

A large unintegrated retrovirus DNA species present in a dermal tumor of walleye *Stizostedion vitreum*

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ABSTRACT: Throughout North America, up to 27% of adult walleye (Pisces: *Stizostedion vitreum*) are seasonally affected by a dermal tumor termed walleye dermal sarcoma (WDS) which is associated with C-type retrovirus-like particles. A homogenate from 20 pooled tumors was fractionated by sucrose density gradient centrifugation and retrovirus particles were identified by electron microscopy in fractions with high reverse transcriptase (RT) activity. RNA was isolated from a sucrose density gradient fraction which contained a major portion of the RT activity and which had a density of 1.18 g ml^{-1} . Denaturing gel electrophoresis showed the presence of a minor 12 kb RNA species, presumed to represent the undegraded genomic RNA of walleye dermal sarcoma virus (WDSV). Digoxigenin-labeled cDNA synthesized from this viral RNA preparation specifically hybridized with a 13 kb linear unintegrated viral DNA species present only in DNA from walleye tumors. These findings strongly suggest that WDS is the result of an infection caused by a unique exogenous retrovirus of which the unusually large genome is predominantly unintegrated in tumor cells.

INTRODUCTION

Retroviruses have long been suspected to be the causative agents of some fish neoplasms (Walker 1969, Yamamoto et al. 1976, Duncan 1978, Yamamoto et al. 1983, 1985, Masahito et al. 1988). The most convincing evidence has been provided by studies of the northern pike *Esox lucius* lymphosarcoma, probably the most frequent malignant neoplasm found in wild vertebrates (Sonstegard 1976). Retrovirus-like particles have been observed in homogenates from this tumor (Papass et al. 1976) and experimental transmission by inoculation of cell-free tumor filtrate has been successful (Mulcahy & O'Leary 1970, Brown et al. 1976). In addition, reverse transcriptase (RT) activity has been detected in sucrose gradient fractions of tumor homogenates with a density consistent with that of retroviruses (Papass et al. 1976). The narrow range of temperature in which experimentally-induced tumors arose and the seasonal prevalence of the tumor support the important role of water

temperature in the induction of this neoplasm (Brown et al. 1976).

Walleye dermal sarcoma (WDS), a tumor originating in the dermis from the superficial surface of scales (Martineau et al. 1990b), seasonally affects up to 27% of adult walleye *Stizostedion vitreum* from Oneida lake, New York, USA (Bowser et al. 1988), and from various parts of North America (Yamamoto et al. 1976, Yamamoto et al. 1985). This neoplasm shares many features with the northern pike lymphosarcoma. First, type-C particles have been observed in experimentally transmitted and spontaneous tumors (Walker 1969, Papass et al. 1976, Yamamoto et al. 1976, Yamamoto et al. 1985). Secondly, the transmissibility of WDS has been demonstrated by inoculation of cell-free tumor homogenate into walleye fingerlings (Martineau et al. 1990a). Thirdly, water temperature has been shown to have a major influence on tumor development (Brown et al. 1976, Bowser et al. 1988, Bowser et al. 1990). Finally, in contrast with their often anaplastic appearance, both the northern pike lymphosarcoma and WDS seem to regress seasonally (Sonstegard 1976, Bowser et al. 1988). However, unlike the northern pike lym-

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phosarcoma, WDS neither invades locally nor metastasizes (Walker 1969, Yamamoto et al. 1976, Yamamoto et al. 1985, Martineau et al. 1990b).

Efforts to further characterize these retroviral agents have been hindered by the lack of an *in vitro* system to support viral propagation. In order to overcome this limitation, we have used labeled cDNA synthesized from viral RNA isolated from partially purified WDS virions. This allowed us to detect the presence of a homologous viral DNA species present only in walleye dermal sarcoma tissue.

MATERIAL AND METHODS

Tumor samples. A total of 14 g of tumor tissue from 20 adult walleyes and 10 g of normal dermal tissue from 3 different normal walleyes as negative control tissues were collected in April 1989. All tissues were stored at -70°C . Additionally, samples of the collected tumors were fixed in 10% buffered formalin for histologic confirmation of the diagnosis.

Purification of viral particles by sucrose density gradient centrifugation. Tissues were homogenized in 40 ml of TNES (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 5% sucrose), then centrifuged for 30 min at $10\,000 \times g$. The resulting supernatant fluids were centrifuged for 2 h at $100\,000 \times g$ (L8-80M, SW28 rotor, Beckman Instruments, Inc., USA). The pellets were suspended in 0.5 ml of TNES with 6 mM dithiothreitol (DTT) and layered on a 36 ml, 15% to 60% (w/v) sucrose density gradient. Following centrifugation for 16 h at $100\,000 \times g$ (Beckman, SW28 rotor), fractions (2 ml) were collected from the bottom of the tubes. The density and absorbance of individual fractions at 280 nm were determined by measurement of refractive index and by spectrophotometry, respec-

tively. RT activity was measured as described below. Fractions with the highest RT activity were pooled and recentrifuged under identical conditions. All fractions were subsequently stored at -70°C .

Electron microscopy. Three fractions with high RT activity obtained from a different identical purification procedure were diluted with TNE to ca 10% sucrose (final concentration) and pelleted at $100\,000 \times g$ for 12 min (TL 100, TLA 100.3 rotor, Beckman). The resulting pellet was resuspended in 0.5 ml TNE. From this suspension, several 10 μl aliquots were sequentially placed on Formvar-coated grids and were allowed to dry between each application. One drop of a mixture of phosphotungstic acid and bacitracin ($100 \mu\text{g ml}^{-1}$) as wetting agent (5:1) was placed on the grid and the excess was blotted off with filter paper. The sample was examined with an electron microscope (Philips EM-301) at 80 kV.

Reverse transcriptase assay. An aliquot (10 μl) of each sucrose gradient fraction was assayed in a final volume of 50 μl containing 50 mM Tris-HCl, pH 7.8, 60 mM KCl, 0.6 mM MnCl_2 , 2 mM DTT, 20 $\mu\text{g ml}^{-1}$ polyriboadenylic acid, 0.34 unit ml^{-1} oligo-deoxythymidylic acid₁₂₋₁₈ (Pharmacia LKB Biotechnology Inc., USA), 0.2% Triton X-100, and 2 μM [^3H]thymidine triphosphate ([^3H]dTTP) (sp. act. 46 Ci mM^{-1}) (Amersham/Searle, USA). The mixture was incubated for 2 h at 25°C and spotted directly onto strips of ion exchange filter paper (DE-81, Whatman Int. Ltd, UK). The strips were then washed 5 times with gentle rocking at room temperature for 5 min in 2% sodium phosphate followed by 1 min in distilled water and 1 min in 95% ethanol. The paper strips were dried and the amount of incorporated [^3H]dTTP was measured with a scintillation counter (LS 1801, Beckman).

RNA isolation. Total RNA isolated from 1 ml of one of the 2 sucrose gradient fractions (4 ml) with the highest

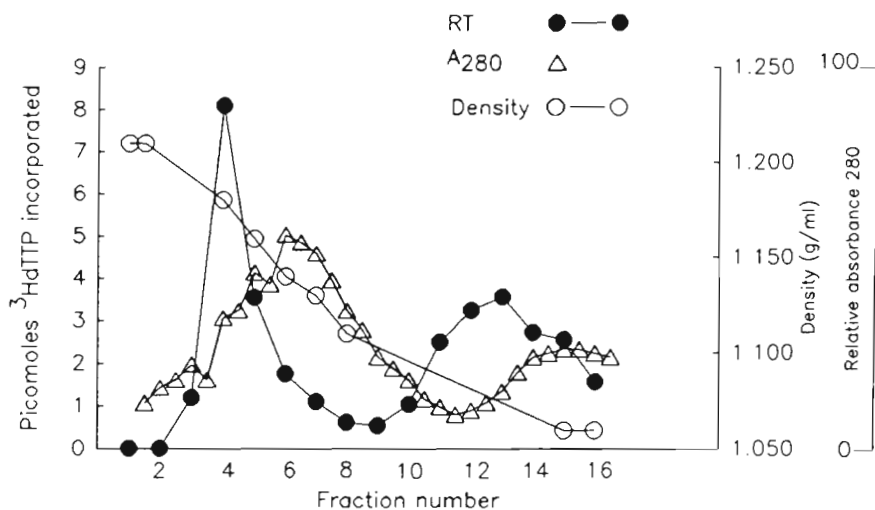


Fig. 1. Sucrose density gradient (36 ml, 15% to 60% (w/v)) purification of viral particles from a homogenate of WDS. Strong reverse transcriptase activity is present in fractions with a density of 1.18 g ml^{-1} . RT activity is expressed as the amount of incorporated [^3H]dTTP.

RT activity using sodium dodecyl sulfate (SDS) (0.5%) and proteinase K ($100 \mu\text{g ml}^{-1}$) digestion followed by phenol extraction. The RNA pellet was dissolved in 0.4 ml of diethylpyrocarbonate (DEPC)-treated water and ethanol-precipitated twice. The final pellet was resuspended in 100 μl of TE (10 mM Tris-HCl, 1 mM ethylene-diaminetetraacetate (EDTA), pH 7.8).

cDNA synthesis and labeling. cDNA synthesized from purified RNA was labeled using a non-radioisotopic method based on the incorporation of digoxigenin-labeled deoxyuridine-triphosphate conjugate (GeniusTM, Boehringer Mannheim Biochemicals, USA). This procedure provides single copy sensitivity as assessed with bovine leukemia virus specific probes and known copy number standards (Renshaw & Casey unpubl. obs.). Briefly, 0.5 μg of viral RNA was mixed with RT buffer (50 mM M Tris-HCl, pH 8.3, 40 mM KCl, 7 mM MgCl_2 , 1 mM DTT, 0.1 mg ml^{-1} bovine serum albumin), 1 μg of random calf thymus oligonucleotides primers (average size of 10 nucleotides), 7 units of avian myeloblastosis RT (Promega, USA) and deoxynucleotide triphosphates (dATP, dCTP, and dGTP at 100 μM , and dig-dUTP at 35 μM) in a total of 20 μl . The mixture was incubated for 2 h at 37 °C.

Formaldehyde agarose gel electrophoresis of RNA. RNA (3 μg) was electrophoresed on a vertical apparatus in a 1% agarose gel prepared with DEPC water, Tris-borate (45 mM Tris-borate, pH 8.0, 1 mM EDTA) and 2.2 M formaldehyde. Electrophoresis was carried out as described (Sambrook et al. 1989). To confirm that the bands visualized under UV illumination were RNA, the gel was incubated 2 h at room temperature in 250 ml of 100 mM Tris-HCl, pH 7.5, containing bovine pancreatic RNase ($40 \mu\text{g ml}^{-1}$) (Sigma, USA). Molecular sizes (kb) of RNA species were calculated using a 0.24 to 9.4 kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD, USA).

DNA isolation. DNA was isolated from hepatic tissue of 5 normal walleye fingerlings (0.7 g per fingerling) collected from the South Otselic Fish Hatchery of the New York State Department of Environmental Conservation and from tumor tissue collected from 2 adult walleyes in April 1989. Tissues were homogenized in extraction buffer (10 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, proteinase K ($100 \mu\text{g ml}^{-1}$), 1% w/v SDS). After overnight digestion, the lysate was extracted once with an equal volume of chloroform/isoamyl alcohol (24:1 v/v) and ethanol-precipitated. After treatment with RNase ($100 \mu\text{g ml}^{-1}$), DNA was extracted twice more with chloroform:isoamyl alcohol, ethanol-precipitated and quantified by UV spectrophotometry.

Agarose gel electrophoresis of DNA and Southern blot analysis. DNA (10 μg) isolated from WDS and from

normal walleye fingerling hepatic tissue was digested with EcoRI restriction enzyme as described by the manufacturer (New England Biolabs, Inc., USA), fractionated on a 0.9% agarose gel and transferred to a nitrocellulose sheet following the procedure described (Sambrook et al. 1989). The blot was incubated for 12 h at 65 °C in 10 ml of prehybridization solution containing 5 \times SSC (15 mM sodium citrate, 150 mM sodium chloride, pH 7.0), 0.5% blocking reagent (GeniusTM, Boehringer-Mannheim), 0.1% N-lauroyl-sarcosine, 0.02% SDS and 100 $\mu\text{g ml}^{-1}$ calf thymus RNA (Sigma). Hybridization was carried out at 65 °C for 60 h in 10 ml of the above solution containing the labeled denatured cDNA probe (20 μl).

RESULTS

Purification of viral particles

After 2 successive purifications by equilibrium density gradient centrifugation, a major peak of RT activity was associated with 2 fractions (4 ml) with a density of 1.18 g ml^{-1} (Fig. 1). This fractionation scheme was effective at separating cellular debris from virus particles as indicated by the peak of UV light absorbance which was found in fractions of lower density.

Electron microscopy

In the fractions with highest enzymatic activity, electron microscopic examination (EM) verified that the purification scheme effectively separated viral particles from cell debris. EM revealed the presence of numerous, often clustered, 90 nm in diameter, viral particles with prominent spikes protruding from the viral envelope (Fig. 2). Negative stain which had penetrated into the particles allowed internal structures to be observed: a round, centrally located, electron-dense nucleoid surrounded by a wide electron-lucent space. An outer thin electron-dense envelope was sometimes present around this space. Some empty collapsed envelopes were also present.

RNA isolated from fractions with RT activity

Formaldehyde agarose gel electrophoretic analysis of RNA prepared from sucrose-banded virions is shown in Fig. 3. A discrete RNA band, ca 12 kb in length, was visualized. In addition, heterogenous RNA species were detected in the 0.25 kb size range. Both the 12 kb and 0.25 kb RNA species were sensitive to pancreatic RNase treatment. This material was subsequently used

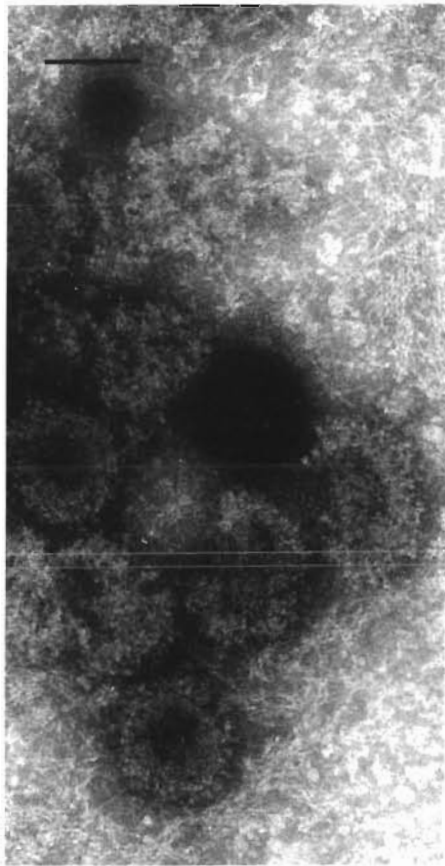


Fig. 2. Pellet from 3 pooled, purified, RT active fractions from sucrose density gradient. Negatively stained viral particles are present. Clusters of viral particles (100 nm) are present amongst debris, presumably collagen fibrils. Viral particles have a circular and central electron-dense core surrounded by a wide electron-lucent space. Spikes conspicuously protrude from the viral envelope. Stain: phosphotungstic acid. Bar = 100 nm

for synthesis of labeled cDNA and employed as a probe for hybridization with genomic DNA from 2 different walleye dermal sarcomas and from normal walleye fingerlings. The absence of visually detectable 28S or 18S ribosomal RNA in the preparation suggests that this procedure was effective at eliminating contaminant cellular RNA.

Southern blot analysis

Hybridization of the digoxigenin-labeled cDNA probe with undigested genomic DNA from 2 different WDS-affected walleyes showed the presence of a homologous, unintegrated 13 kb DNA species. This DNA species was not detected in genomic DNA of normal walleye fingerling hepatic tissue (Fig. 4a). In additional experiments, there was no hybridization with

undigested genomic DNA from a channel catfish ovary cell line nor with DNA from bovine lymphosarcoma (data not shown). Hybridization of the cDNA probe with EcoRI digested genomic DNA from another tumor demonstrated the presence of 2 DNA fragments, 5.9 and 6.1 kb in size (Fig. 4b). The sum of the 2 fragments closely approximated the anticipated size of a linear full-length viral genome. In contrast, the labeled probe did not detect any homologous DNA species in EcoRI digested DNA from the other aforementioned animal species.

DISCUSSION

We have previously demonstrated the transmissibility of WDS by inoculation of cell-free tumor homogenates (Martineau et al. 1990a). In spontaneous tumors, we have now detected viral particles having a morphology and a density similar to those of oncornaviruses. In addition, we have demonstrated that a high RT activity is associated with the purified virions. The diameter (90 nm) and the morphology of these negatively stained retrovirus-like particles are similar to those of retrovirus type C particles observed in thin

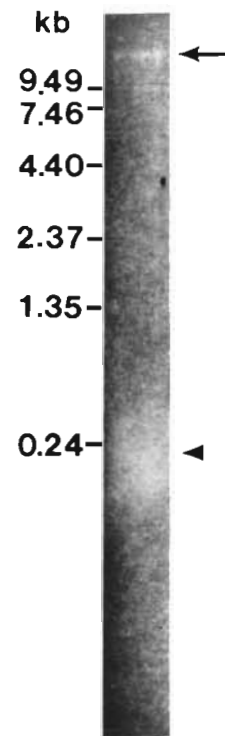


Fig. 3. Formaldehyde agarose gel electrophoretic analysis of total RNA isolated from a RT active fraction. A discrete RNA species, ca 12 kb in size, is present (arrow). Smaller heterogeneous RNA species are present in the 0.25 kb size range (arrowhead). Total RNA was isolated from a fraction with high RT activity. Molecular sizes at left in kilobases (kb) are represented by a 0.24 to 9.4 kb RNA ladder

sections of tumors transmitted in walleye fingerlings by inoculation of cell-free tumor homogenate (Martineau et

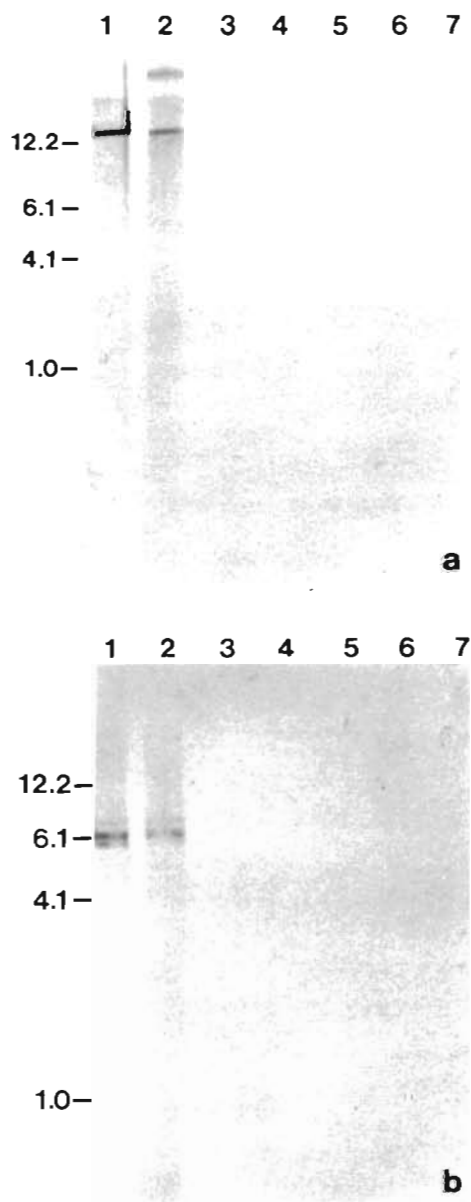


Fig. 4. (a) Southern analysis of undigested DNA from WDS from 2 different fish (lanes 1 and 2) and from 5 normal walleye fingerling livers (lanes 3 to 7). The probe was digoxigenin-labeled cDNA synthesized from viral RNA isolated from RT active fractions. This probe detects the presence of a homologous, unintegrated 13 kb DNA species in tumor tissue only (lanes 1 and 2). Molecular size markers at left in kilobases (kb) correspond to 1 to 12 repeats of a 1.018 kb DNA fragment. (b) Southern analysis of EcoRI digested DNA from WDS from 2 different fish (lanes 1 and 2) and from 5 normal fingerling livers (lanes 3 to 7). The probe was digoxigenin-labeled cDNA synthesized from viral RNA isolated from RT active fractions. The probe detects the presence of 2 DNA fragments (5.9 and 6.1 kb) (lanes 1 and 2). Molecular size markers at left in kilobases (kb) correspond to 1 to 12 repeats of a 1.018 kb DNA fragment

al. 1990a). Differences in the preparation techniques may account for the 10 nm smaller size of the viruses described here (Fig. 2). Morphologically, the particles that we observed differ somewhat from prototypic type C particles in that they have finer spikes and a smaller core.

An unexpected finding relates to the number of viruses present in tumors and to the integrity of viral RNA. At least 10% of the total RNA recovered represented undegraded genome length molecule (Fig. 3). Since the total amount of viral RNA present in the 2 most RT active fractions is ca 40 μ g and assuming that the viral genome is 12 kb in length (24 kb diploid), we conservatively calculate from the estimated amount of full length viral RNA (4 μ g) that 17×10^{10} viral particles were present in 14 g of tumor. Since 1 g of tumor contains ca 10^9 tumor cells (Tannock 1983), WDS contains a minimum average of 12 viruses per tumor cell. Further, the large amount of intact viral RNA present in thawed tumors submitted to a relatively extensive purification procedure suggests that WDSV may not be as labile as mammalian retroviruses. This unusual viral stability, possibly required for virus transmission in nature, needs to be further investigated.

The specificity of the labeled cDNA probe synthesized from viral RNA was demonstrated by its hybridization with a 13 kb unintegrated DNA species found only in dermal sarcoma. This DNA species was not detected in tissue from normal walleye fingerlings (Fig. 4a, b). The fragments resulting from EcoRI digestion of genomic tumor DNA add to ca 13 kb. Considered together, these findings suggest that the 13 kb DNA detected in tumors represent the unintegrated linear form of WDSV DNA. The present study generated the information and the reagents that will be necessary to molecularly clone the WDSV. In turn, the availability of WDSV molecular clones will allow us to more fully characterize the retroviral etiology of WDS.

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