Properties of a Transforming Virus, KiMSV(RHHV), Isolated from a Co-culture of Rat HTC-H1 Cells with K-NRK Cells

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SUMMARY

Some morphological, biological, immunological and biochemical characterizations of a virus, rat helper virus pseudotype Kirsten sarcoma virus, KiMSV(RHHV), have been presented here. KiMSV(RHHV) has a type C virus ultrastructure. It is strictly rat tropic and is able to transform rat cells in vitro. The possibility of its being a xenotropic mouse virus has been carefully ruled out by exhaustive analyses of host range and immunological studies. Antigenically KiMSV (RHHV) demonstrates cross reactivity with an antiserum specific against rat leukaemia virus, no cross reactivity with antiserum against Moloney leukaemia virus, and only minor cross reactivity with antiserum against cat leukaemia virus. Analysis of virus proteins and glycoproteins by equilibrium acrylamide gradient gel electrophoresis showed that the virus complex possesses both a gp70 fraction and a p30 fraction. KiMSV(RHHV) sediments isopycnically in a linear sucrose gradient at 1.145 to 1.155 g/ml and possesses RNA and reverse transcriptase activity.

INTRODUCTION

The appearance of type C viruses in various chemically induced rat hepatomas, both in vivo (Karasaki, 1969; Klement, Nicolson & Huebner, 1971) and when cultured in vitro (Weinstein et al. 1972; Orenstein & Weinstein, 1973), has stimulated a great deal of interest and speculation as to the possible aetiological significance of these viruses. Further study of these viruses, however, was handicapped by the paucity of their replications in vitro. Recently, co-cultivation of non-producer rat kidney cells (K-NRK) transformed by Kirsten murine sarcoma virus with hepatoma tissue culture cells, HTC-H1, has been shown to result in the rescue of a rat helper virus pseudotype Kirsten sarcoma virus KiMSV(RHHV). This virus can be produced in tissue culture at a titre of $10^4$ focus forming units (f.f.u.) of culture media (Yang, Chan & Thompson, 1976). The cell transforming activity, replication pattern, and some immunological characteristics of KiMSV(RHHV) were compared to an endogenous K-NRK virus induced by treating K-NRK cells alone with 5'-iododeoxyuridine (IdUrd) and dexamethasone phosphate (DXP). The differences between KiMSV(RHHV) and the endogenous K-NRK virus in these studies suggest that the helper virus of the KiMSV(RHHV) complex might have originated from a different source. The current studies were designed to examine in detail the morphological, biological, immunological and some biochemical characteristics of KiMSV(RHHV). Some pertinent comparisons are also made with other type C viruses.

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METHODS

Viruses and cells. KiMSV(RHHV) was cultured in Fisher rat embryonic cells (FEC) at a titre reaching 10^4 f.f.u./ml in Dulbecco modified Eagle's medium supplemented with 10% heat-inactivated (56 °C, 30 min) foetal calf serum plus penicillin (50 units/ml) and streptomycin (25 µg/ml) in a 5% CO2 atmosphere at 37 °C. In order to maintain this high rate of virus replication, freshly prepared secondary FEC were added to the KiMSV(RHHV) transformed culture, KiMSV(RHHV)/FEC, every few passages. A 5% cell lysate in medium of a KiMSV(RHHV)/FEC culture was clarified at 15,000 g for 10 min, and stored in aliquots at −70 °C prior to their use in virus assays. Moloney murine sarcoma virus rat leukaemia virus (Ting, 1968; Aaronson, 1971), MSV(RaLV), and Theilen feline leukaemia virus (FeLV) were gifts from Dr R. C. Ting, Biotech, Rockville, Md. Moloney strain of mouse leukaemia-sarcoma virus, MSV(MoLV) and MoLV were obtained from Electronucleonics, Bethesda, Md. All tissue culture materials were purchased from GIBCO, Grand Island, N.Y.

NRK 153 and NRK-Bt01, normal rat kidney cell lines derived from the original NRK line (Duc-Nguyen, Rosenblum & Zeigel, 1966), C81 (S^+L−) cat cell line, human osteosarcoma cells (HOS), human rhabdomyosarcoma (A204) cell line, and canine thymus cells (A7573), were supplied by Biotech, Rockville, Md. NRK cells were used from passages 11 to 14, and were kept in medium supplemented with 5% FCS. Mink 1516 and Mink S^+L− cells were kindly provided by Dr J. Hartley of the National Institutes of Health. XC cells were kindly supplied by the laboratory of Dr W. Rowe. RBL-1, Buffalo rat liver cell line, rat embryonic cells, and the above mentioned cell lines were all fed medium supplemented with 10% foetal calf serum (FCS). Cultures of Fisher or Osborn-Mendel rat embryonic cells and of NIH Swiss or BALB/c mouse embryonic cells were established by trypsinization of 18- to 20-day-old embryos. These cultures were kept and used for four passages.

Partial purification of virus. Approximately 500 ml of medium was harvested from a logarithmic culture of KiMSV(RHHV)/FEC, labelled with 14C-uridine (25 µCi/ml medium; sp. act. 350 mCi/mmol; New England Nuclear, Boston, Ma.). The culture medium was centrifuged at 30,000 g for 20 min to remove cell debris. The clarified medium was further sedimented through a solution containing 32% sucrose, tris-HCl (pH 7.2, 50 mM), NaCl (0.15 M), at 105,000 g for 3 h. The virus pellets were pooled in phosphate-buffered saline (PBS). This was used for both equilibrium sucrose gradient centrifugation and electron microscopy.

Virus infection and neutralization assays. Indicator cells such as NRK^153, RBL-1, rat secondary embryonic cells and mouse secondary embryo fibroblasts, were seeded at 25,000 cells/60 mm plate (Falcon, Baltimore Biological Assoc., Md.), in 2 µg/ml of polybrene (Aldrich Chemical Co., Pa.) overnight. The media were removed and 0.3 ml of a virus preparation was added directly to cells for infection at 37 °C. At the end of a 30 min adsorption period, the cells were fed with 4 ml of medium. Change of culture medium was carried out every other day. Scoring of f.f.u. was carried out on day 6 by phase microscopy.

Neutralization of virus was carried out with an equal volume of a known dilution of specific antiserum at 37 °C for 30 min. The virus-serum suspension was then further diluted prior to its use in infection experiments.

Virus-specific antisera. Pre-immunization goat serum and goat anti-sera against rat leukaemia virus (anti-RaLV), against Moloney murine leukaemia virus (anti-MoLV), against Gross leukaemia virus (anti-GLV), and against Theilen feline leukaemia virus (anti-FLV), were kindly supplied by Dr R. Wilsnack via the Office of Program Resources.
Fig. 1. (a) Partially purified pellet of KiMSV(RHHV) in thin section. (b) Higher magnification of (a), × 167 000. (c, d, e) Fisher embryonic cells infected with KiMSV(RHHV). This series shows the budding and maturation sequence of the virus. (c) An early bud in which a dense crescent-shaped nucleoid is beginning to form beneath a protuberance in the cell’s plasma membrane. A thin intermediate membrane (I) can be seen close to the nucleoid between it and the plasma membrane. (d) A later budding stage. An intermediate membrane (I) is still seen and the core is still less dense than the ring-shaped periphery of the nucleoid. (e) A mature virus particle in which the nucleoid is dense throughout and the intermediate membrane is no longer discernible.

and Logistics, Viral Oncology of NCI. Preparation of these specific antisera has been described earlier (Yang et al. 1976).

**Fluorescent-antibody (FA) assay.** Some FA assays were kindly performed by Dr J. Hartley of NIH, others were performed in our laboratory as follows. An indicator cell culture infected with virus was grown on slides (Cel-line, New Jersey) for immunofluorescence. At 80% confluency, the slides were washed with PBS, acetone fixed at 0 °C and stored at −70 °C until use. For FA assay, the slide for immunofluorescence with the cell sample was incubated with the appropriate dilutions of control or antiserum at 37 °C for 60 min in
a moist chamber. It was then rinsed extensively with PBS containing 0.001% Triton X-100 (Packard, Ill.) and treated with anti-goat fluorescein-conjugated IgG (Cappel Lab., Pa.). The sample was finally counter-stained with 0.004% Evans Blue (Fisher Scientific, Ma.) and examined under a Zeiss fluorescent microscope. Generally, in a virus-infected culture that showed a positive reaction, 60% of the cells exhibited bright fluorescence.

Electron microscopy. A logarithmic culture of KiMSV(RHHV)/FEC producing 10^6 f.f.u./ml of virus was rinsed in PBS, collected as a cell pellet and fixed for 40 min in 2% glutaraldehyde in 0.05 M-Na-cacodylate buffer, pH 7.2, at 4°C. After a 40 min rinse in the same buffer, the cells were post-fixed for 40 min in Dalton chrome-osmium fixative (Dalton, 1955). The sample was stained for 60 min with 0.5% aqueous uranyl acetate in 0.5% sucrose, pH 5.0. After dehydration in a graded series of ethanol and propylene oxide, the cells were embedded in an Epon-Araldite mixture (Mollenhauer, 1964). Thin sections were cut and stained with ethanolic uranyl acetate and lead citrate. They were examined using a Hitachi HU 11E electron microscope at 75 kV accelerating voltage with a 50 μm objective aperture. The microscope was calibrated with a germanium-shadowed carbon replica calibration grid (Ladd Industries, Burlington, Vt.).

A pellet of partially purified KiMSV(RHHV) was fixed, embedded and examined by electron microscopy as described above. Virus dimensions were determined by measurement of 100 viruses in thin section.

Equilibrium gel electrophoresis. A sample of gradient purified virus consisting of approx. 25,000 ct/min, labelled in either 3H-amino acid or 35S-methionine, was first precipitated in 10% TCA at 0°C. The precipitates were collected by centrifugation at 3000 g for 10 min, washed extensively with 5% TCA, acetone, and then air dried. The precipitates were then dissolved in a buffer consisting of 1% SDS, 1% mercaptoethanol, 10% glycerol and 50 mM-tris-HCl, pH 6.8, at 100°C for 1 min. The sample was then applied to a 7.5 to 25% gradient polyacrylamide gel. The gradient slab gel was prepared according to the method of Maizel (1971). Standards that we ran against the radioactive KiMSV(RHHV) protein sample consisted of purified gp70 and p30 of R-MLV (Oroszlan et al. 1974), kindly provided by Dr M. Rogers of the National Cancer Institute, and 35S-adenovirus 2, generously supplied by Dr W. Chin. Other protein standards were purchased from Sigma Chemicals, Mo. The electrophoresis was carried out in a running buffer consisting of 0.1% SDS, and tris-glycine (4 g: 28.8 g/litre), at 50 V for 18 h at room temperature. At the end of the electrophoresis, the slab gel, which generally carried eight samples, was divided and processed for (a) identification of protein bands, (b) radioactivity determination by a liquid scintillation method and (c) fluorography.

For identification of protein bands, the slab gel sample was stained in 0.05% Coomassie blue in acid methanol, and then destained electrophoretically. For radioactivity determination, the slab gel sample was sectioned into about 80 fractions of 1 mm each. The gel slice was homogenized and extracted in 0.5 ml of 5% ammonium hydroxide at 60°C for 30 min, neutralized with HCl and brought to 1 ml volume. Beckman Biosolv, BBS3 (2ml), was added to the sample before counting by a liquid scintillation method.

Virus glycoproteins were analysed also by equilibrium gel electrophoresis using gradient-purified virus labelled with either 3H-glucosamine or 14C-galactosamine. The virus pellet was first lysed with 100 μl of a buffer consisting of 20 mM-tris-HCl, pH 7.2, 50 mM-NaCl, 0.5% Nonidet (NP40; Shell) and 0.5% deoxycholate (Sigma, Missouri). Ten μl of anti-RaLV serum, previously adsorbed with FEC, was added to precipitate the virus components at 37°C for 60 min. Five μg of protein A from Staphylococcus aureus (Pharmacia, New Jersey) was then added to the immune complex and the reaction was allowed to continue
Table 1. A comparison of the host specificities of KiMSV(RHHV), of the IdUrd/DXP induced virus from K-NRK cells, and of the Moloney strain of MSV(MLV)

<table>
<thead>
<tr>
<th>Host culture</th>
<th>KiMSV(RHHV) f.f.u./ml</th>
<th>IdUrd/DXP induced virus* f.f.u./ml</th>
<th>Mo-MSV(MLV) f.f.u./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRK*16a</td>
<td>32 × 10^2</td>
<td>456</td>
<td>87 × 10^2</td>
</tr>
<tr>
<td>Fisher embryonic cells</td>
<td>78 × 10^2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Osborn-Mendel embryonic cells</td>
<td>12 × 10^4</td>
<td>62</td>
<td>0</td>
</tr>
<tr>
<td>RBL-1</td>
<td>950</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BALB/c mouse EF</td>
<td>0</td>
<td>0</td>
<td>12 × 10^8</td>
</tr>
<tr>
<td>NIH Swiss mouse EF</td>
<td>0</td>
<td>0</td>
<td>9.8 × 10^3</td>
</tr>
<tr>
<td>Mink 1516</td>
<td>0</td>
<td>0</td>
<td>n.t.†</td>
</tr>
<tr>
<td>Mink S-L</td>
<td>0</td>
<td>6</td>
<td>n.t.</td>
</tr>
<tr>
<td>IIEC</td>
<td>0</td>
<td>6</td>
<td>n.t.</td>
</tr>
<tr>
<td>IIEC-P4</td>
<td>NRK</td>
<td>0</td>
<td>n.t.</td>
</tr>
<tr>
<td>Mink 1516-P4</td>
<td>NRK</td>
<td>0</td>
<td>n.t.</td>
</tr>
<tr>
<td>HOS</td>
<td>0</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>HOS-P4</td>
<td>0</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>HEK</td>
<td>0</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>HEK-P4</td>
<td>NRK</td>
<td>0</td>
<td>n.t.</td>
</tr>
<tr>
<td>A204</td>
<td>0</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>A204-P4</td>
<td>NRK</td>
<td>0</td>
<td>n.t.</td>
</tr>
<tr>
<td>A7573</td>
<td>0</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>A7573-P4</td>
<td>NRK</td>
<td>0</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

* IdUrd/DXP induced endogenous virus from K-NRK culture on day 6 after the IdUrd/DXP treatment when the virus titre was the highest. The virus was prepared as a 10% cell extract in culture media. The clarified virus suspension was then concentrated 10 × before use.

† n.t. denotes not tested.

‡ Primary host cells were treated with 2 μg/ml of polybrene overnight and then infected with the virus. After 6 days of incubation f.f.u. were determined. Where P4, or passage 4, was indicated, the infected culture was passed blindly four times prior to infection of a second NRK culture with the culture media and then assayed in the same manner.

at 4 °C overnight. The immune precipitates were then collected by centrifugation, washed twice with the same buffer, and then dissolved in 100 μl of a buffer consisting of 20 mM-tris-HCl, pH 8.0, 20 mM-EDTA, 1% SDS, and 1% 2-mercaptoethanol, and analysed in a 5 to 20 % gradient slab gel.

Fluorography. Radioactivity in the slab gel was also detected directly by fluorography as described by Bonner & Laskey (1974) and Laskey & Mills (1975).

DNA polymerase assays. Details of DNA polymerase assay have been described earlier (Yang et al. 1972). Synthetic template, oligo (dT)·poly (rA) (dT12-18·rA18), was used for the poly (rA) directed incorporation of 3H-TTP. Twenty μl of gradient purified KiMSV(RHHV) treated in 0.02% of Triton X-100 (Packard Instrument, Ill.); dT12-18·rA18, 50 μg/ml; dATP at 8 × 10^-6 M; and 3H-TTP at 100 μCi/ml (56 Ci/mmol, New England Nuclear) were used in each assay at 32 °C for 15 min.
Table 2. Virus gs antigens associated with KiMSV(RHHV) transformed cells as demonstrated by indirect fluorescent-antibody assays*

<table>
<thead>
<tr>
<th>Infected cultures</th>
<th>Pre-bleed</th>
<th>Anti-RaLV</th>
<th>Anti-MoMLV</th>
<th>Anti-FeLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>KiMSV(RHHV)/NRK</td>
<td>negative</td>
<td>5120</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>MSV (RaLV)/NRK</td>
<td>negative</td>
<td>5120</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>Mo-MSV(MLV)/NRK</td>
<td>negative</td>
<td>80</td>
<td>2560</td>
<td>40</td>
</tr>
<tr>
<td>FeLV/C81(S'xL')/NRK</td>
<td>negative</td>
<td>320</td>
<td>40</td>
<td>2560</td>
</tr>
<tr>
<td>NRK uninfected</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

* Indirect fluorescent-antibody assays were carried out as described in Methods. FA titre is expressed as the reciprocal of the terminal dilution that yielded positive immunofluorescence. All sources of antisera and preparations were as described in Methods.

RESULTS

Ultrastructure of KiMSV(RHHV)

The viruses in a partially purified pellet isolated from the KiMSV(RHHV)/FEC culture media are predominantly mature type C viruses with outer membrane envelope and dense centrally located nucleoid (Fig. 1a). These viruses are 90 ± 7 nm in diam. with 56 ± 6 nm nucleoids. This is consistent with the morphology and the diam. of about 100 nm reported by Valentine & Bader (1968) for the type C particles found in the MSV(MoLV) infected BN rat embryo cells. Only a few immature forms are seen with ring-shaped nucleoid and electron lucent core (arrow). Immature forms in the virus pellet usually have an incomplete outer envelope or nucleoid suggesting that these are predominantly buds that have separated prematurely, perhaps because of cell lysis incurred during isolation procedures.

At high magnification the mature particles are seen to have a smooth outer envelope with a unit membrane. The centrally located nucleoid can be very dense (D) or loosely granular (G) in structure. No intermediate membrane can be distinguished in the mature virus (Fig. 1b).

On examination of the KiMSV(RHHV) infected Fisher rat embryonic cells, budding forms of the virus can be seen (Fig. 1c and d). The nucleoid of the virus bud is crescent or ring shaped. A closely adherent intermediate membrane (I) can be discerned and this cannot be seen in mature particles. They thus resemble the immature forms of the murine type C virus. The presence of mature forms (Fig. 1e) between the whole cells suggests that the pelleted viruses from culture media were derived from the infected embryonic cells.

Host specificity of KiMSV(RHHV)

The host specificity of KiMSV(RHHV) was compared to other known type C viruses, such as Mo-MSV(MoLV) and the endogenous K-NRK virus, induced from K-NRK cells by IdUrd and DXP treatment. Results of this comparative study are summarised in Table 1. KiMSV(RHHV) infects and transforms all four rat cell cultures tested, which includes NRK158, Fisher and Osborn–Mendel secondary rat embryonic cells and RBL-1, Buffalo rat liver cell line. The transformed foci consisted of small refractile, round and spindle cells, piled on top of the contact-inhibited monolayer of test cells. It is interesting to note that HTC-H1 cells were originally established from a Buffalo rat hepatoma (Morris, 1965; Thompson, Tomkins & Curran, 1966). The IdUrd/DPX induced endogenous virus from K-NRK cells, however, shows a restricted host range, limited to NRK153 and Osborn–Mendel rat embryonic cells. This is consistent with the observation made on an induced
Properties of KiMSV(RHHV) transforming virus

Fig. 2. Major structural proteins associated with KiMSV(RHHV). Virus proteins extracted from KiMSV(RHHV), labelled in either \(^{3}H\)-amino acid (●—●) or in \(^{35}S\)-methionine (○··○), were analysed by gradient polyacrylamide gel electrophoresis in the presence of SDS. All experimental details were described in Methods. \(^{3}H\)-amino acid mixture was purchased from New England Nuclear, Ma., and has an average specific activity of 33 Ci/mmol. \(^{35}S\)-methionine, purchased from the same source, has a specific activity of 400 Ci/mmol. The culture medium was adjusted to 25 \(\mu\)Ci/ml. Repeated experiments with \(^{35}S\)-cystine and \(^{35}S\)-cysteine also yielded profiles similar to that of \(^{35}S\)-methionine. Protein standards run simultaneously in the same slab gel are: H, the hexon (mol. wt. 120,000) of adenovirus 2; GP70 (mol. wt. 70,000) of RLV; BSA (mol. wt. 69,000), bovine serum albumin; OA (mol. wt. 45,000), ovalbumin; P30 (mol. wt. 30,000) of RLV; C (mol. wt. 19,000), core protein of adenovirus 2; cyt c (mol. wt. 12,270), cytochrome c. ▲—▲, mol. wt.

endogenous virus from a different K-NRK line, 1255 B-7, which transformed NRK cells (Klement et al. 1971). Repeated attempts to infect Fisher embryonic cells or RBL-1 cells with the IdUrd/DXP induced endogenous virus from our K-NRK line resulted in negative findings. Because infectivity is primarily a function of the host range of the helper virus, it therefore shows that the helper virus of the KiMSV(RHHV) complex differs from the K-NRK endogenous virus induced by IdUrd/DXP treatment.

KiMSV(RHHV) is strictly rat tropic and showed no infectivity on either NIH Swiss or BALB/c mouse embryonic cells; nor did it infect any of the known supportive cell lines for xenotropic mouse virus, such as feral mouse cell line, 116C (Hartley & Rowe, 1975), Mink 1516 and Mink S+L\(^{-}\) (Peebles, 1975), human embryonic kidney cells (HEK), human osteosarcoma (HOS) cells, human rhabdomyosarcoma cells (A204), and canine thymus cells (A7573), which are generally considered as suitable hosts for xenotropic viruses (Table 1). This, however, does not necessarily rule out that KiMSV(RHHV), or a component thereof, may infect these cells. In order to rule out definitively the possible existence of a xenotropic virus component in KiMSV(RHHV), a number of host cells known to augment the replication of mouse xenotropic viruses were infected with KiMSV(RHHV) and carried blindly for four passages. Media from these ‘infected’ cells were then used for reverse transcriptase assay and also to infect other secondary host cells. The ‘infected’ cultures were
Fig. 3. Virus glycoproteins associated with KiMSV(RHHV). A KiMSV(RHHV)/FEC culture was chronically labelled with either 3H-galactosamine (O——O) at 2.5 μCi/ml of medium (sp. act. 55 mCi/mmol) or 3H-glucosamine (●——●) at 5 μCi/ml of medium (sp. act. 15 Ci/mmol). The radioactive virus particles were isolated from 24 h culture medium and purified by linear sucrose gradient. Virus-specific glycoproteins and proteins were precipitated with anti-RaLV serum. The 14C- and 3H-samples were run together and the electrophoresis was carried out in a 5 to 20% gradient polyacrylamide slab gel for 20 h. Protein standards run simultaneously in the same slab gel are indicated by arrows only. These are the dimer (mol. wt. 138000) and monomer (mol. wt. 69000) of bovine serum albumin; ovalbumin (mol. wt. 45000); myoglobin (mol. wt. 17300); and cytochrome c (mol. wt. 12270). Details of all methods are described in the text; ••••, mol. wt.

also monitored for mouse gs antigens by indirect fluorescent-antibody assays using a broadly reactive anti-MoLV serum. The results of the bioassays were all negative (Table 1). No significant level of reverse transcriptase activity in the medium was observed in any of the augmented cultures. Neither did these 'infected' cultures show any cross reactivity with the broadly reactive anti-MoLV serum in the indirect fluorescent-antibody assays. The overall negative findings from the diversity of the augmentation procedures used for xenotropic virus demonstrate that KiMSV(RHHV) is a rat virus with no mouse xenotropic contaminant.

Type of virus gs antigens associated with KiMSV(RHHV) infected cells

The presence of virus gs antigens in KiMSV(RHHV) infected cells was tested by indirect fluorescent-antibody assays with the following specific antisera: anti-RaLV, anti-MoLV and anti-FeLV (Theilen). The results, summarized in Table 2, show that a high fluorescent-antibody (FA) titre (5120) was obtained with NRK cultures infected with either MSV(RaLV) or KiMSV(RHHV), when anti-RaLV serum was used. Anti-RaLV serum also demonstrated a minor cross reactivity with the FeLV infected C81(S+L−) cells (FA = 320) and little or no reactivity with Mo-MSV(MLV) infected NRK cells (FA ≤ 40). Reciprocally both
Properties of KiMSV(RHHV) transforming virus

Table 3. Neutralization studies on KiMSV(RHHV) and other type C viruses with specific antisera*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>f.f.u./plate*</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>KiMSV(RHHV) + Pre-bleed serum</td>
<td>231</td>
<td>0</td>
</tr>
<tr>
<td>KiMSV(RHHV) + Anti-RaLV serum</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>KiMSV(RHHV) + Anti-MoLV serum</td>
<td>212</td>
<td>8</td>
</tr>
<tr>
<td>KiMSV(RHHV) + Anti-GLV serum</td>
<td>238</td>
<td>0</td>
</tr>
<tr>
<td>KiMSV(RHHV) + Anti-FeLV serum</td>
<td>192</td>
<td>17</td>
</tr>
<tr>
<td>MSV(RaLV) + Pre-bleed serum</td>
<td>268</td>
<td>0</td>
</tr>
<tr>
<td>MSV(RaLV) + Anti-RaLV serum</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>MSV(RaLV) + Anti-MoLV serum</td>
<td>232</td>
<td>13</td>
</tr>
<tr>
<td>MSV(RaLV) + Anti-GLV serum</td>
<td>264</td>
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<tr>
<td>MSV(RaLV) + Anti-FeLV serum</td>
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<td>15</td>
</tr>
<tr>
<td>MoMSV(MLV) + Pre-bleed</td>
<td>246</td>
<td>0</td>
</tr>
<tr>
<td>MoMSV(MLV) + Anti-RaLV serum</td>
<td>251</td>
<td>0</td>
</tr>
<tr>
<td>MoMSV(MLV) + Anti-MoLV serum</td>
<td>5</td>
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<tr>
<td>FeLV* + Pre-bleed serum</td>
<td>403</td>
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<td>FeLV* + Anti-RaLV serum</td>
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<tr>
<td>FeLV* + Anti-FeLV serum</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* All antisera sources and preparations were as described in Methods. Final concentration of all pre-bleed and antisera used in the neutralization assays was set at 1/40 dilution, which showed no non-specific cytotoxicity to the test cells. Neutralization assay was described in detail in Methods. NRK 153 cells were used for assay of KiMSV(RHHV) and MoMSV(MLV). C81 (S-L-) cells were used for the focus formation assay of FeLV according to the method of Fischinger, Blevins & Nomura (1974).

anti-MoLV and anti-FeLV sera showed little or some minor reactivity with both KiMSV(RHHV) and MSV(RaLV) transformed NRK cells. These observations suggest that, upon infection, the KiMSV(RHHV) transformed cells exhibited a major RaLV specific gs antigen besides a minor common antigenic determinant shared with the other type C viruses (FeLV) tested here.

Major proteins and glycoproteins associated with KiMSV(RHHV)

We further examined the major proteins associated with the purified virus preparation of KiMSV(RHHV). Fig. 2 shows the profile of proteins solubilized from KiMSV(RHHV) labelled in either 3H-amino acid or 35S-methionine as analysed by equilibrium polyacrylamide gel electrophoresis. Three major and one minor protein components were seen in both the 35S- and 3H-profiles (Fig. 2). This was confirmed by direct fluorography on X-ray film exposed directly by contact with the gel. The proteins of current interest are the smaller peak coinciding with the standard gp70 from Rauscher LV (RLV) and one major peak that migrated along with the standard p30 also from Rauscher LV. Our observation of components the size of 70 and 30 agrees with the earlier report by Parks et al. (1976) that rat type C viruses also possess p30. The high mol. wt. major peak (p110–120) that migrated near the 120000 hexon of adenovirus 2 (Maizel, 1971) is most likely a glycoprotein since both 3H-glucosamine and 14C-galactosamine were also incorporated into such a fraction (Fig. 3). Another major 3H-protein peak migrated ahead of both standards cytochrome c and the core protein (mol. wt. 19000) of adenovirus 2 (Maizel, 1971). It has a mol. wt. of about 7000 to 10000.

Three distinct glycoprotein peaks were associated with KiMSV(RHHV): two major peaks of gp69–71 (gp70), gp40–45 (gp40), and a minor peak of gp85 (Fig. 3). Both galactosamine
and glucosamine were incorporated into these peaks equally well since the $^{14}$C and $^3$H profiles coincided with each other. A virus-associated glycoprotein of gp180 was resolved when 4 to 30% gel was used in the analysis. It migrates as a distinct peak separate from the residual radioactivity at the top of the gel. A peak of glycoprotein gp110-120 was also occasionally seen using 4 to 30% gel. The origin of this gp110-120, whether viral or cellular and the precursor and product relationship of these glycoproteins are currently under investigation.

**Neutralization of KiMSV(RHHV) infectivity**

Infectivity of KiMSV(RHHV) can be blocked by neutralization with anti-RaLV serum. The results of neutralization studies on KiMSV(RHHV) as compared to other type C viruses such as MSV(RaLV), Mo-MSV(MLV) and FeLV, using specific anti-type C virus sera as described above, are summarized in Table 3. It is evident that all biological activity of KiMSV(RHHV) can be neutralized by anti-RaLV serum. Some inhibition (17%) was
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also observed on KiMSV(RHHV) infectivity when anti-FeLV serum was used for neutralization. A reciprocal 11 % inhibition on FeLV infectivity of C81 S-L− cells was also obtained when anti-RaLV serum was used for neutralization of FeLV. No inhibitory effect was seen with anti-GLV serum on both the rat and feline viruses in this study. Only a slight inhibitory effect (8 %) on KiMSV(RHHV) infectivity was observed with anti-MoLV serum. Similar results were obtained in the neutralization study of MSV(RaLV) infectivity using the same antisera. However, no reciprocal inhibition was seen with Mo-MSV(MLV) infectivity when anti-RaLV serum was used for neutralization of Mo-MSV(MLV). Considering that the amount of antiserum used in the neutralization process was at excess, the lack of inhibition cannot be explained by an apparent greater number of MoLV in the Mo-MSV(MLV) stock.

Density of KiMSV(RHHV)

KiMSV(RHHV) sediments at a density of 1.145 to 1.155 g/ml in a neutral linear sucrose gradient after equilibrium centrifugation (Fig. 4). This value is in agreement with that of MSV(RaLV) labelled in 3H-uridine and run in the same tube as that of 14C-KiMSV(RHHV). 14C-KiMSV(RHHV) is, however, physically less dense than 3H-Rauscher LV, which shows a typical value of 1.155 to 1.165 g/ml when the two viruses were compared in a linear sucrose gradient equilibrium centrifugation.

In a separate density centrifugation analysis of KiMSV(RHHV) alone, the gradient samples were assayed for poly (rA)-directed polymerase activity using the DNA-RNA hybrid, oligo (dT)12-18-poly (rA) as template and 3H-TTP as substrate in the presence of Mn2+ (Fig. 4). It is evident that the activity of KiMSV(RHHV) DNA polymerase coincides with the physical sedimentation profile of KiMSV(RHHV) and appears in a uniform peak.

DISCUSSION

Although there has been much discussion about possible co-carcinogenesis between chemical carcinogens and the appearance of oncogenic viruses as the causation of rat hepatomas, relatively little is understood regarding their mode of interaction or the characteristics of these viruses. It has also been argued that their appearance in the tumour cell may represent no more than passenger type C viruses from an extrinsic source; neither has the oncogenicity of these type C viruses associated with chemically induced rat hepatomas been investigated. With the establishment of a KiMSV(RHHV)/FEC system replicating virus particles at 104 f.f.u./ml of medium, we are now able to examine not only some basic characteristics of this hepatoma-associated virus in the form of a pseudotype Kirsten sarcoma virus complex, but also to assess its role as a helper virus to the sarcoma virus complex in cell transformation.

Morphologically KiMSV(RHHV) is a typical type C virion. The ultrastructure of KiMSV(RHHV) infected culture showed mostly mature type C virions with relatively few buds. The same type C virion morphology was infrequently seen in HTC-H1 cultures, indicating the existence of a slow release of a possible helper virus. Our results on host specificity, neutralization studies with specific antisera and indirect immunofluorescence demonstrated that KiMSV(RHHV) is a rat-tropic and cell-transforming virus complex. It possesses a major RaLV-specific gs antigen, gp70, and a p30 antigen when analysed by indirect fluorescent-antibody assays. This observation supports the earlier reports on two rat viruses, V-NRK and RT21c, isolated from Osborn–Mendel derived rat cells by Scolnick, Maryak & Parks (1974), which established the existence of a p30 antigen in rat type C viruses. The reciprocal cross reactivity in FA assays and neutralizations of infectivity by anti-RaLV
serum on FeLV and by anti-FeLV serum on KiMSV(RHHV), though moderate, tend to substantiate the observations reported on radioimmunoprecipitation study with p30 antigen by Charman et al. (1976) and nucleic acid hybridization by Benveniste, Sherr & Todaro (1975), in which FeLV and RaLV shared considerable common genetic information.

It has been established that murine Rauscher leukaemia virus possesses gp180, gp80, gp69–71 and gp60 as its envelope specific glycoproteins (Duesberg, Martin & Vogt, 1970; Strand & August, 1973; Arcement et al. 1976). By equilibrium gradient polyacrylamide gel electrophoresis we also succeeded in resolving the glycoproteins of this rat virus complex into gp180, gp85, gp69–71 and a smaller gp40–45. Although the molecular weights of the RLV glycoproteins and those of KiMSV(RHHV) are similar except for the gp40–45, it should be emphasized that RLV and KiMSV(RHHV) envelope glycoproteins share no common antigenic determinants as evidenced in the results of the neutralization assays.

The structural proteins associated with the rat virus complex have also been identified as p110–120, p70, p30 and p7–10. Among these, p30 is the major non-glycosylated structural protein of KiMSV(RHHV). The p110–120 and p70 proteins are both glycosylated proteins of the virion. The nature of p7–10 is not well understood. Our current research effort is directed towards a better understanding of the synthesis and assembly of these virus gene products, especially in the aspect of precursor-product relationship.

It is also relevant to resolve the genetic constituents of the KiMSV(RHHV) complex so that questions such as (1) the possible role, if any, of such a helper virus in a chemically induced hepatoma, and (2) the possible recombination of genetic information in the formation of a virus genome may be answered.

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REFERENCES


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