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Pneumovirus-like characteristics of the mRNA and proteins of turkey rhinotracheitis virus

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Summary

Electronmicroscopy has indicated that turkey rhinotracheitis virus (TRTV), the causative agent of an acute repiratory disease in turkeys, is a member of the Paramyxoviridae family. To determine if TRTV belongs to one of the three defined genera of this family (*Paramyxovirus, Morbillivirus* and *Pneumovirus*) we have analysed the RNA and proteins induced during replication of TRTV in Vero cells. Following replication in the presence of actinomycin D 10 polyadenylated RNA bands, ranging in M_r from 0.22 to 2.0×10^6, were detected in infected cells; some bands probably contained 2 or more RNA species. Viral proteins were studied after radiolabelling in the presence of [^{35}S]methionine and [^{3}H]glucosamine. Comparison of the polypeptides in mock-infected and infected cells, virions and nucleocapsids and after lentil-lectin chromatography and immunoprecipitation revealed seven virus-specific polypeptides (p), some of which were glycosylated (gp): gp82 (M_r 82K), gp68, gp53, gp15, p43, p40 and p35. These are considered to be analogous to the large glycopolypeptide (HN, H and G), fusion protein precursor F0, the F protein cleavage products F1 and F2, nucleocapsid (N), phosphorylated (P) and matrix (M) polypeptides, respectively, of the Paramyxoviridae. Two other polypeptides (M_r 200K and 22K) were also detected, as was a glycopolypeptide of M_r 97K, probably related to gp82. Tunicamycin inhibited glycosylation of gp53 and gp15 but gp82 was little affected, most glycans still being present on a glycopolypeptide of approximately 79K. This finding, indicating that gp82 has mostly

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O-linked glycans, considered with the mRNA profile and the molecular weight of the N protein shows that of the three genera in this family, TRTV most closely resembles the *Pneumovirus* genus.

Pneumovirus; Paramyxoviridae; RNA; Protein; Turkey

Introduction

Turkey rhinotracheitis, an acute highly contagious respiratory disease of turkeys, has been present in Europe for several years but was first reported in the U.K. in the summer of 1985 after which it rapidly spread to turkey flocks throughout England and Wales, causing considerable economic loss (McDougall and Cook, 1986). A virus, turkey rhinotracheitis virus (TRTV), capable of reproducing the disease symptoms, was isolated by several laboratories and was shown to be an enveloped virus with virions 80–600 nm in diameter but also with elongated forms (McDougall and Cook, 1986; Wilding et al., 1986; Wyeth et al., 1986; Giraud et al., 1986). Protruding from the virus surface were projections 13–15 nm long, and nucleocapsids 15 nm in diameter have been seen in disrupted particles. The virus failed to haemagglutinate turkey, chicken, human O and guinea pig erythrocytes. These observations, together with preliminary analysis of the polypeptides of virus grown in chicken tracheal organ cultures (Collins et al., 1986), have suggested that TRTV is a member of the Paramyxoviridae, possibly of the *Pneumovirus* genus.

Materials and Methods

Viruses and cells

The 3B strain of TRTV was used (McDougall and Cook, 1986). It was titrated in chicken embryo tracheal organ cultures (Cook et al., 1976), the end-points being established at 10 days after inoculation and the titres expressed as $\log_{10}$ median ciliostatic doses/ml (CD50/ml). TRTV was propagated in Vero cells (ATCC No. CCL 81; Flow Laboratories, Irvine, Scotland) which were grown with M199 medium (Flow Laboratories) supplemented with 10% foetal calf serum (FCS) and maintained, with or without virus, in M199 and 1% FCS in an atmosphere of 5% carbon dioxide. Virus stocks were prepared by inoculating 25 cm^2 flasks of Vero cells with 1 $\log_{10}$ CD50/ml of virus. After 3 days incubation at 37°C the resultant virus was diluted to 2 $\log_{10}$ CD50/ml and used to infect more Vero cells. After incubation for 2 days, and at daily intervals thereafter, the medium was removed for use as a virus stock and was replaced with fresh medium. Maximum virus titres (about 4 $\log_{10}$ CD50/ml) were obtained at 4–5 days after infection. A large plaque variant of the Onderstepoort vaccine strain of canine distemper virus (CDV) (from Dr. B. Rima, The Queen's University of Belfast) was produced in Vero cells.

Radiolabelling of viral RNA and proteins

For both RNA and protein studies 25 cm^2 flasks of confluent Vero cells were inoculated with approximately 4 $\log_{10}$ CD50/ml of TRTV. After 40 min the inoculum was replaced with maintenance medium and incubation continued at 37°C. For RNA labelling the medium was replaced 1 day after infection with phosphate-free medium (Flow Laboratories) containing 1 μg/ml of actinomycin D (AMD; Sigma, Poole, England). Incubation was continued after 5 h with phosphate-free medium containing 10 μg/ml of AMD and 250 μCi/ml of [^{32}P]orthophosphate (Amersham International, Amersham, England). RNA was extracted 12–13 h later. This procedure was repeated with other flasks at 2, 3 and 4 days after infection. For CDV RNA cells were infected for 3 h with 0.1 PFU of virus/cell and then incubation continued in fresh medium. Phosphate-free medium containing 10 μg/ml of AMD was substituted for the existing medium after 23 h. Five hours later [^{32}P]orthophosphate (250 μCi/ml) was added for a further 6 h incubation.

For radiolabelling the proteins of virions and ribonucleoprotein (RNP) medium was replaced 3 days after infection with either M199 plus 0.2% bovine serum albumin (BSA) for use with D-[6-^{3}H]glucosamine hydrochloride or methionine-free medium (Flow Laboratories) with BSA when L-[^{35}S]methionine (Amersham International) was used. Cells were incubated in methionine-free medium for 1 h prior to addition of [^{35}S]methionine. For labelling medium containing 50 μCi/ml of [^{35}S]methionine or 50 μCi/ml of [^{3}H]glucosamine was used. Where appropriate AMD and tunicamycin (Sigma) dissolved in ethanol (1 mg/ml) and dimethylsulphoxide (100 μg/ml), respectively, were both used at 1 μg/ml. After 24 h the medium and cells were processed separately for the recovery of virions and RNP, respectively (see below).

For analysis of cell-associated proteins the medium was replaced 3 days after infection with methionine-free medium containing 10 μg/ml of AMD. After 5 h the medium was replaced with 0.75 ml of methionine-free medium containing 10 μg/ml of AMD and 75 μCi of [^{35}S]methionine. Four hours later the cells were washed and proteins extracted as described below.

Analysis of mRNA

Cytoplasmic RNA was extracted from infected and mock-infected Vero cells as previously described (Barrett and Mahy, 1984) and polyadenylated RNA selected using messenger RNA affinity paper (Hybond-mAP, Amersham International) as instructed by the manufacturer. The resulting mRNA was mixed with 20 μg of yeast tRNA (Boehringer, Lewes), precipitated with ethanol and dissolved in 50 μl of water. Samples of 10–20 μl were analyzed in 1.5% agarose-formaldehyde gels as described by Barrett and Mahy, 1984). After electrophoresis the gel was soaked in 20 × SSC (175.3 g NaCl and 88.2 g sodium citrate in 1 l of water, adjusted to pH 7.0 with NaOH) for 1 h at 4°C and then blotted onto nitrocellulose (Hybond N, Amersham International). Autoradiographs were made with Fuji RX film at −70°C with an intensifying screen. For treatment with RNase H 30 μl of polyadenylated TRTV RNA was mixed with 30 μl of 2 × RNase H buffer (1 × RNase H buffer: 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 10%

glycerol), 2 μl of oligo-dT12-18 (2.5 μg/ml; Pharmacia, Milton Keynes) and 8 μl of 1 × RNase H buffer.

Ten microlitres were removed (zero time) and then 3 μl of RNase H (6 units; Boehringer, Lewes) added for incubation at 30 °C. At intervals 12 μl samples were removed and mixed with 180 μl of water, 20 μg tRNA, 10 μl 3 M sodium acetate and precipitated with 2.5 volumes of ethanol at −20 °C. Pelleted RNA was dissolved in 10 μl of water prior to electrophoresis. For pancreatic RNase treatment the RNase (25 mg/ml; Sigma, Poole) was heated at 65 °C for 10 min to inactivate any DNase present. Then 10 μl was mixed with 10 μl of RNA and incubated at 37 °C for 30 min prior to electrophoresis.

Analysis of cell-associated TRTV proteins

Infected and mock-infected cells were washed with phosphate-buffered saline (PBS) and then lysed using 1 ml of RIP buffer for 10 min on ice; 10 mM Tris-HCl, pH 7.8, 2% Triton X-100 (TX100), 150 mM NaCl, 600 mM KCl, 5 mM EDTA, 2.5 mM iodoacetamide, 3 mM phenylmethylsulphonyl fluoride (PMSF) and, when used for immunoprecipitations, 1% aprotinin (Sigma, Poole). The lysate was then clarified using a refrigerated microfuge and the supernatant aliquoted and stored at −70 °C. For affinity selection of glycoproteins cell lysates were mixed with 1/5th volume of lentil-lectin-Sepharose 4B (Sigma) on ice for 2–3 h. The Sepharose was then washed 6 times with RIP buffer and twice with PBS before heating at 100 °C prior to electrophoresis. For immunoprecipitation 20 μl of extract was mixed on ice with 30 μl of RIP buffer and 10 μl of a pool of preimmune serum of convalescent serum taken from turkeys that had been inoculated intranasally once with TRTV. After 1 h, 10 μl of goat anti-turkey IgG (Sera-Lab, Crawley Down, Sussex) was added at 0 °C and then 25 μl of a 20% w/v suspension of protein-A Sepharose (Sigma) in RIP buffer was added for a further hour on ice. The Sepharose was then washed 6 times with RIP buffer, twice with PBS and then heated at 100 °C in dissociation buffer containing SDS and 2-mercaptoethanol prior to electrophoresis.

SDS-polyacrylamide gel electrophoresis

Proteins were dissociated and analysed in polyacrylamide gels as described by Laemmli (1970). After electrophoresis gels were fixed and stained (20% methanol, 10% acetic acid, 0.01% Coomassie Brilliant Blue) and then dried for autoradiography or soaked for 20 min in water and then in 10 volumes of 1 M sodium salicylate prior to drying for fluorography (Chamberlain, 1979) using Kodak XAR or Fuji RX film at −70 °C. For further analysis of some polypeptides these were cut out of the dried gel, swollen for 2 min in dissociation buffer with or without 2-mercaptoethanol, excess fluid removed and the gel pieces heated at 100 °C for 2 min. The pieces of gel were then placed in the sample wells of a 10% polyacrylamide gel, surrounded by dissociation buffer with or without 2-mercaptoethanol and then electrophoresis performed. For estimation of molecular weight the following ^{14}C-labelled polypeptides (Amersham International) were electrophoresed in some gels; myosin (200K), phosphorylase b (93K), BSA (69K), ovalbumin (46K) and carbonic anhydrase (30K). In other gels the following unlabelled proteins were used (all from Sigma); phosphorylase b, BSA, carbonic anhydrase and lysozyme (14.3K).

Preparation of virions and nucleocapsids

Culture fluid containing radiolabelled virus was clarified, calf serum added (2%) and virus precipitated by addition of an equal volume of saturated ammonium sulphate for 2 h on ice. The precipitate was dissolved in 1 ml of NET buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.2, 100 μg/ml BSA) and placed on top of a discontinuous gradient comprising 1 ml of 60% (w/w) sucrose and 8 ml of 25% (w/w) sucrose in NET. After centrifugation at $65000 \times g_{av}$ for 2 h at 4° C in a Sorvall TH641 swing out rotor the material at the 25/60% surcrose interphase was located by scintillation counting, diluted and placed on top of a 25–55% w/w sucrose gradient in NET. After centrifugation at $31000 \times g_{av}$ for 16 h at 4° C the gradients were fractionated and radiolabelled material detected by scintillation counting. Nucleocapsids were prepared as described by Ward et al. (1983). Briefly, infected and mock-infected cells were lysed with nonionic detergent, the nucleocapsids, pelleted, further purified by isopycnic centrifugation in a potassium tartrate gradient and finally pelleted.

Results

Virus-induced RNA

To analyse the mRNAs induced by TRTV, Vero cells were infected with 0.001 CD50 of virus/cell. At 1, 2, 3 and 4 days after infection the maintenance medium was replaced with medium containing AMD and [^{32}P]orthophosphate and 12–13 h later cytoplasmic nucleic acid was extracted. Autoradiographs made after agarose gel electrophoresis of polyadenylated (poly-A +) RNA revealed 10 bands, maximum synthesis of RNA having occurred between days 3 and 4 after infection (Fig. 1, tracks c–e). No labelled RNA could be detected under similar conditions in uninfected cells (Fig. 1, track b). All the nucleic acid species were suspecceptible to pancreatic ribonuclease, showing them to be RNA (data not shown), and were reduced in size by hydrolysis with RNase H in the presence of oligo-dT, showing that the RNA had poly-A tracts characteristic of mRNAs (Fig. 1, tracks g–l). The appearance of some of the bands suggested that they contained 2 or more RNA species. The RNA profile was very different from that of canine distemper virus (CDV), a member of the *Morbillivirus* genus of the Paramyxoviridae (Fig. 1, tracks m and n), whose smallest mRNA has a M_r of 0.46×10^6 (Barrett and Mahy, 1984). The TRTV RNA bands were estimated to have M_r values of 0.22, 0.26, 0.39, 0.48, 0.53, 0.57, 0.69, 0.86, 1.1 and 2.0 (all $\times 10^6$).

Cell-associated virus-specific polypeptides

Polyacrylamide gel electrophoresis of cytoplasmic extracts of mock-infected and TRTV-infected cells labelled with [^{35}S]methionine in the presence of actinomycin-D revealed 3 virus-induced polypeptides of M_r 43K (p43), 40K (p40) and 35K (p35) (Fig. 2, track c). Similar labelling of uninfected cells is shown in track b for comparison. In an attempt to detect glycoproteins cell extracts were mixed with lentil-lectin Sepharose which has an affinity for glycoproteins containing glycans

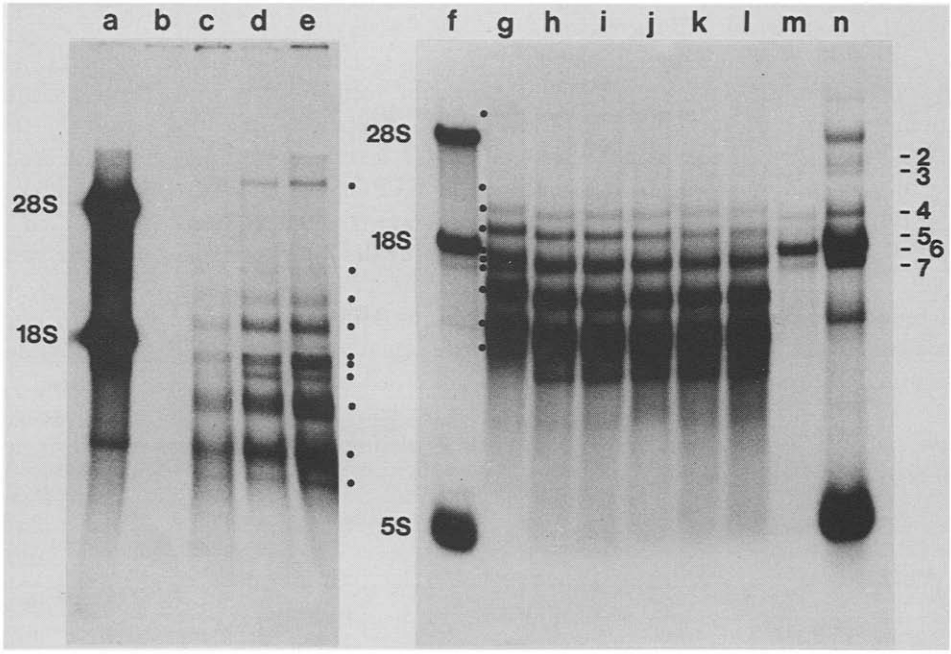

Fig. 1. Agarose gel electrophoretic analysis of poly-A+ RNAs induced by TRTV and labelled using [^{32}P]orthophosphate. (a) Uninfected cell RNA labelled without AMD and without poly-A+ selection showing 28S and 18S ribosomal RNA; (b) poly-A+ uninfected cell RNA labelled with AMD present; (c–e) poly-A+ TRTV-infected cell RNA labelled at (c) 1, (d) 2 and (e) 3 days after infection in the presence of AMD. The dots mark the positions of the 10 reproducibly detected RNA bands. The band above the topmost dot resulted from incomplete denaturation of the sample. (f–n) Effect of ribonuclease H on TRTV poly-A+ RNA and comparison with CDV-induced RNA: (f) as (a) showing 5S transfer RNA; TRTV poly-A+ RNA before (g) and after incubation with ribonuclease H for (h) 5, (i) 10, (j) 20, (k) 40 and (l) 60 min; CDV-induced RNA (m) after and (n) before poly-A+ RNA selection. The CDV RNA bands numbered 2–7, estimated to have M_r values of 1.0, 0.96, 0.70, 0.62, 0.52 and 0.46×10^6 (Barrett and Mahy, 1984), were used to estimate the M_r values of the TRTV RNAs.

with α-D-mannosyl residues. Virus-induced polypeptides of M_r 68K, 53K, 43K, 35K and 22K were detected (Fig. 2, track e). The amount of p43 and p35 recovered by the procedure was small when compared to the enrichment of p68 and p53 (Fig. 2; compare tracks c and e). The proteins precipitated by lectin from uninfected cells are shown in track d. This result strongly indicated that p68 and p53 were glycosylated but the situation with respect to p43, p35 and p22 was unclear until resolved by radiolabelling of the glycoproteins with [^{3}H]glucosamine. A pool of convalescent turkey sera immunoprecipitated [^{35}S]methionine-labelled p68, p53, p43 and a small amount of p35 (Fig. 3, track c). In addition a polypeptide of M_r approximately 200K was precipitated; the identity of this polypeptide is unclear, but it possibly represents the L protein.

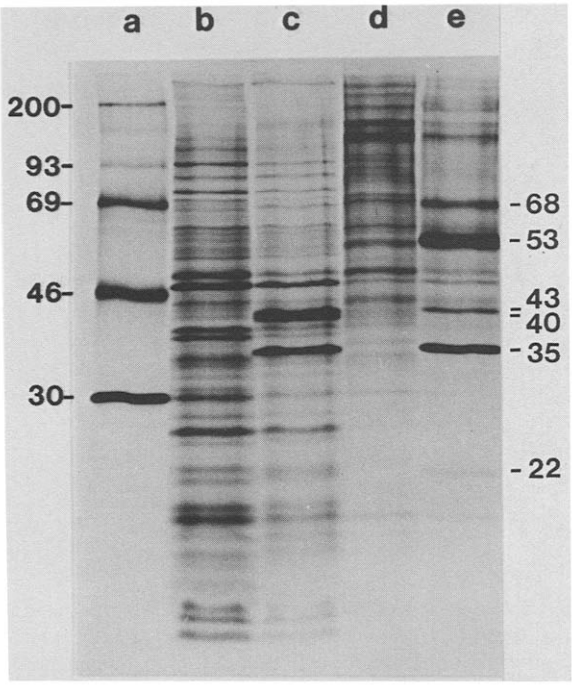

Fig. 2. Lentil-lectin affinity selection of proteins from Vero cells labelled with [^{35}S]methionine and analysis by SDS-polyacrylamide gel electrophoresis. (a) M_r marker polypeptides ($M_r \times 10^{-3}$); (b) uninfected, and (c) TRTV-infected cell extracts before lentil-lectin selection, showing the 43K, 40K and 35K TRTV-induced polypeptides. Uninfected (d) and infected (e) cell extracts after lentil-lectin selection, showing the enrichment of the 68K and 53K polypeptides.

Virion-associated polypeptides

To further identify virus-specific polypeptides and those which were virus structural proteins, virions labelled with [^{35}S]methionine were purified by sedimentation in sucrose gradients in which the virus had a buoyant density of approximately 1.18 g/ml. Electrophoretic analysis of material released into the medium from mock-infected and infected cells is shown in Fig. 4. This revealed that p53, p43, p40 and p35 were virion-associated. In addition a polypeptide of approximately 200K was associated with virions.

When [^{3}H[glucosamine-labelled material released from cells was examined in the same way virtually no glycopolypeptides were detected from mock-infected cells in the sucrose gradient fractions (Fig. 5, tracks a–f) corresponding to those that contained virions (Fig. 5, tracks g–l). The major band was diffuse and had a M_r of 82K (p82). Above this was another diffuse band of M_r 97K while [^{3}H]glucosamine-labelled material of M_r approximately 35–45K was also reproducibly detected. Dissociation of virus in the absence of 2-mercaptoethanol resulted in p82 migrating with an apparent M_r of 115K (p115) (Fig. 5, tracks n and o). When p82 and p115 were recovered from a gel and re-electrophoresed without reduction they migrated

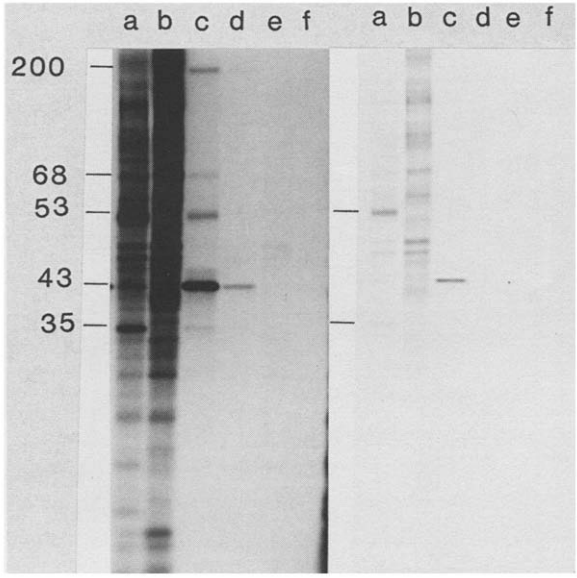

Fig. 3. Radioimmunoprecipitation of TRTV proteins. The two panels represent the same polyacrylamide gel exposed for different times. (a, b) extracts of (a) TRTV-infected and (b) non-infected cells, labelled with [^{35}S]methionine, after lentil-lectin affinity selection. (c–f) Immunoprecipitates obtained after immunoprecipitation of infected-cell extract with (c) turkey anti-TRTV serum and (d) pre-immune turkey serum, and uninfected cell extract with (e) turkey anti-TRTV serum and (f) pre-immune turkey

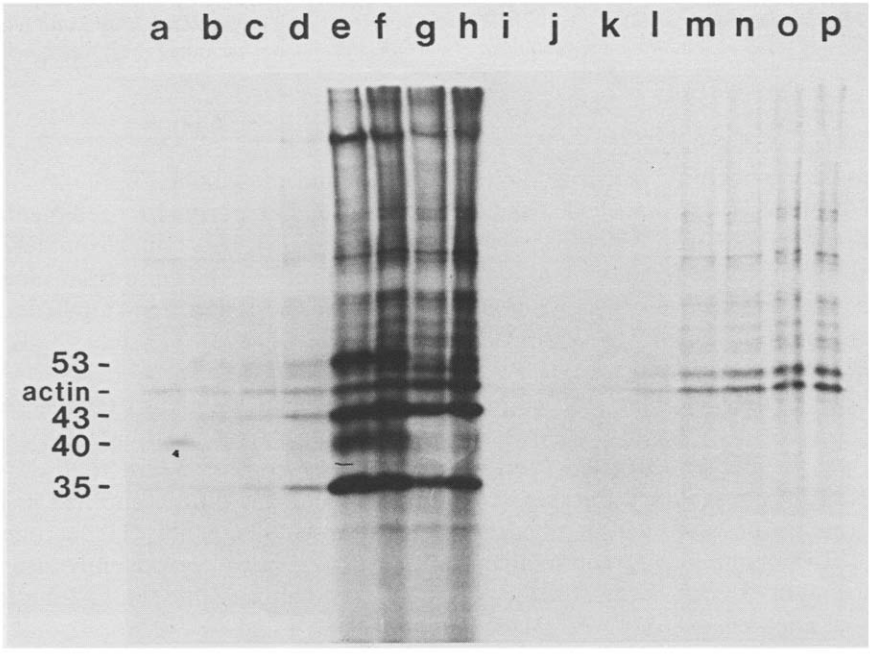

Fig. 4. SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labelled polypeptides associated with TRTV virions. Material released from infected and uninfected cells cultured with AMD was sedimented in 25–55% sucrose gradients for 16 h. Alternate fractions, starting with fraction 6 and ending with fraction 20, were analysed; material from (a–h) infected and (i–p) uninfected cells. Sedimentation was from left to right. The numbers show the M_r ($\times 10^{-3}$) of virion proteins.

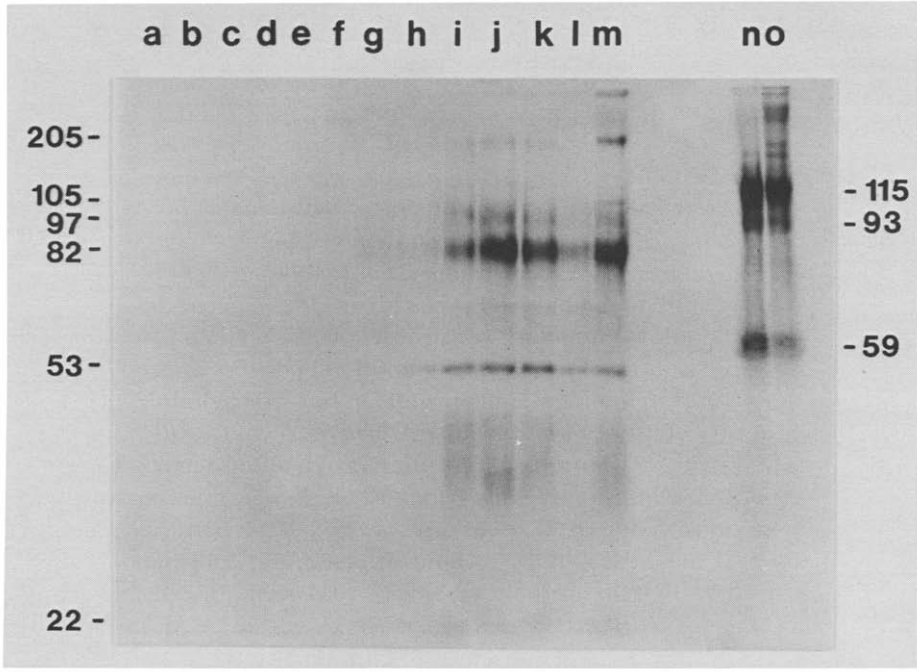

Fig. 5. SDS-polyacrylamide gel electrophoresis of [^{3}H]glucosamine-labelled polypeptides associated with TRTV virions. Material released from infected and uninfected cells cultured with AMD was sedimented in 25–55% sucrose gradients for 16 h. Fractions 11–16 were analysed after dissociation at 100 °C with SDS and mercaptoethanol; material from (a–f) uninfected and (g–l) infected cells. Sedimentation was from left to right; (m) same as track (j) but dissociated at room temperature with SDS and mercaptoethanol. Material in fraction 14 (track j) was also dissociated with SDS but without mercaptoethanol at (n) 100 °C and (o) room temperature. The numbers show the M_r ($\times 10^{-3}$) of virion proteins.

with unchanged M_r values of 82K and 115K (Fig. 6, tracks a and b). However, when the recovered polypeptides were reduced most of p115 comigrated with p82 (Fig. 6, tracks d and e). The 115K material may be a dimer of p82 which comigrates anomalously fast; BSA (M_r 69K) electrophoresed with an apparent M_r of only 57K following denaturation with SDS but without reduction. Alternatively the tertiary structure of p82 may be stabilised by disulphide bonds in such a way that electrophoretic migration is retarded when these bonds are not broken by reduction.

Coelectrophoresis of [^{3}H]glucosamine-labelled and [^{35}S]methionine-labelled virus clearly showed that the p53 glucosamine-labelled polypeptide comigrated with the [^{35}S]methionine-labelled p53 polypeptide (data not shown). We have also shown that ^{3}H-labelled p53 coelectrophoreses with [^{35}S]methionine labelled p53 which bound to lentild-lectin (not shown). When virus was dissociated with SDS and mercaptoethanol at room temperature instead of at 100 °C the amount of p53 diminished concomitant with an increase of material of M_r 205K and the appearance of a band of M_r 105K (Fig. 5, compare track m with j). These bands may

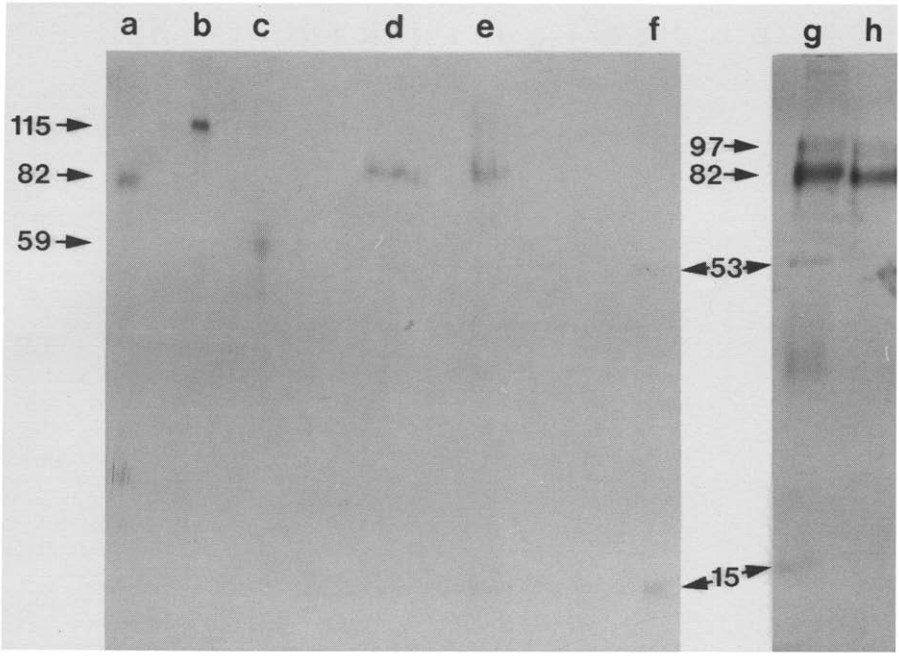

Fig. 6. Re-electrophoresis in SDS-polyacrylamide gels of selected [^{3}H]glucosamine-labelled TRTV polypeptides with and without reduction by mercaptoethanol. Bands of M_r 115K, 82K and 59K were cut out of the gel shown in Fig. 4, re-hydrated in buffer containing SDS without (a–c) or with (d–f) mercaptoethanol, and re-electrophoresed: (a and d) 82K, (b and e) 115K, (c and f) 59K bands. (g, h) Effect of tunicamycin on the glycosylation of TRTV proteins. TRTV virions were radiolabelled with [^{3}H]glucosamine in the absence (g) or (h) presence of tunicamycin. The numbers show the M_r ($\times 10^{-3}$) of virion proteins.

represent multimers of p53. In addition to the feint 22K [^{3}H]glucosamine-labelled polypeptide (Fig. 5) an additional ^{3}H- but not ^{35}S-labelled glycopolypeptide (M_r 15K) was detected when the electrophoresis time was decreased such that no polypeptides migrated from the gel (data not shown). When virus was dissociated at 100 °C with SDS but without mercaptoethanol (Fig. 5, track n) no p53 or the feint band of 22K was detected. Instead there were diffuse bands of M_r 59K (p59) and 93K (p93). Denaturation at room temperature without reduction (Fig. 5, track o) resulted in a decrease of p59 and p93 and an increase in material near the top of the gel. Reduction of p59, excised from a gel, resulted in the conversion of p59 to p53 and p15 (Fig. 6, compare tracks c and f). The most likely explanation for these results is that p59 corresponds to the fusion glycoprotein of members of the Paramyxoviridae family, p53 and p15 corresponding to the fusion protein glycopolypeptides F1 and F2, respectively. In the absence of reduction the cleaved fusion protein (F1,2) has an apparent M_r of 59K and comprises F1 and F2 linked by a disulphide bond. The p68 present in infected cell lysates and detected by electrophoresis under reducing conditions (Fig. 2) is most probably the uncleaved F

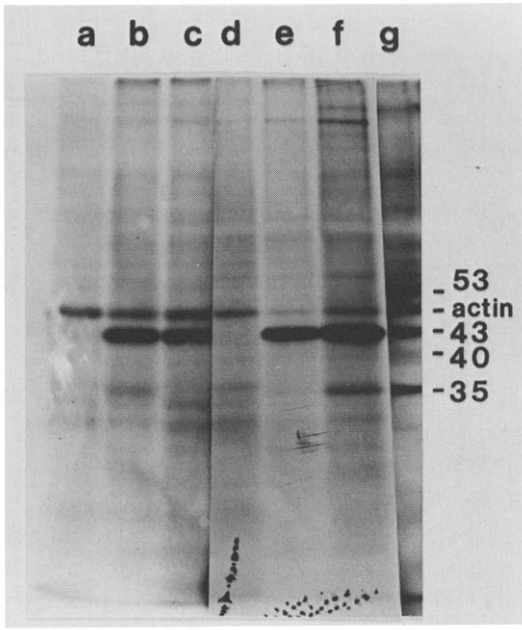

Fig. 7. Polypeptide composition of TRTV nucleocapsids. (a, d) Uninfected and (b, c, e, f) infected cells were labelled with [^{35}S]methionine in the presence (a, b, d, f) or absence (c, e) of AMD. After solubilization of the cells with non-ionic detergent and clarification the extracts were centrifuged at 100000 × *g*. Some of the pelleted material was electrophoresed (a–c) and the remainder was centrifuged to equilibrium in a potassium tartrate gradient. Peak fractions were pooled, the nucleocapsids pelleted and then electrophoresed (d–f). (g) [^{35}S]methionine-labelled virion polypeptides. The figure is a composite of two fluorograms exposed for different lengths of time. The numbers show the M_r ($\times 10^{-3}$) of virion proteins.

protein (F0). The material of 93K and > 200K probably represent aggregates of p59 (F1,2).

Pelleted nucleocapsids from detergent-solubilised TRTV-infected cells had p43 as the major protein component, with a small amount of p35 (Fig. 7, tracks b, c). Isopycnic sedimentation of this preparation through a potassium tartrate gradient gave a peak of density 1.25 g/ml, p43 being the major component (Fig. 7, tracks e, f).

Effect of tunicamycin on glycosylation of TRTV proteins

The nature of the glycans associated with p82, p53 and p15 was investigated by growth of TRTV in the presence and absence of 1 μg/ml of tunicamycin. This drug inhibits the N-glycosylation of asparagine residues but not the O-linked glycosylation of serine and threonine residues. The potency of the tunicamycin was verified by [^{35}S]methionine-labelling of avian infectious bronchitis coronavirus (IBV), strain Beaudette, in Vero cells. The tunicamycin inhibited glycosylation of the matrix protein and resulted in virions which lacked spike protein, as reported for the effect of tunicamycin on the replication of IBV in chick kidney cells (Stern and Sefton,

1982) (data not shown). Tunicamycin resulted in an approximately 3K decrease in the M_r of [^{3}H]glucosamine-labelled p82, the resultant protein still being associated with glycans (Fig. 6, tracks g and h). In addition the M_r of p97 had decreased to a similar extent, further indicating its relationship with p82. The glycosylated material in the M_r range 35–45K was no longer detectable after replication in the presence of tunicamycin.

No p53 or p15 was detected by [^{3}H]glucosamine-labelling after TRTV replication with tunicamycin (Fig. 6, tracks g and h) indicating that all of the [^{3}H]glucosamine of p53 and p15 was associated with tunicamycin-sensitive N-linked glycans.

Discussion

The mRNA profile of TRTV closely resembles that of respiratory syncytial (RS) virus, a pneumovirus (Huang and Wertz, 1983). Seven major RNA species have been reported in RS virus infected cells, ranging in M_r from 0.26 to 2.5×10^6, but some of these bands represent two or three mRNAs. In addition there are several other bands which are polycistronic read-through transcripts (Huang and Wertz, 1983; Dickens et al., 1984). As shown in Fig. 1 and elsewhere (Barrett and Underwood, 1985) the mRNA profile of the morbilliviruses is distinct from that of the pneumoviruses, as is that of the paramyxoviruses, the other genus of the Paramyxoviridae (Wilde and Morrison, 1984), particularly with regard to the absence with the morbilliviruses and paramyxoviruses of mRNAs of less than 0.4×10^6.

The results of our analysis of the proteins induced by TRTV, supported by the RNA data, indicate that this virus is a member of the Paramyxoviridae family and that it most closely resembles members of the *Pneumovirus* genus. Thus comparison of the polypeptides in mock-infected and infected cells, lentil-lectin affinity selection, analysis of purified virions and nucleocapsids, tunicamycin inhibition of glycosylation and immunoprecipitation have shown that p82, p68, p53, p15, p43, p40, p35 and polypeptides of M_r 22K and about 200K are virus specified proteins. Our results complement and extend those recently published by Collins and Gough (1988) and Ling and Pringle (1988). Collins and Gough (1988) analysed TRTV grown in chick embryo fibroblasts by Coomassie Blue and Schiff's staining of virion polypeptides separated by SDS-polyacrylamide gel electrophoresis (Table 1). Schiff's staining showed that polypeptides of M_r 84K and 54K were glycosylated although it did not detect glycans on the 15K polypeptide. Ling and Pringle (1988) observed that two glycosylated polypeptides, M_r 45K and 15K, ran as a single 57K band under non-reducing conditions, which indicates that these are analogous to p53, p15 and p59 described in this communication (Table 1). A comparison of the structural polypeptides of the three genera of the Paramyxoviridae is shown in Table 1. On the basis of molecular weight alone three polypeptides discriminate the pneumoviruses from the other two genera, p24/25 (not possessed by the *morbilli-* and *paramyxovirus* genera) N and P (Wunner and Pringle, 1976; Levine, 1977; Cash et al., 1977). TRTV had a 22K polypeptide detected after lentil-lectin affinity selection of

TABLE 1

COMPARISON OF THE M_r VALUES, DETERMINED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE VIRION PROTEINS OF TRTV AND THE THREE GENERA OF THE PARAMYXOVIRIDAE

Polypeptide	*TRTV*			*Pneumovirus*	*Morbillivirus*	*Paramyxovirus*
	(a) [1]	(b) [2]	(c) [3]			
L	200 [a]	200 [a]		160–200	160–200	>160
HNO	97 [a]					82
G, H, HN	82	84	83	84–90	76–85	65–74
F0	68			66–70	60–62	60–68
F1	53	54	45	43–50	40–41	48–59
F2	15	14	15	19–24	20–25	10–15
N	43	42	38	41–45	60	56–61
P	40	37	35	32–37	70–73	75–84; 54 (NDV) 45 (mumps virus)
M	35	31	30	27–33	34–38	34–41
Other				24–25		

[a] The identity of these polypeptides awaits confirmation.

[1] Data from Cavanagh and Barrett.

[2] Data from Collins and Gough (1988).

[3] Data from Ling and Pringle (1988).

References: pneumoviruses: Cash et al., 1977; Levine, 1977; Peeples and Levine, 1979; Pringle et al., 1981; Dubovi, 1982; Gruber and Levine, 1983; morbilliviruses: Campbell et al., 1980; Rima, 1983; paramyxoviruses: Mountcastle et al., 1971; Lamb et al., 1976; Waxham and Wolinsky, 1986.

[^{35}S]methionine-labelled cell-associated proteins which may be analogous to the p24/25 virion-associated polypeptide of RS virus. A 19K polypeptide was detected by Ling and Pringle after both in vivo and in vitro translation of TRTV mRNA. The 22K polypeptide and p43 and p35 which bound to lentil-lectin (Fig. 2) may have done so either non-specifically or because they were associated with the glycoproteins p68 and p53. As with RS virus (Wunner and Pringle, 1976; Peeples and Levine, 1979) nucleocapsid preparations of TRTV consisted predominantly of p42, clearly showing that p42 is the nucleocapsid (N) protein. Characteristically the pneumovirus N protein has a significantly lower M_r than that of the morbilliviruses and paramyxoviruses (Table 1). So, too, has the phosphorylated (P) protein. Although we have no direct evidence to identify p40 it is likely that this is the P protein of TRTV. P35, by virtue of its M_r, its abundance in infected cells and its presence in small quantities in nucleocapsid preparations (Peeples and Levine, 1979) is likely to be the matrix (M) protein.

The largest glycopolypeptide of the Paramyxoviridae is the HN, H and G protein of the *Paramyxovirus, Morbillivirus* and *Pneumovirus* genera, respectively. Features of the TRTV p82 indicate that this is analogous to the G protein of pneumoviruses. As with G of RS virus, p82 was not usually detected in cell extracts or virions labelled with [^{35}S]methionine (Wunner and Pringle, 1976; Cash et al., 1977; Pringle et al., 1981; Dubovi, 1982; Gruber and Levine, 1983) although the RS virus G

protein could be detected when [^{3}H]leucine or a mixture of radiolabelled amino acids was used (Levine, 1977; Peeples and Levine, 1979; Dubovi, 1982). Cloning and sequencing of the RS virus G protein has shown that this is due to the presence of only 2 methionine residues (Wertz et al., 1985; Satake et al., 1985). The p82 of TRTV was readily detected by radiolabelled glucosamine; indeed it was the major glycopolypeptide detected in this way, an observation also made by Ling and Pringle (1988) and by Collins and Gough using Schiff's reagent and with RS virus (Levine, 1977; Dubovi, 1982; Pringle et al., 1981; Gruber and Levine, 1983). A further indication that p82 is structurally more closely related to G of RS virus than to the H or HN of the paramyxoviruses and morbilliviruses is our observation that tunicamycin reduced only slightly glycosylation of p82. Whereas the H and HN glycoproteins are believed to be associated solely with N-linked glycans the G protein of RS virus, whose polypeptide moiety is only 32.5K, has largely O-linked glycans and only 4 potential glycosylation sites for N-linked glycans. Consequently tunicamycin resulted in only a small M_r decrease leaving a protein which was still substantially glycosylated (Wertz et al., 1985; Satake et al., 1985). Tunicamycin sensitivity of the glycosylation of the TRTV 35–45K glycosylated material showed that the glycans were of the N-linked type. While the identity of this material is unclear it is possible that it is the polypeptide moiety of p82 associated with different amounts of only N-linked glycans. Ling and Pringle reported a broad, minor [^{3}H]glucosamine-labelled band, referred to as having a M_r of 31K, in immunoprecipitates of TRTV-infected cell lysates. A broad band of similar M_r was detected by Collins and Gough (1988) using Schiff's reagent but was not commented upon. Ball et al. (1986) observed that a vaccinia virus recombinant containing the RS virus G protein gene sometimes expressed minor glycopolypeptide species of lower M_r. They suggested that this might correspond to the 46K species observed by Gruber and Levine (1985) which was proposed as a precursor to the G protein.

Although sequencing has shown that G of RS virus is structurally very different from the HN and H glycoproteins, we observed that p82 of TRTV appeared to migrate as a dimer following denaturation without reduction, a feature of the HN protein of Newcastle disease virus (Garten et al., 1980) which has not been observed with RS virus G protein (Gruber and Levine, 1983). Alternatively, non-reduced p82 might have migrated more slowly than reduced p82 because of differences in tertiary structure. Some strains of Newcastle disease paramyxovirus produce virions which contain a precursor to HN, termed HNO, which has a M_r of about 8K greater than HN (Garten et al., 1980). The similarity between p97 and p82 with respect to the diffuse nature of the bands and the extent of their M_r decrease following replication in the presence of tunicamycin suggests that p97 is related to p82. Whether p97 is a precursor of p82 or simply a differently glycosylated form remains to be investigated.

Strains of all the Paramyxoviridae genera have a fusion protein precursor, FO, which is usually cleaved to produce a small aminoterminal F2 moiety with up to 4 N-linked glycosylation sites, and a large F1 moiety containing only 1 glycosylation site and a hydrophobic carboxy terminus. [^{35}S]methionine-labelled gp53 of TRTV

was readily detected in virions but not in infected cell extracts, as has been reported for F1 of RS virus (Cash et al., 1977) and the F protein of RS virus has been shown to dimerise when virions are dissociated at room temperature (Walsh et al., 1985; Walsh et al., 1986). Our observations of p68, p53 and p15, the aggregates of p53 and the association of p53 with p15 in the absence of reduction lead us to propose that p68, p54 and p15 are analogous to F0, F1 and F2, respectively, of other viruses of the Paramyxoviridae family, and that the 59K glycopolypeptide is the form in which cleaved F migrates in non-reducing gels. The absence of p68 in virions indicates that TRTV from Vero cells has F in the completely cleaved form. The inhibition of glycosylation of p53 and p15 by tunicamycin shows that their glycans are of the N-linked type, a feature common to the F proteins of all three genera of the Paramyxoviridae.

Further insight into the relationships of TRTV with other members of the *Pneumovirus* genus will be forthcoming from comparisons of gene organisation and the sequence of individual genes. Such studies are in progress.

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