution of helper virus. It was observed that early (3 to 4 days) enumeration of foci resulted in a non-linearity of foci versus virus dilution which approached two-hit kinetics. When counted at days 7 to 9, one-hit kinetics were displayed. The early foci counts, by virtue of the very small size of the foci, may be spurious. These observations are interesting in light of the fact that the murine sarcoma virus (MSV) seems to exhibit two-hit kinetics (Hartley & Rowe, 1966; O' Connor & Fischinger, 1968, 1969), due to the requirement of a murine leukaemia virus helper (MLV); one-hit kinetics are observed in the presence of excess helper. It was shown by Aaronson et al. (1970) that MSV titrated in Balb/c cells with both one- and two-hit dose responses since the foci counted 3 to 5 days after infection were due to MSV (MLV) and MLV, while if the foci were counted 7 days or more after infection, small foci due to MSV (MLV) alone could be seen which corresponded to a one-hit response. In the case of HaRV, the requirement for a helper virus is not ruled out by the fact that all four different cells lines were transformed with similar efficiencies, although a variation in efficiency might be seen if a helper was involved.

The inability of HaRV to induce tumours when injected into hamsters does not rule out the possibility that tumours might appear under different conditions or after a prolonged
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latent period. It is not uncommon for malignancies (either solid tumours or leukaemias) induced by type-C viruses to incubate for a year before appearing. The tumours produced by HaRV-transformed hamster embryo cells produced numerous type-C viruses in addition to R particles and were transplantable to other hamsters. The presence of pre-melanosomes and melanin in 3% of the cells in these tumours indicates the involvement of melanocytes, which can be explained in several ways. Since whole embryos were used for infections, some pigmented cells may have been transformed in vitro and contributed to the tumours. Or, melanocytes already present near the injection site may have become 'innocent bystanders' within the tumours. Finally, it is possible that virus released by the tumours transformed local melanocytes which then became part of the tumours.

A number of type-C like viruses have been previously isolated from hamster cells, most of these from tumours induced by murine sarcoma viruses (Bassinet et al. 1968; Kelloff et al. 1970). Various manipulations have yielded the hamster murine pseudotype viruses called hamster sarcoma virus (HaSV or B-34) and hamster leukaemia virus (HaLV or GLOH-') which appear, in some respects, to be 'Hamster' in that radioimmunoassays using p30 proteins from the pseudotypes reveal that their p30 is indistinguishable from that of the Graffi endogenous hamster virus (Charman et al. 1974). However, it has been shown by nucleic acid hybridizations that HaLV contains considerable murine-specific sequences in addition to hamster and that HaSV is predominantly composed of murine sequences with some hamster and rat. The Graffi hamster virus does not possess detectable rat or murine sequences (Okabe et al. 1974). For these reasons, the HaLV-HaSV pseudotypes are not particularly appropriate examples of hamster retroviruses and it is difficult to determine the origins of their proteins without extensive characterization. With the exception of their polymerases, HaLV and HaSV appeared to be classical type-C viruses.

Another endogenous hamster virus, the 'reverse transcriptase-defective' D-9 virus, has been isolated from the cells of a chemically-induced lymphoma (Somers et al. 1973). The D-9 virus was non-infectious, a trait possibly due to its inactive or non-existent polymerase. The characteristics distinguishing HaRV polymerase from those of the other hamster viruses will be presented in greater detail in the following paper (Gregerson et al. 1979).

The spontaneous appearance of HaRV after prolonged culture suggests that it is an endogenous virus and is similar to the spontaneous appearance of endogenous viruses from other cell lines (Aaronson et al. 1969; Barbacid et al. 1978) although confirmation by nucleic acid hybridizations has not yet been carried out.

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REFERENCES


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