Arch Virol (1991) 119: 153–163

Archives of Virology

The free nucleocapsids of the viral haemorrhagic septicaemia virus contain two antigenically related nucleoproteins

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Accepted January 8, 1991

Summary. A protein of 34 kDa (Nx) was induced in vitro by the infection of fish cell cultures with the rhabdovirus agent of viral haemorrhagic septicaemia (VHS) of the trout. This protein only appeared as a major component in concentrated or intracellular labeled VHS virus but not in purified VHS or in the related infectious haematopoietic necrosis virus. That Nx protein is antigenically related to the nucleoprotein of purified virus was shown by its reaction with four antinucleoprotein monoclonal antibodies (at least 3 of them reacting non competitivly against different epitopes) and by immunoprecipitation with polyvalent international reference sera. The Nx protein was shown to be specifically associated with free non-infective particles isolated by ultracentrifugation which were confirmed to be nucleocapsids by electron microscopy.

Introduction

There are two salmonid rhabdoviruses (family *Rhabdoviridae*, genus *Lyssavirus*) of economic importance, which cause infectious haematopoitetic necrosis (IHN) and viral haemorrhagic septicaemia (VHS). Their structural proteins are designated L (the RNA-dependent RNA polymerase of 200 kDa), N (the phosphorylated nucleoprotein of 45–50 kDa), M_1 and M_2 (the matrix proteins of 22–28 kDa) and G (the glycoprotein of the spikes of 60–80 kDa). This protein composition closely resembles that of rabies virus rather than vesicular stomatitis virus in that there are two M proteins [8, 14, 17, 19].

Mammalian rhabdoviruses also contain small amounts of a phosphorylated nucleoprotein of 38–45 kDa called Ns, which is distinct from the N protein [23]. No protein of similar molecular weight is present in IHN virus, but a minor protein component was found in purified VHS virus and called Nx by De Kinkelin et al. [10]. Nx was also detected by immunoblotting of purified VHS virus with homologous monoclonal antibodies [18]. In contrast to the small proportion of Nx found [10, 18], we recently reported the presence of

Nx in higher concentrations (equivalent to N) in intracellular [^{35}S]methionine labeled virus. Five different isolates and the reference strains (F_1, F_2, and 23.75) of VHS virus showed such high amounts of labeled Nx whereas non-infected cells or IHN virus lacked any similar protein.

In this work we report the presence of Nx at high concentrations in concentrated but not in purified virus and have demonstrated the antigenic similarities between the N and Nx proteins, together with the specific association of the latter with free nucleocapsids of VHS virus.

Material and methods

Viruses

The strains of virus used were, VHS-F_1 and VHS-F_2, provided by Dr. P. E. Vestergard-Jorgensen, VHS-23.75 provided by Dr. De Kinkelin and IHN provided by Dr. Hedrick. Five VHS-F_1 isolates [16] were obtained from fish tissue samples in Spain [1]. These virus were isolated from: rainbow trout, *Salmo gairdneri* Richardson (689, from Galicia in 1984; 471, from Navarra in 1986; and 144, from Salamanca in 1984); *Salmo salar* Linneus (472, from Cantabria in 1986), and *Barbus graellsi* Steindachner (798, from Aragón in 1986). Unless otherwise indicated isolate 144 was used throughout experiments. It was cloned 3 times, frozen in aliquots and used to infect cultures at low multiplicity of infection (0.001 m.o.i.) to avoid the generation of defective particles.

Cells, media, and virus purification

Epithelioma papillosum cyprini (EPC) cells were used throughout the experiments. Other cells used were RTG-2 (rainbow trout gonad), CHSE (Chinook salmo embryo) and BF-2 (bluegill fibroblast). Cell culture techniques and media (RPMI-1640 medium, Flow, Ayrshire, Scotland) were essentially as reported by Basurco and Coll [1].

Cell culture supernatants from infected cell cultures were centrifuged at 3,000 **g** for 30 min and the pellet discarded. The supernatant was made, 7% polyethylenglycol 6,000, 2.3% NaCl and agitated at 4° C for at least 2 h. After centrifugation at 10,000 **g**, 45 min, the pellet was resuspended in a small volume of TNE (0.15 M Tris, 0.15 m NaCl and 1 mM EDTA, pH 7.6). Analysis of this fraction by gel electrophoresis showed to be 90% composed of viral proteins [1]. This fraction is referred to as concentrated virus. To further purify the concentrated virus it was ultracentrifuged through a 15 to 45% sucrose gradient in TNE at 80,000 **g** for 270 min in a Beckman 25-75 rotor SW 27. The band containing viral activity was collected. This fraction is termed purified virus. Analysis of this fraction by gel electrophoresis showed it to be 98% composed of viral proteins [1].

Virus plaque forming assays were performed as described [16] by using ultra-low gelling temperature agarose type X (Sigma Chemical Co., St. Louis, Mo.). The protein content of purified virus was estimated both by staining the whole preparation with Coomassie blue G-250, using BSA as standard protein (Biorad, Richmond, Va.) and by staining the viral protein bands separated by gel electrophoresis with Coomassie blue and comparing them to standards. Protein concentration was also determined by absorption at 280 nm using an extinction coefficient of 1.4.

Isotopic labelling of intracellular virion proteins

To label with [^{35}S]methionine, EPC cell monolayers at 90% confluence cultivated in 96 microwell plates (0.15 ml per well) were infected at a m.o.i. of 1–5 $TCID_{50}$/cell in medium containing 2% foetal calf serum (FSC). After 1 h of absorption at 14 °C, 150 µl of cell

culture media with 2% FCS was added to each well. Cells were incubated at 14 °C until 70% cytopathic effect (about 36–48 h). At this time, the culture medium was removed, and 40 µl of methionine free media were added. 15 min later, media was replaced with 40 µl of methionine free media containing 200 µCi of [^{35}S]methionine/ml (Amersham, Paris, France) and maintained for 2 to 3 days at 14 °C. The label was removed and the remaining cells were washed twice with 50 µl of serum free media, pH 7.4. The cells were then lysed with 40 µl of gel electrophoresis buffer and stored at −70 °C. The immunoprecipitations were carried out in dissociating buffer as described [1]. The separation of the SDS and mercapoethanol dissociated proteins was performed by SDS-polyacrylamide gel electrophoresis. The gels were composed of a slab of 15–20% polyacrylamide gradient with a stacking gel of 6% polyacrylamide. The gels were stained with Coomassie blue and dried onto cellophane. Dried gels of isotope labelled samples were exposed to Agfa Curix RP-2 X-ray film for 1–4 days at room temperature before development [1].

To label with [^{32}P]phosphorous, EPC cells in 96-well microtiter plates were infected with VHS virus at m.o.i. of 0.4. One day after infection, cells were washed twice with medium lacking phosphorous, then 50 µl of the same medium containing 100 µCi of carrier free [^{32}P] (Amersham, The Netherlands) were added and the cells were incubated until 70% cytopathic effect was obtained. Then remaining cells were washed, resuspended in electrophoresis buffer and electrophoresed as above.

Production of anti-VHS monoclonal antibodies (MAbs)

Female mice (BALB/c) were given 9 intraperitoneal injections of 25 µg of concentrated VHS viral protein over a period of 9 months [20]. Antibodies against VHS virus were detected by indirect ELISA and immunoblotting [1]. Spleen cells from the immunized mice were fused with the myeloma celine P3-X63-Ag8653 as described [15]. Fusion, cloning twice by limiting dilution and cultivation of hybridoma cells and ascites production were performed as described previously [20, 22].

The assay used for the screening of the hybridoma supernatants was essentially as described [15, 20]. Briefly, microtiter plates (Dynatech, Plochingen, Federal Republic of Gemany) were coated to dryness with 0.5 µg of viral VHS proteins (concentrated virus), washed for 15 min and kept dried for weeks. Plates coated with concentrated EPC cell extracts were used as controls. Horseradish peroxidase-conjugated rabbit immunoglobulin to mouse immunoglobulins (Nordic, Tilburg, The Netherlands) was used to develop the reaction between hybridoma supernatants and the virus-coated solid-phase. An ascites pool from immunized mice [5] at a dilution of 1,000-fold was used as positive control. Development was with o-phenylenediamine, using the low background buffer as described previously [7]. A well was considered positive when its absorbance at 492 nm in the virus coated wells was above 0.3, compared with the absorbance in the well coated with non-infected cells.

Immunoblotting

The protein bands were transferred from 12% polyacrylamide gels to nitrocellulose membranes as described [6]. After blocking with dilution buffer (0.5% bovine albumin, 0.1% Tween-20, 0.01% merthiolate, 5 mg/l phenol red in 10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.6) the strips were incubated for 1 h with the hybridoma supernatants diluted 1:2 in dilution buffer. The bands were developed with 1 mg/ml diaminobenzidine in citrate buffer as described [7].

Competition among MAbs

Anti-N MAbs numbers 4, 5, and 6 were obtained as mouse ascites as described [5], purified by affinity chromatography over immobilized protein A sepharose (Pharmacia, Uppsala, Sweden) and conjugated to horseradish peroxidase (E.C. 1.11.1.7.) by the one-step glutar-

aldehyde method as described [6]. Only the conjugates of MAbs numbers 5 and 6 gave enough titre to perform competition experiments by using a similar ELISA assay as the one described above for screening the hybridoma supernatants. Briefly, the wells coated with viral VHS proteins (1 μg/well) were allowed to react with MAbs 4, 5, or 6 (1 μg/well) and then each one was reacted with conjugates 5 or 6 (0.2 μg/well). Only the homologous MAb was able to compete the binding of their respective conjugate (not shown). MAb 4 did not compete with the MAb conjugates 5 or 6.

Electron microscopy

Specimens (never frozen) for electron microscopy were fixed in 6% buffered glutaraldehyde, treated with 1% osmium tetroxide, dehydrated in ethanol, stained with uranyl acetate and lead citrate [21] and observed with JEOL 100-B electron microscope.

Results

Presence of large amonts of Nx

The presence of the Nx protein was demonstrated in concentrated virus obtained from each of the cell lines, EPC, CHSE, BF or RTG-2, infected with VHS virus. In all the cases the estimated molecular weight was 34 kDa.

To measure the variations of the relative amounts of Nx the supernatants obtained from EPC cell cultures after infection with 5 isolates of VHS virus, 3 reference strains of VHS virus and 1 strain of IHN virus, were concentrated and pelleted through a 20% sucrose cushion by ultracentrifugation. By densitometry of the Coomassie blue stained bands after electrophoresis the viral particles isolated in the above way contained between 38 to 42% of the total protein as N (38 kDa) and 7 to 20% of the total protein as Nx (34 kDa). The IHN virus had N but showed no detectable Nx. The VHS virus serotype F_2,

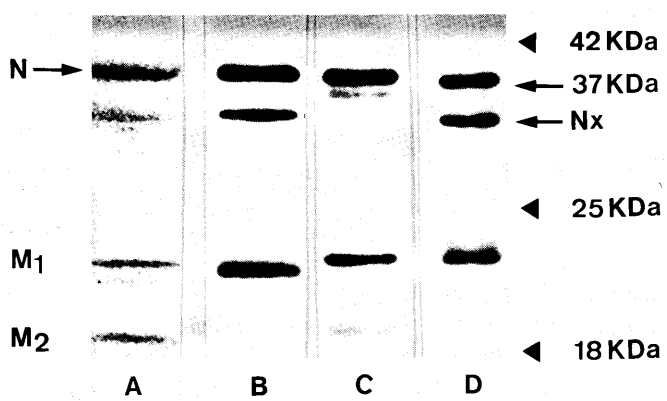

Fig. 1. Electropherogram of VHS virus in the 18–42 kDa region. **A** Coomassie-blue stained concentrated virus ultracentrifuged through a 20% sucrose cushion. **B** Autoradiography of [^{35}S]methionine labeled supernatant from infected cultures immunoprecipitated with standard international reference antisera. **C** Autoradiography of 2 h [^{35}S]methionine labeled infected cells. **D** Autoradiography of 3 day [^{35}S]methionine labeled infected cells. *N*, M_1, M_2 Viral proteins of VHS virus as identified by the molecular weight markers run in parallel (right) and by purified virus. The long arrows to the right indicate the position of the Nx and the 37 kDa proteins

consistently contained the highest proportion of Nx. The Nx protein remained associated with concentrated particles after the virus was treated with 1% of either Triton-X 100 or Tween 20 for 1 h and then pelleted through a 20% sucrose cushion by ultracentrifugation, whereas the G protein was lost from these particles (not shown).

The synthesis of Nx was studied by labeling intracellular virus, starting with infected cell cultures at 70% of cytopathic effect to minimize the background of host cellular synthesis. Large amounts of Nx appeared labelled after 3 days (Fig. 1 D), however only N was labelled with short 2 h pulses. After these short pulses a protein of intermediate molecular weight (about 37 kDa) appeared also labelled instead of Nx (Fig. 1 C). A 3–5 fold increase in the label of Nx could be detected if the 2 h pulses were chased with cold methionine for 24 additional hours. By using [^{32}P]phosphorous in similar experiments, the N protein was labelled (not shown), but no label could be demonstrated in Nx or 32 kDa protein regions.

Antigenic relationship between the N and Nx proteins

Simultaneous immunoprecipitation of N and Nx occurred with anti-VHS virus antisera (Fig. 1 B). Both standard international reference antisera raised in rabbits (anti-VHS virus serotypes $F_1+F_2+23.75$) and ascites raised in mice (anti-VHS isolate 144) coprecipitated N and Nx.

To demonstrate that the simultaneous immunoprecipitation was not due to interaction between N and Nx, we raised 3 anti-N MAbs against concentrated VHS virus and obtained 1 anti-N MAb raised against purified VHS virus (gift

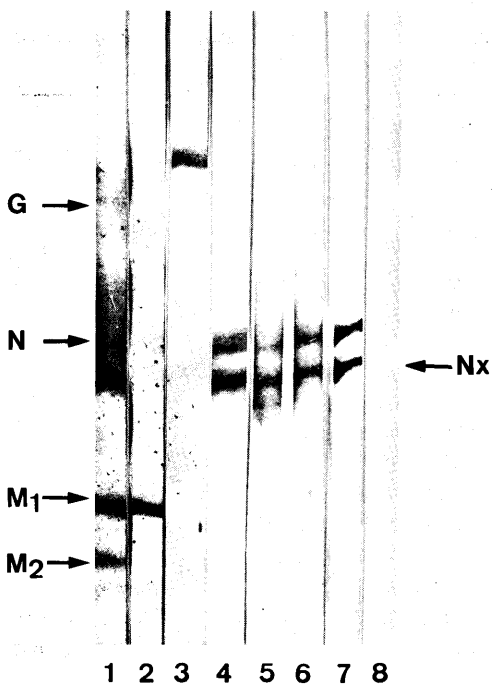

Fig. 2. Simultaneous recognition of N and Nx proteins with anti-N monoclonal antibodies by immunoblotting. Concentrated VHS virus was electrophoresed and transferred to nitrocellulose. The strips of nitrocellulose were incubated with 3-fold diluted hybridoma supernatants and developed as indicated. Viral proteins (*G, N, M_1, M_2*) were identified by Coomassie blue staining of the gel and amidoblack staining of the nitrocellulose filter. *1* Polyclonal ascites anti-VHS raised in mice; *2–7* supernatants from anti-VHS selected hybridomas: *2* anti-M; *3* anti-G; *4–6* anti-N; *7* anti-N from Dr. Vestergard-Jorgensen [18]; *8* supernatant from non-producer hybridoma

of Dr. Vestergard-Jorgensen). All 4 anti-N MAbs reacted by immunoblotting with both N and Nx proteins after separation by electrophoresis of concentrated virus under denaturing conditions (Fig. 2). To confirm the immunoblotting results, N and Nx were isolated by electroelution after preparative electrophoresis. Purity, as estimated by densitometry of the silver nitrate stained gels following reelectrophoresis of the isolated proteins, was 90% for N and 73% for Nx (the rest of the protein being Nx and N, respectively).

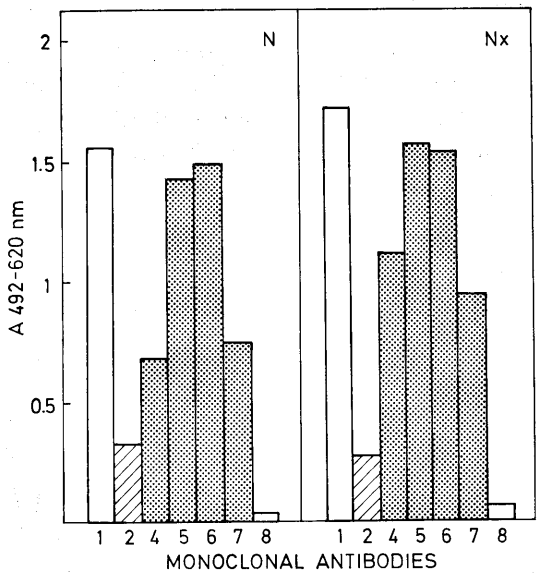

Fig. 3. Reaction between purified N or Nx with anti-N monoclonal antibodies by ELISA. Proteins N and Nx were isolated by electroelution of polyacrylamide gel electrophoresed bands of concentrated VHS virus. Purified N (90% purity) and Nx (73% purity) were used to coat plates at 0.3 μg of protein per well and hybridoma supernatants diluted 10-fold, were used to react with the wells. *1* Polyclonal ascites anti-VHS; *2* anti-M monoclonal antibody; *4–6* anti-N monoclonal antibodies; *7* anti-N from Dr. Vestergard-Jorgensen; *8* non-producer hybridoma

Table 1. Reaction of anti-N monoclonal antibodies with 5 VHS virus isolates and 3 reference VHS virus strains

Anti-N monoclonal antibodies	Purified/concentrated virus							
	789	471	472	689	144	F_1	F_2	23.75
4	2.0/1.9	2.2/1.9	2.0/2.0	1.9/1.9	1.8/1.5	2.2/1.5	1.9/1.6	1.8/1.4
5	2.4/1.9	2.5/1.9	2.0/2.4	2.2/2.0	2.2/1.8	2.2/1.5	2.0/2.0	2.2/1.5
6	2.2/1.9	2.3/1.9	1.9/2.4	2.1/2.4	2.1/1.9	1.9/1.6	2.0/2.1	2.0/1.6
7	2.0/1.2	1.8/1.3	1.8/1.4	1.8/1.2	1.8/1.0	1.3/1.0	1.6/1.2	1.5/1.0

The ELISA assays were performed by coating the wells with either 0.3 μg of protein from purified virus or concentrated virus. The supernatants of the hybridomas were diluted 20-fold in dilution buffer so as to produce an ELISA titre of the same order of magnitude. The results were finally normalized by the ELISA absorbances obtained in parallel with rabbit polyvalent antisera ($F_1+F_2+23.75$) used as international reference standard. The correction factor varied between 1–2 fold. The results were similar whether rabbit anti-VHS 144 antisera or mouse polyclonal anti-VHS ascites were used for normalization. Background values obtained by coating the wells with uninfected cells were 0.1. Numbers of monoclonal antibodies as shown by the immunoblotting (Fig. 2). IHN virus did not show any reactivity

ELISA performed with isolated N or Nx coated directly to the solid-phase and the MAbs, did not show any significant differences between them (Fig. 3). Furthermore, the 4 anti-N MAbs recognized to the same extent purified (99.5% N and 0.5% Nx) and concentrated virus (60% N and 40% Nx) from each of 5 isolates and 3 reference VHS viral strains (Table 1).

Association of Nx with free nucleocapsids

Concentrated VHS-144 virus contained 12% of the total protein as Nx. Since the purified virus contained less than 0.5% of Nx (as estimated by densitometry of Coomassie blue stained gels), we searched for the rest of Nx throughout the sucrose gradient used for the ultracentrifugation. The gradient was divided in 6 fractions and aliquots of each fraction were examined by electrophoresis and by virus titration. Fractions 2 and 5 had 48% of the total sedimenting protein (32 and 16%, respectively).

Fraction 2 banded at 31% sucrose and contained L, G, N, M_1, and M_2 proteins whereas fraction 5 banded at 26% sucrose and contained N and Nx proteins (Fig. 4). The relative amounts of Nx protein increased from $\leqslant 0.5\%$ (fractions 1 and 2) to 8% (fraction 3), 36% (fraction 4) and 58% (fractions 5 and 6). Only one protein band, presumably albumin (67 kDa) was found in the supernatant initially overlayed on the sucrose gradient. The percentages of the total $TCID_{50}$ recovered from the gradient were distributed as follows, 2.7, 94.5, 2.3, 0.2, 0.1 and 0.1 in fractions 1 to 6, respectively. No detectable viral infectivity

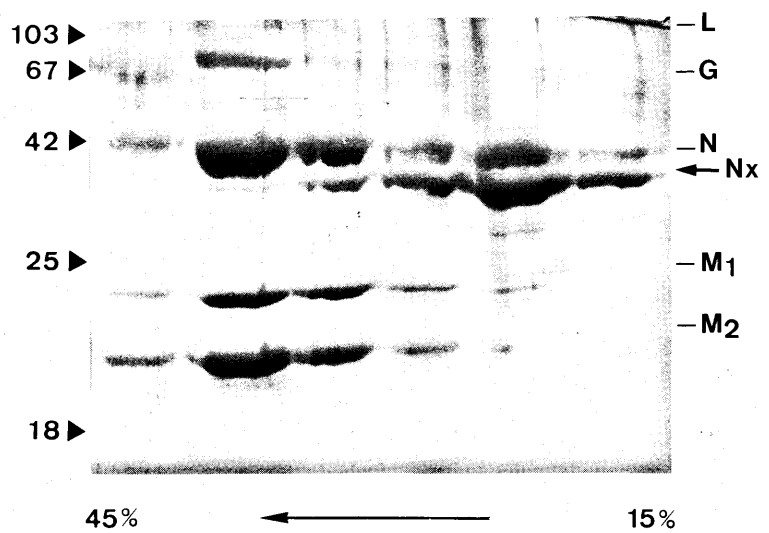

Fig. 4. Protein profile of ultracentrifuged VHS virus fractions by gel electrophoresis. Concentrated VHS-144 virus was ultracentrifuged in a sucrose gradient. Fractions of 3 ml were collected and concentrated by cold acetone precipitation overnight. Numbers to the left are the positions of molecular weight markers run in parallel (kDa). Letters to the right indicate the postion of viral proteins. The 34 kDa protein (Nx) position is indicated by the horizontal arrow. The direction of the sucrose gradient is indicated in the bottom of the figure

could be found in the overlayed supernatant. The residual infectivity (0.1%) could be removed from fraction 5 by reultracentrifugation without altering its protein composition (not shown).

Fractions 2 and 5, prepared for electron microscopy by negative staining, appeared as shown in Fig. 5. Fraction 2 contained complete viral particles of average length of 169 nm. Fraction 5, however, contained two different structures, small circles of average diameter of 22.2 nm surrounded by a thin apparent coat (Fig. 5, A) and cylinders with striations parallel to the axis of the particle with different lengths (Fig. 5, B). Long filaments connecting the small circles were also visible. The released structures were highly heterogeneous in lenght and appeared distributed throughout the gradient in three different experiments (not shown).

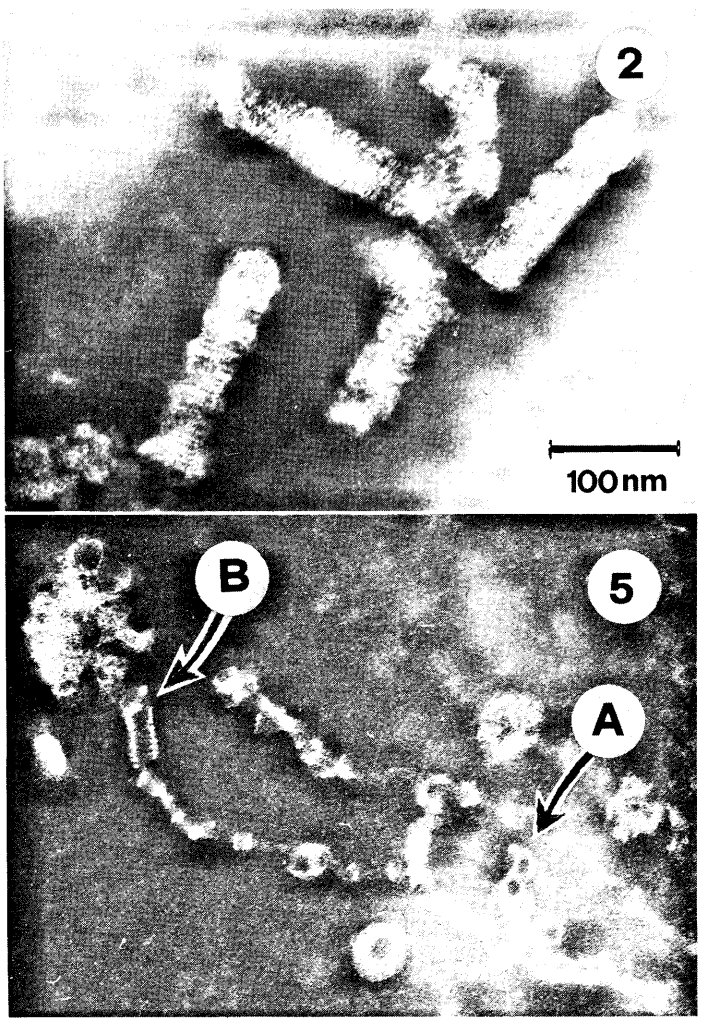

Fig. 5. Electron microscopy of negative-stained ultracentrifuged VHS virus bands numbers 2 (*2*) and 5 (*5*) from Fig. 4. Arrows indicate, *A* small circles of average diameter 22.2 nm and *B* cylinders

That both fraction 2 and 5 contained nucleic acids was shown by labeling with [^{32}P]. Both fractions contained radioactivity, of which at least 50% was recovered in the aqueous phase after phenol extraction (not shown).

To investigate the possible generation of Nx from N by artefactual handling, the particles isolated in fractions 2 and 5 were kept frozen during 4 months at −30 °C, thawed, heated at 37 °C for 24 h and studied by gel electrophoresis. The Nx neither appeared (fraction 2) nor increased or disappeared (fraction 5) after this treatment. In contrast to these results, the amount of M_2 decreased significatively in fraction 2 after the above treatment (not shown).

Discussion

A major protein component of 34 kDa (Nx) was found in all the concentrated VHS virus studied, including the international reference serotypes, F_1, F_2, and 23.75, and was independent of the cell line used to grow the virus. The Nx protein could also be demonstrated by intracellular [^{35}S]methionine labeling and by immunoprecipitation of infected cell supernatants with standard international reference antisera. A similar protein was absent from parallel studies with IHN virus. Because a protein of the same molecular weight, although much less abundant, was observed before and called Nx [10] this major protein has been also called Nx. A similar, though faint band of protein was found when analyzing anti-N VHS virus MAbs by immunoblotting with purified virus proteins [18].

Whether or not the Nx is equivalent to the Ns of other rhabdoviruses is not known. However, Ns is phosphorylated and encoded by a different gene than N [11, 23]. In contrast to Ns, Nx does not seem to be phosphorylated [8, 14, 17, 19] and we have demonstrated that Nx is antigenically related to N since 4 different MAbs (3 raised in our laboratory against concentrated virus and 1 raised outside against purified virus) reacted with both N and Nx (Figs. 2 and 3 and Table 1). Very recently, Bernard et al. [2] described the recognition of N and Nx proteins by an independent anti-N MAb. No other anti-N MAb is available at present. The use of concentrated rather than purified virus [18] for the immunoblotting explains the higher intensity of Nx found in our studies with the same anti-N MAb used by others [18]. The Nx and N proteins do not seem to be easily degraded or converted into each other following storage. The results of the experiments using radio labels suggested some kind of higher molecular weight precursor-product relationship (37 kDa protein) in which phosphorylation could also be involved.

There were no significant variations among the molecular weights of the N or Nx proteins from different VHS virus strains [1], in contrast to IHN virus, in which the size of N varies among different strains. There is no indication, however, of the existence of a Nx-like protein in IHN virus, or whether the variations in electrophoretic mobility of N result from differences in phosphorylation or size of the polypeptide chain [14]. These differences further distinguish IHN and VHS viruses [13] and could serve for diagnostic purposes.

The Nx protein was found associated with non-infectious particles isolated by ultracentrifugation and of nucleocapsid appearance in electron microscopy [14]. Its specific location in the upper part of the sucrose gradient after ultracentrifugation would explain why Nx has been only found in trace amounts in purified virus banding in the lower part of the gradient [9, 10, 18]. Nucleocapsids seem to be in excess in the cytoplasm because of their high content of Nx [1] and as infection lyses the cell they are released into the supernatant together with the whole virus. Only free nucleocapsids without matrix and G contain Nx in contrast to nucleocapsids within the whole virus.

A common characteristic of rhabdoviruses is the occurrence of defective truncated virions which are non-infectious because part of the RNA genome is deleted [9, 23]. The possibility existed, therefore, that the Nx was associated with defective viruses present in the same band of the sucrose gradient with the nucleocapsids [9]. However, the generation of defective particles, was minimized by infecting at low multiplicity [9] and no defective viruses could be found by electron microscopy in the fraction containing the nucleocapsids. Furthermore, the absence of interfering particles was confirmed by the addition of the fraction containing the nucleocapsids (fraction 5) to purified virus and testing the infectious capacity of the mixture as measured by PFUs (not shown).

Thus nucleocapsids containing a high content of Nx do not appear to be incorporated into mature virus particles. Further study of this phenomenon may be of value to understanding the mechanisms of virus assembly. On the other hand, attention has been recently focused on the role of nucleoproteins of mammalian rhabdoviruses in the immune response to viral infection and immunization [3, 12]. The Nx protein may give some clues about these processes in the case of VHS virus. It is also possible that Nx-like proteins may exist in other rhabdoviruses.

Acknowledgements

This work was supported by Instituto Nacional de Investigaciones Agrarias grant 8568 from the Ministero de Agricultura of Spain. Thanks are given to Dr. Vestergard-Jorgensen for the generous gift of anti-N monoclonal antibody. The rest of the monoclonal antibodies used were raised with the help of M. Balbin and Dr. J. Dominguez Juncal. The technical assistance of C. Hernandez, D. Frías, and P. Parrilla is appreciated.

References

1. Basurco B, Coll JM (1989) Spanish isolates and reference strains of viral haemorrhagic septicaemia virus show similar protein size patterns. Bull Eur Assoc Fish Pathol 9: 92–95
2. Bernard J, Lecocq-Xhonneux F, Rossius M, Thiry ME, De Kinkelin P (1990) Cloning and sequencing the messenger RNA of the N gene of viral haemorrhagic septicaemia virus. J Gen Virol 71: 1669–1674
3. Bunschoten H, Klapmuts RJ, Claassen IJTM, Reyneveld SD, Osterhaus ADME, Uytdehaag FGCM (1989) Rabies virus-specific human T cells clones provide help for an in vitro antibody response against neutralizing antibody-inducing determinants of the viral glycoprotein. J Gen Virol 70: 1513–1521

4. Cohen J, Lenoir G (1974) Ultrastructure et morphologie de quatre rhabdovirus de poissons. Ann Rech Vet 5: 443–455
5. Coll JM (1987) Injection of physiological saline facilitates recovery of ascitic fluids for monoclonal antibody production. J Immunol Methods 104: 219–222
6. Coll JM (1988) Immunochemical recognition of the binding of C-reactive protein to solid-phase phosphorylethanolamine. Rev Esp Fisiol 44: 169–178
7. Coll JM (1989) Addition of reducing agents to the peroxidase-o-phenylenediamine buffer reduces background of enzymeimmunoassays. Rev Esp Fisiol 45: 41–46
8. Deuter A, Enzmann PJ (1986) Comparative biochemical and serological studies on two fish-pathogenic rhabdoviruses (VHS-V and SVC-V). J Vet Med 33: 36–46
9. De Kinkelin P (1972) Le virus d'Egtved. II. Purification. Ann Rech Vet 3: 199–208
10. De Kineklin P, Berre MB, Bernard J (1980) Viral haemorrhagic septicaemia of rainbow trout: selection of a thermoresistant virus variant and comparison of polypeptide synthesis with the wild-type virus strain. J Virol 36: 652–658
11. Dietzschold B, Lafon M, Wang H, Otvos L, Celis E, Wunner WH, Koprowski H (1987) Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. Virus Res 8: 103–125
12. Ertl HCJ, Dietzschold B, Gore M, Otvos L, Larson JK, Wunner WH, Koprowski H (1989) Induction of rabies virus-specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. J Virol 63: 2885–2892
13. Gilmore RD, Leong JC (1988) The nucleocapsid gene of infectious hematopoietic necrosis virus, a fish rhabdovirus. Virology 167: 644–648
14. Hsu YL, Engelking HM, Leong JC (1985) Analysis of the quantity and synthesis of the virion proteins of infectious hematopoietic necrosis virus. Fish Pathol 20: 331–338
15. Iturralde M, Coll JM (1984) High stability hybrids producing monoclonal antibodies against human C-reactive protein. Rev Esp Fisiol 40: 279–288
16. Jimenez de la Fuente J, Marcotegui MA, San Juan ML, Basaurco B (1988) Diagnosis of viral diseases in salmonid farms in Spain. Bull Eur Assoc Fish Pathol 271: 3–4
17. Lenoir G, De Kinkelin P (1975) Fish rhabdoviruses comparative study of protein structure. J Virol 16: 259–262
18. Lorenzen N, Olesen NJ, Jorgensen PEV (1988) Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. Dis Aquat Organ 4: 35–42
19. Mc Allister PE, Wagner RR (1975) Structural proteins of two salmonid rhabdoviruses. J Virol 15: 733–738
20. Martinez J, Coll JM (1988) Selection and clinical performance of monoclonal anti C-reactive proteins in ELISA quantitative assay. Clin Chim Acta 176: 123–132
21. Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17: 208–212
22. Rueda AZ, Coll JM (1988) Cloning of myelomas and hybridomas in fibrin clots. J Immunol Methods 114: 213–217
23. Wagner RR, Schnaitman TC, Snyder RM (1969) Structural proteins of vesicular stomatitis viruses. J Virol 3: 395–399

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Received August 27, 1990