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Molecular Characterization of a Unique Retrovirus Associated with a Fish Tumor

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The walleye dermal sarcoma is a mesenchymal tumor which seasonally affects up to 27% of adult walleye fish (*Stizostedion vitreum*). It arises multicentrically in the dermis, in which its development remains restricted. We report the molecular cloning of a type C retrovirus from this tumor. The genome of this virus (13.2 kb) is larger than that of all retroviruses and in that respect is approximated only by the recently characterized spumaviruses. In tumors, the predominantly unintegrated linear viral DNA has a single-stranded gap region which is similar to the structure found in some lentiviruses and all spumaviruses. The presence of at least four viral transcripts suggests that this virus has the capacity to encode accessory functions and is reminiscent of the transcriptional complexity of lentiviruses and spumaviruses.

The *Retroviridae* family of viruses is composed of three subfamilies, *Oncovirinae*, *Lentivirinae*, and *Spumavirinae*, which all replicate via a DNA intermediate. The effects they exert upon their respective hosts span a broad spectrum: oncoviruses are involved in induction of tumors and lentiviruses induce severe but slowly developing diseases, while spumaviruses have not to date been associated with any specific disease (5). While the genome sizes of oncoviruses and lentiviruses are in the 8- to 10-kb range, the genomes of spumaviruses are larger (about 12 kb) (13, 20, 21). The DNA intermediate of spumaviruses and of some lentiviruses is distinct from that of oncoviruses in that the major form is often unintegrated in infected cells and in that it can have a central single-stranded gap structure. An additional distinction between most oncoviruses and other retroviruses is that oncoviruses usually express two transcripts that direct the synthesis of viral structural proteins. Lentiviruses, spumaviruses, and the human T-cell lymphotrophic virus type I–bovine leukemia virus subgroup of oncoviruses express additional messages corresponding to nonstructural accessory genes that regulate viral expression (7, 9, 17, 20, 25).

Some fish populations have a high prevalence of neoplasms, probably the highest among wild vertebrates (2, 12, 18, 23, 26). Type C oncoviruses have been observed in only three fish tumors (8, 19, 27). One of these, the walleye dermal sarcoma (WDS), affects up to 27% of adults in the spring and regresses in summer (2, 3). Although WDS often appears histologically malignant and arises in a multicentric manner, this tumor neither invades nor metastasizes (15). Experimental transmissibility of WDS by inoculation of cell-free tumor homogenate has been demonstrated (14); water temperature influences both the extent of tumors and the time required for their development (1). Isolation of a retrovirus, termed walleye dermal sarcoma virus (WDSV), from a naturally occurring tumor has been also reported, and preliminary hybridization studies indicated that a linear unintegrated species is the major form of viral DNA in tumors (16).

Our preliminary analysis of WDSV in tumors showed that the majority of viral DNA was linear and unintegrated (16). We took advantage of these findings to clone complete viral DNA directly from total tumor DNA; the addition of *Eco*RI adaptors (Promega, Madison, Wis.) to high-molecular-weight tumor DNA results in the genesis of viral genomes that can be ligated and packaged in the EMBL4 lambda vector. Since high-molecular-weight tumor DNA is beyond the lambda packaging limits, a biological enrichment for viral genomes is obtained. A similar protocol has been recently used in our laboratory to clone the bovine syncytial virus genome (20).

After ligation of *Eco*RI synthetic adaptors, undigested DNA (10 μg) from tumors was ligated to *Eco*RI-digested bacteriophage vector lambda EMBL4 DNA. The ligation mixture was packaged in vitro according to the manufacturer's instructions (Gigapack; Stratagene, La Jolla, Calif.). In order to screen the genomic library, a digoxigenin-labeled cDNA probe was synthesized with random primers from partially purified virion RNA (16). The genomic library contained approximately 3,100 plaques, of which four clones hybridized with the cDNA probe. One clone (WDSV-1) appeared to be full length (13.2 kb) and was selected for further study. Fragments of this clone were subcloned into Bluescript plasmid vector according to standard procedures. The orientation of the clone and the presence of long terminal repeats were determined by hybridization of a 3′-enriched cDNA probe to various restriction enzyme digests of WDSV-1. The restriction map of WDSV-1 is presented in Fig. 1a.

Tumors and organs of affected and unaffected walleyes were analyzed by Southern blot by using digoxigenin-labeled probes representative of the entire WDSV genome and synthesized by random priming (16). Viral DNA was readily detected by Southern blot in all of the six tumors investigated (Fig. 1b and c). By comparison with the copy number standards, we estimated that the number of viral copies per tumor cell ranged from 7 to more than 50 copies, assuming that all cells in each tumor contain the same amount of viral DNA (Fig. 1c). Twelve of 14 additional tumors also contained viral DNA (data not shown). Analysis of undigested tumor DNA revealed strong hybridization to a DNA species migrating at an apparent size of 13.2 kb (Fig. 1b, lanes 4 to 9). Digestion of tumor DNA with *Bam*HI resulted in five fragments totaling 13.2 kb and identical to the *Bam*HI

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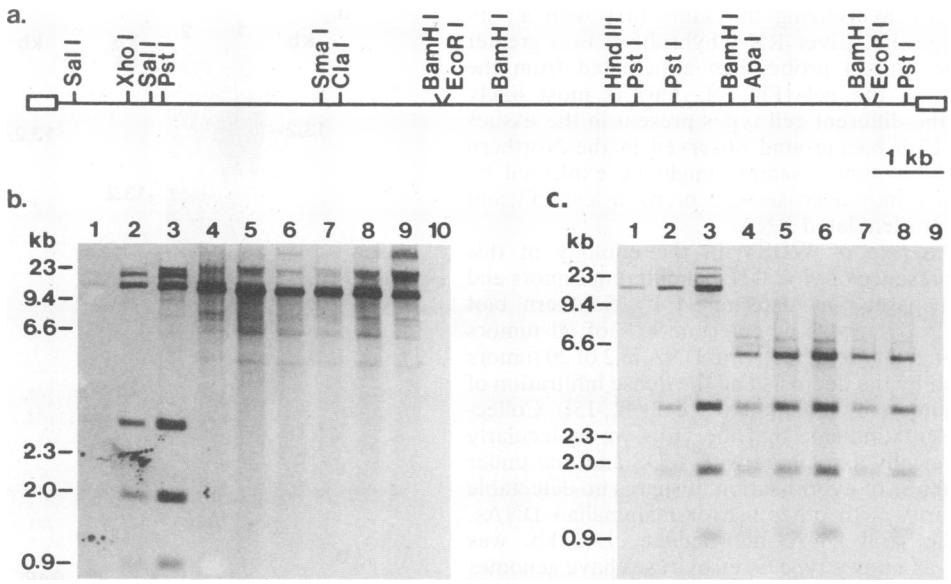

FIG. 1. Structure of the recombinant clone WDSV-1 and Southern blot analysis of WDSV in tumors. (a) Restriction map of recombinant phage clone (WDSV-1). (b) Southern blot analysis of undigested tumor DNAs. Copy number standards of *Bam*HI-digested WDSV-1 (1, 10, and 50 copies per cell; lanes 1, 2, and 3, respectively) were used. Lanes contain undigested DNA from six different tumors (lanes 4 to 9) and sperm DNA from unaffected walleye (lane 10). (c) Southern blot analysis of *Bam*HI-digested tumor DNAs (lanes 1 to 3, same as for panel b; lanes 4 to 8, same as for panel b except digested with *Bam*HI; lane 9, sperm DNA digested with *Bam*HI).

fragments of WDSV-1 (Fig. 1c, lanes 4 to 8). This result further supported the major unintegrated state of viral DNA in tumors. The observation of additional faintly hybridizing bands in both digested and undigested tumor DNA samples is consistent with the presence of covalently closed circular intermediates. Viral DNA was not detected by Southern blot analysis of DNAs from internal organs of unaffected or tumor-bearing walleyes. Similarly, DNA from sperm of an unaffected walleye did not hybridize with WDSV DNA (Fig. 1b, lane 10 and Fig. 1c, lane 9). DNAs from two different fish species (fathead minnow cell line FHM [ATCC CCL 42] and carp liver) and from a canine cell line (Cf2Th) also tested negative (data not shown).

Considering the large size of the WDSV genome and the unintegrated status of viral DNA in tumors, we attempted to determine whether other similarities might exist among spumaviruses, lentiviruses, and WDSV. Since the DNA intermediates of some lentiviruses and all spumaviruses contain a central single-stranded gap structure, we tested for the presence of a similar structure in WDSV DNA intermediates. Southern blot analysis of heat-denatured tumor DNA, hybridized with a randomly primed ^{32}P-labeled probe representative of the entire viral genome, revealed the presence of three virus-specific single-stranded DNA species of 13.2, 7.4, and 5.6 kb. A randomly primed and ^{32}P-labeled 3′-biased probe, encompassing the 1.05-kb *Eco*RI 3′ fragment, hybridized with a stronger intensity to the smaller 5.6-kb fragment. Since this probe contains approximately 500 nucleotides of 3′ viral sequence in addition to the long terminal repeat, this stronger signal allowed the location of the gap to be assigned at 5.6 kb from the 3′ end of the genome. Additionally, digestion of total DNA with S1 nuclease yielded two fragments with lengths similar to those detected by the analysis of heat-denatured tumor DNA (data not shown).

Polyadenylated RNA from a single tumor and from liver tissue of a tumor-bearing adult walleye were examined by Northern (RNA) blot analysis (Fig. 2a, lanes 1 and 2) with a ^{32}P-labeled representative probe synthesized by random priming. The strongest hybridization signals corresponded to two transcripts with molecular lengths of 13 and 7.4 kb. Two less intense smaller transcripts, 2.8 and 1.8 kb in length, were also observed. Identical patterns of hybridization were obtained with viral RNA from a pool of 10 tumors and from two single tumors (data not shown). No viral transcripts were detected in liver RNA from a tumor-bearing walleye.

The quantity and integrity of the polyadenylated RNA

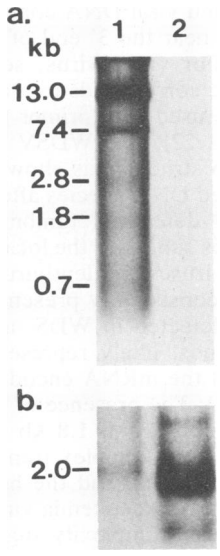

FIG. 2. Northern blot analysis of poly(A)-selected RNA from a single tumor (2 μg) and from liver (7 μg) (lanes 1 and 2, respectively). A duplicate filter was hybridized with a β-actin probe.

were assessed by hybridizing the same blot with a ^{32}P-labeled β-actin probe. Liver RNA hybridized to a greater extent with the β-actin probe than anticipated from the amount loaded on the gel (Fig. 2). This is most likely attributable to the different cell types present in the tissues analyzed. The high background observed in the Northern blot analysis of the tumor sample might be explained by some degree of tumor necrosis (15) or by a less efficient selection for polyadenylated RNA.

To assess the role of WDSV in the etiology of this neoplasm, the presence of viral DNA in different tumors and in organs and sperm was determined by Southern blot analysis. WDSV DNA was detected in 90% of all tumors examined (our inability to detect viral DNA in 2 of 20 tumors can be explained by the necrosis and the dense infiltration of some of these tumors by inflammatory cells [2, 15]). Collectively, these results indicate that the virus we molecularly cloned is etiologically associated with WDS and that under moderate stringency of hybridization, it shares no detectable sequence similarity with other fish or mammalian DNAs. The size of the viral DNA intermediate, 13.2 kb, was surprising since all known type C retroviruses have genomes ranging from about 8 to 10 kb in length. Only spumaviruses have genomes which are comparable in length to that of WDSV (13, 20, 21).

The predominance of unintegrated viral DNA in WDS closely resembles the status of viral DNA in lentivirus and spumavirus infections and clearly differs from the general pattern of type C oncovirus infections. Accumulation of unintegrated viral DNA in cells infected with retroviruses is believed to result from superinfection. In turn, superinfection is thought to occur because the initial viral infection fails to block the viral receptor present at (or targeted to) the plasma cell membrane (24). We propose that the high numbers of virions present in walleye tumors, as assessed by electron microscopy (28) and hybridization studies (16), might contribute to superinfection. The abundance of virions might be due to depression of the humoral immune response of fish in cold water (6), to the stress associated with spawning, and perhaps to a direct effect of water temperature on viral replication.

In some lentiviruses and in all spumaviruses examined thus far, unintegrated viral DNA contains a single-stranded gap region located near the 3′ end of the polymerase gene (11, 13, 20, 21). For visna virus, sequence analysis has shown that this site corresponds to a duplicated polypurine tract which can be used as a primer to initiate plus-strand DNA synthesis (11, 22). The WDSV DNA also contains a single-stranded gap structure as shown by the presence of three single-stranded DNA species after Southern blot analysis of undigested denatured tumor DNA (Fig. 3). The location of the gap is similar to the location of gap structures detected in spumaviruses and lentiviruses.

Viral RNA was consistently present in tumors. The two major transcripts detected in WDS, approximately 13 and 7.4 kb in length, most likely represent the full-length genomic message and the mRNA encoding envelope protein, respectively (Fig. 2). The presence of two additional faintly hybridizing mRNAs, 2.8 and 1.8 kb in length (Fig. 2), is reminiscent of the more complex transcriptional pattern of lentiviruses, spumaviruses, and the human T-cell lymphotropic virus type I–bovine leukemia virus subgroup of oncoviruses. This apparent complexity suggests that WDSV, in contrast with most type C oncoviruses, has the capacity to encode nonstructural accessory proteins (9, 25).

The classification of retroviruses into three subfamilies

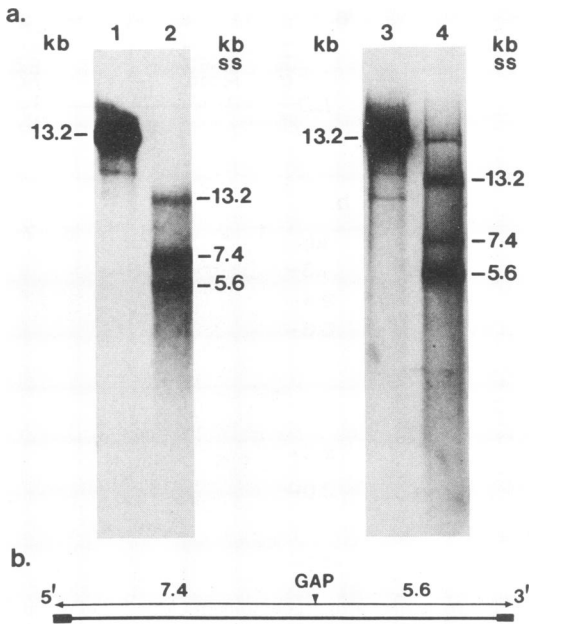

FIG. 3. Gap structure according to Southern blot analysis of native and denatured tumor DNA. (a) Ten micrograms each of native tumor DNAs (lanes 1 and 3) and heat-denatured (2 min at 100°C) tumor DNAs (lanes 2 and 4) was electrophoresed in parallel and hybridized with either a representative WDSV probe (lanes 1 and 2) or a 3′-biased probe (lanes 3 and 4). (b) Schematic representation of WDSV and location of the gap structure. SS, single stranded.

was originally based on the type of cellular lesion induced by viral infection both in vivo and in vitro, on the morphology of virions, and later on genetic structure (7). WDSV, the first fish retrovirus to be molecularly cloned, currently stands as an exception to these conventions because of its morphology, the size and structure of its genome, its transcriptional profile, and the proliferative lesion it induces. However, WDSV may not represent a unique exception to the present scheme of retroviral classification but rather may be the first member of a new subfamily yet to be recognized. There are more fish species than all other vertebrates combined, and only a few have been investigated with regard to the presence of retroviruses in their tissues. Possibly, the characterization of other type C retroviruses visualized in malignant tumors such as the pike lymphosarcoma (19) and the Atlantic salmon swim bladder fibrosarcoma (8) will result in the addition of new members to this potential new subfamily.

With the exception of (rare) multicentric fibrosarcomas of young cats due to feline sarcoma viruses (10), WDS is the only sarcoma caused by a type C oncovirus which occurs in nature with a significant prevalence; further, besides Kaposi's sarcoma, WDS is the only sarcoma endemic in a human or animal population whose etiology implicates a retrovirus.

The internal temperature of fish is primarily determined by the environment (poikilothermy) and is a critical aspect in which the dynamics of the virus-host interaction differ from those of the well-studied avian and mammalian retrovirus systems. This additional parameter plays a major role in the development of WDS (3) and northern pike lymphosarcoma (4). The molecular cloning of WDSV will facilitate further investigation of the mechanisms involved in tumor formation and might provide insights into the evolutionary history of retroviruses.

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