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Walleye Retroviruses Associated with Skin Tumors and Hyperplasias Encode Cyclin D Homologs

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Walleye dermal sarcoma (WDS) and walleye epidermal hyperplasia (WEH) are skin diseases of walleye fish that appear and regress on a seasonal basis. We report here that the complex retroviruses etiologically associated with WDS (WDS virus [WDSV]) and WEH (WEH viruses 1 and 2 [WEHV1 and WEHV2, respectively]) encode D-type cyclin homologs. The retroviral cyclins (rv-cyclins) are distantly related to one another and to known cyclins and are not closely related to any walleye cellular gene based on low-stringency Southern blotting. Since aberrant expression of D-type cyclins occurs in many human tumors, we suggest that expression of the rv-cyclins may contribute to the development of WDS or WEH. In support of this hypothesis, we show that rv-cyclin transcripts are made in developing WDS and WEH and that the rv-cyclin of WDSV induces cell cycle progression in yeast (Saccharomyces cerevisiae). WEHV1, WEHV2, and WDSV are the first examples of retroviruses that encode cyclin homologs. WEH and WDS and their associated retroviruses represent a novel paradigm of retroviral tumor induction and, importantly, tumor regression.

Studies of tumor induction by avian and murine simple retroviruses have led to key advances in the understanding of cell proliferation and oncogenesis (reviewed in references 47 and 49). Tumor induction by these oncoviruses occurs by the activation of proto-oncogenes resulting from proviral insertion (non-acutely transforming viruses) and by the transduction of oncogenes (acutely transforming viruses). The discovery that viral oncogenes were derived from cellular proto-oncogenes has provided important clues about the roles of proto-oncogenes in normal cell proliferation and in tumor induction (56). Several classes of oncogenes have been identified by retroviral transduction and/or proviral insertion, including those encoding nonreceptor and receptor protein tyrosine kinases (src and erkB, respectively), G proteins (ras), serine-threonine kinases (raf), growth factors (sis), and transcription factors (myc) (49). In addition, numerous genes that encode proteins with yet unknown function have been identified in various tumors resulting from proviral insertional mutagenesis (49).

Tumor induction by complex retroviruses, i.e., members of the human T-cell leukemia virus (HTLV)-bovine leukemia virus (BLV) group, is less well understood. These viruses lack identifiable cell-derived oncogenes and are not known to activate cellular proto-oncogenes by proviral insertion (49). HTLV and BLV contain two accessory genes at the 3′ end of their genomes, tax and rex, that control viral gene expression (12). Tax also activates the expression of many cellular genes, including those that stimulate T-cell growth (e.g., interleukin 2), and considerable attention has focused on its role in cell transformation (50, 63). Tax of HTLV-1 has been shown to induce mesenchymal tumors in transgenic mice (43) and is essential for transformation of human T lymphocytes in cell culture (51). Although Tax is likely to be important for transformation, in vitro studies and analysis of transgenic mice show that Tax alone is not sufficient for maintenance of the transformed phenotype (49). Rather, Tax may stimulate abnormal replication early in the disease process, thereby leading to the transformation of infected T cells.

Retroviruses have also been implicated in several neoplastic diseases of lower vertebrate animals, such as fish, amphibians, and reptiles (45). The best characterized of these viruses are walleye dermal sarcoma virus (WDSV) and, more recently, walleye epidermal hyperplasia virus types 1 and 2 (WEHV1 and WEHV2, respectively) (25, 32, 38, 60). These related, large complex retroviruses are etiologically associated with neoplastic and hyperproliferative skin diseases of walleye fish, walleye dermal sarcoma (WDS) and walleye epidermal hyperplasia (WEH), respectively (7, 10, 38, 39). Most interestingly, these diseases are among several neoplastic and proliferative diseases of poikilotherms that are seasonal in nature, presenting an opportunity to study both tumorigenesis and tumor regression (1, 8, 45). WDS and WEH have been observed on 10 to 30% of walleyes in a given year from late autumn until early spring, when they completely regress (8, 9). These diseases have not been observed to progress to invasive or metastatic tumors on adult feral fish, but injection of cell-free filtrates of WDS into walleye fingerlings less than 12 weeks of age can cause invasive tumors in 12 to 16 weeks, suggesting that WDSV has oncogenic potential (19, 40).

The mechanisms by which WDSV and WEHV1 and WEHV2 induce WDS and WEH are not known. These viruses contain three open reading frames in addition to gag, pol, and env (25). orfA and orfB are located between env and the 3′ long terminal repeat (LTR), and orfC is located between the 5′ LTR and gag. We report here that the orfA genes of these viruses encode cyclin D homologs. Cellular cyclins associate with and activate cyclin-dependent kinases (Cdks) to promote cell cycle progression (54, 55). D-type cyclin-Cdk complexes regulate the cell-cycle G1-S transition and have been proposed to induce cell proliferation by phosphorylating and thereby inactivating the retinoblastoma tumor suppressor protein (Rb) (18, 20, 30). Aberrant expression of human cyclin D1 has been demonstrated in various human tumors, including parathyroid adenomas (41), breast and squamous cell carcinomas (31), and esophageal carcinomas (28). Cyclin genes have not been pre-
viously identified as oncogenes in acutely transforming viruses, but integration of murine leukemia virus (MLV) into the cyclin D1 (Fis1) and cyclin D2 (Vin1) loci results in T-cell lymphomas (42, 59). WDSV, WEHV1, and WEHV2 are the first examples of retroviruses to encode cyclin homologs, and the possible roles of retroviral cyclins (rv-cyclins) in tumor induction and viral replication are discussed.

**RESULTS**

WDSV, WEHV1, and WEHV2 encode cyclin homologs. The genomic organization of WDSV, WEHV1, and WEHV2 is shown in Fig. 1A (25). Database searches with the WDSV orfA gene and all three orfB and orfC genes by using the BlastX algorithm did not identify proteins with obvious homology. However, BlastX searches with the WEHV1 and WEHV2 orfAAs suggested a distant relationship to D-type cyclins. Visual alignment of the WDSV, WEHV1, and WEHV2 OrfA proteins (297, 233, and 231 amino acid residues, respectively) with an array of cellular cyclins showed that each was a cyclin D homolog, with the WDSV cyclin being the most distantly related (Fig. 1B). The similarity of the retroviral cyclins (rv-cyclins) and D cyclins is limited to the conserved 10-α-helical (A to E) cyclin box motif (4, 44), with the greatest similarity being in the α-helices C and E, which form the cyclin surface that interacts with cell division kinases (Cdks) (11, 27). Additionally, the lysine and glutamate residues in these regions necessary for Cdk binding are conserved in the rv-cyclins (Fig. 1B) (27). The WEHV1 and WEHV2 rv-cyclins have about 20% amino acid identity with D-type cyclins and about 12% amino acid identity with human cyclin A or E (Table 1). The WEHV1 rv-cyclin has the greatest similarity (35%) to human cyclin D3 and Kaposi's sarcoma herpesvirus (KSHV) cyclin (13, 14), whereas the WEHV2 and WDSV rv-cyclins are most similar to human D1 (35 and 29% amino acid similarity, respectively). The WDSV rv-cyclin is the most divergent from cellular cyclins, having 19% amino acid identity to human D1 and about 16% amino acid identity to other D-type cyclins. In general, the rv-cyclins are more similar to one another than they are to human cyclin D1 (Table 1). The WEHV1 and WEHV2 rv-cyclins are more closely related to one another (37% amino acid identity) than to WDSV rv-cyclin (28 and 21% amino acid identity, respectively).

**The rv-cyclins are not closely related to walleye cellular cyclins.** Since walleye cellular cyclin sequences were not available for comparison with the rv-cyclins, we used degenerate primer pairs and reverse transcription-PCR to amplify D-type cyclin box sequences from walleye cell lines WF2 and W12 and primary cells. These experiments yielded a cellular cyclin that is most similar to human cyclin D2 having 85% amino acid identity in the cyclin box (Table 1). These data are consistent with earlier studies showing that cyclin subfamily proteins are highly conserved across species; e.g., human cyclin D1 is 79% identical to zebrafish cyclin D1 (27). The WEHV1 and WEHV2 rv-cyclins have about 20% amino acid identity with D-type cyclins and about 12% amino acid identity with human cyclin A or E (Table 1). The WEHV1 rv-cyclin has the greatest similarity (35%) to human cyclin D3 and Kaposi's sarcoma herpesvirus (KSHV) cyclin (13, 14), whereas the WEHV2 and WDSV rv-cyclins are most similar to human D1 (35 and 29% amino acid similarity, respectively). The WDSV rv-cyclin is the most divergent from cellular cyclins, having 19% amino acid identity to human D1 and about 16% amino acid identity to other D-type cyclins. In general, the rv-cyclins are more similar to one another than they are to human cyclin D1 (Table 1). The WEHV1 and WEHV2 rv-cyclins are more closely related to one another (37% amino acid identity) than to WDSV rv-cyclin (28 and 21% amino acid identity, respectively).

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parison, approximately three to five copies of WEHV2 per cell are present in lesions (Fig. 2A, lane 4). In agreement with earlier reports, there are approximately 50 copies of WDSV DNA per cell in spring tumors (Fig. 2A, lane 6). Importantly, only very weak hybridization was detected with the walleye sperm DNA samples with the WDSV cyclin probe (Fig. 2A, lane 7), and no hybridization was detectable with the WEHV1 and WEHV2 probes (Fig. 2A, lanes 3 and 5). As a control for genomic DNA quality, the walleye kinase gene probe (KIN) hybridizes strongly to its cognate sequence at approximately 12 kb and to a related 500-bp restriction fragment in WEH and WDS DNA samples and walleye sperm DNA (Fig. 2B, lanes 1 to 4). These data demonstrate that unlike the oncogenes transduced by simple retroviruses, the walleye retroviral cyclins are divergent from the cellular sequences from which they are presumably derived.

The WDSV rv-cyclin complements cyclin deficiency in yeast.

To assess the ability of rv-cyclins to induce cell cycle progression, we chose to use a standard yeast complementation assay (34). The ability of a putative cyclin to induce cell-cycle progression is indicated by its ability to rescue yeast (S. cerevisiae), which are conditionally deficient in G1 cyclins (Cln genes), from growth arrest. This assay has been used to identify cyclins from diverse organisms, including plants (Arabidopsis), insects.

<table>
<thead>
<tr>
<th>Cyclin type</th>
<th>WEHV1</th>
<th>WEHV2</th>
<th>WDSV</th>
<th>Human D1</th>
<th>Zebrafish D1</th>
<th>Human D2</th>
<th>Walleye D2</th>
<th>Human D3</th>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>65</td>
<td>69</td>
<td>69</td>
<td>68</td>
<td>30/43</td>
</tr>
</tbody>
</table>

* Alignments were done with Megalign and Geneworks software.

* Values represent percent identity/percent similarity when two numbers are shown or percent identity when one number is shown.

* ND, not done.
We expressed each rv-cyclin in a yeast strain (BY613) that is deficient for the synthesis of Cln1, -2, and -3 but grows in the presence of galactose because it contains a plasmid that expresses Cln3 from a galactose-inducible promoter (pGAL::CLN3). BY613 does not grow in the presence of glucose, because the galactose promoter is inactive and Cln3 is not produced. We transfected tryptophan-selectable plasmids containing the WDSV, WEHV1, or WEHV2 cyclin gene under the control of the MET2 promoter into BY613 and plated cells on minimal galactose medium lacking tryptophan. Eight individual transformants from each were plated onto minimal glucose medium lacking tryptophan (Fig. 3). All of the transformants grew well on galactose minimal medium (Fig. 3A). Those expressing the WDSV cyclin grew well on the glucose minimal medium (Fig. 3B, rows 4 and 5), whereas expression of the WEHV1 and WEHV2 cyclins did not support growth (Fig. 3B, rows 2 and 3). Immunoprecipitations and Western blots of crude protein extracts showed that the WEHV1 and WEHV2 cyclins were present in transformants (data not shown) but were unable to complement the cyclin deficiency. The experiment was repeated three times at temperatures ranging from 16 to 33°C to test if the interaction of the WEHV1 and WEHV2 rv-cyclins with CDC28p could be sufficiently active at higher or lower temperatures to support yeast growth, with the same result.

Importantly, the growth of yeast expressing the WDSV rv-cyclin was linked to the input plasmid; transformants did not grow in the presence of glucose (Fig. 3D, rows 4 and 5) on rich plates that contain sufficient methionine to repress the MET3 promoter directing expression of the WDSV rv-cyclin. To test the ability of the WDSV rv-cyclin to support yeast growth in the absence of pGAL::CLN3, we plated transformants on plates containing FOA and uracil (52). Since FOA kills cells capable of synthesizing uracil, only yeast cells lacking pGAL::CLN3 are able to grow. Yeast clones lacking pGAL::CLN3, but expressing the WDSV rv-cyclin, grew well, confirming that the rv-cyclin will support growth. Subsequently, the WDSV rv-cyclin plasmids were isolated from these segregants and sequenced. The sequences were unaltered (data not shown), showing that the native WDSV OrfA protein can function as a cyclin.

rv-cyclin mRNA is present in fall and spring lesions. To assess the role of the rv-cyclins in tumor induction and regression, we characterized viral RNA expression patterns in developing (fall) and regressing (spring) lesions. The Northern blot in Fig. 4 showed that the rv-cyclin probes detect genomic RNA (~13 kb), spliced env mRNA (~7.5 kb), and spliced orfA mRNA.
mRNA (rv-cyclin mRNA) (~2.6 to 2.8 kb) in hyperplasias and dermal sarcomas collected in the spring. As with earlier studies of WDSV RNA expression (10, 46), the expression of viral transcripts was low in fall lesions, requiring isolation of poly(A)^+ RNA for analysis. While the levels of viral gene expression in fall lesions were low, the most abundant WEHV1 and WDSV mRNAs were consistent with those predicted to encode the rv-cyclins (Fig. 4, lanes 1 and 3; WEHV2 mRNA was not detected in these samples [lane 2]). Upon long film exposure times, genomic WDSV RNA and spliced env mRNA can be observed (data not shown). Additionally, spliced orfB transcripts have been previously observed with LTR probes in fall dermal sarcomas (46), but have not been observed with LTR probes in fall hyperplasias (data not shown). The presence of rv-cyclin transcripts in fall lesions suggests that the rv-cyclin proteins may play an important role in tumor induction. These data emphasize that there is differential expression of viral genes at different stages of disease, i.e., only a relatively low level of the rv-cyclin transcripts (and orfB transcripts for WDSV) is detected in developing fall lesions, whereas abundant levels of many viral transcripts are detected in regressing spring lesions. This phenomenon has been previously reported for WDS (10, 46), but not for WEH.

**DISCUSSION**

Sequence analysis of the WEHV1, WEHV2, and WDSV orfA\(\text{A}\)s shows that they encode cyclin D homologs. The homology of the rv-cyclins to D-type cyclins suggests that an ancestral retrovirus acquired a cellular cyclin gene, possibly in a process similar to the transduction of oncogenes by avian and murine simple retroviruses (57), and the virus subsequently evolved into WEHV1, WEHV2, and WDSV. Unlike the oncogenes acquired by the simple oncovirosis, which have substantial homology with their respective proto-oncogenes, the rv-cyclins share only limited homology with known cellular cyclins. WEHV1, WEHV2, and WDSV may represent a new class of oncogenic retroviruses; they are the first examples of retroviruses to encode cyclin homologs and are the only complex retroviruses that encode a protein with homology to a cellular proto-oncogene.

Divergent cyclin D homologs have previously been identified in two oncogenic herpesviruses, the KSHV and herpesvirus saimiri (HVS) (13, 14, 29). These viral cyclins have biochemical properties that differ from those of human D-type cyclins. For example, unlike human cyclin D1-activated Cdk6, HVS and KSHV cyclin-activated Cdk6 phosphorylates histone H1 in addition to Rb in vitro, suggesting that these cyclins alter the substrate preference of Cdk6 (23, 29). In addition, it was recently shown that the KSHV cyclin-Cdk6 complex is resistant to a number of proteins that function to negatively regulate cyclin-Cdk complexes (p16^INK4a, p21^WAF1, and p27^KIP1) (58). The resistance of the KSHV cyclin-Cdk6 complex to these inhibitors suggests that these viruses may use a novel mechanism for deregulating the cell cycle. Interestingly, the herpesvirus cyclins (and rv-cyclins) lack the canonical Rb binding domain (LXCXE) (18), suggesting that a different domain is used for binding to Rb or a different mechanism is used by these proteins to bind Rb. By analogy with the herpesvirus cyclins, the divergent rv-cyclins may have unique biochemical and functional properties from known cyclins.

Analogous to cellular cyclins, we suggest that the rv-cyclins may promote cell cycle progression by activating Cdns to initiate the protein phosphorylation cascade that culminates in cell division (54, 55). This is supported by our experiments showing that WDSV rv-cyclin rescues yeast deficient in G_{1}/S cyclins from growth arrest. These results indicate that WDSV cyclin can activate the yeast kinase, CDC28p, and therefore has the potential to stimulate the growth of walleye cells. While the WEHV rv-cyclins did not complement the cyclin deficiency in yeast, it has previously been observed that cyclins differ in their ability to complement cyclin deficiency in this experimental system, e.g., human cyclins A, B, C, and E work well in this assay, whereas, cyclin D1 works poorly (34). Therefore, we infer from their similarities to the human cyclin D and WDSV rv-cyclin that the WEHV1 and WEHV2 rv-cyclins may affect cell cycle progression in their homologous system. The hypothesis that the rv-cyclins may play a role in the induction of WDS and WEH is supported by (i) our observation that rv-cyclin transcripts are present in developing lesions, (ii) studies demonstrating that expression of human cyclin D1 from tissue-specific promoters in transgenic mice results in cell hyperproliferation in cognate tissues (48, 61), and (iii) the general observation that cyclin overexpression is associated with many types of human tumors (2).

Since most retroviruses require dividing cells for replication, the induction of cell proliferation by the rv-cyclins may be necessary for viral replication. However, other observations suggest the possibility that the rv-cyclins may have other roles in the viral life cycle. The pattern of a low level of viral gene expression that appears limited to orfA (and orfB in WDSV) mRNA in developing WDS and WEH is reminiscent of complex retrovirus accessory gene expression early after infection of tissue culture cells (16, 46). Similarly, the expression of accessory and structural genes observed in regressing WDS and WEH may be analogous to the pattern of human immuno deficiency virus (HIV) gene expression observed in the late stages of cell culture infection (16, 46). While a specific regulatory role for the rv-cyclins in viral gene expression cannot be assigned, it is noteworthy that cyclins and cyclin homologs play important roles in the assembly of transcription complexes. A yeast cyclin-like protein, Srb11, forms a novel complex with a Cdk (SRB10) that is part of the yeast RNA polymerase II holoenzyme (35). Analogously, the Cdk-activating kinase (CAK)-cyclin H complex is a subunit of the basal transcription factor TFIIH in higher eukaryotes (36). There is also precedent for cyclin involvement in regulating viral transcription. A cellular kinase complex has been identified whose activity is modulated by Tat to activate HIV transcription by increasing the elongation properties of RNA polymerase II (24, 37, 64). Recently, Wei et al. (62) identified a novel Cdk9-associated C-type cyclin (cyclin T) as a component of the Tat-associated kinase complex, and they provide evidence to suggest that cyclin T is a TAR RNA-binding cellular cofactor for Tat. This is interesting because the 5' end of WDSV mRNAs is predicted to fold into a stem-loop that is reminiscent of the TAR stem-loop. Additionally, human cyclin D1, independent of Cdk4, interacts directly with the estrogen receptor or the estrogen-estrogen receptor complex to stimulate transcription of cellular genes, thereby enabling estrogen-independent growth of breast tumor cells that overexpress cyclin D1 (65). The latter finding may be particularly relevant, because the development and regression of WDS and WEH and the changes in walleye retrovirus gene expression occur at different stages of the wall eye reproductive cycle.

**The walleye tumor model.** It is most likely that walleyes are infected by WEHV1, WEHV2, and/or WDSV when fish congregate in the spring of the year to spawn. Following initial infection, we presume that viral accessory gene protein expression (the rv-cyclins and possibly the OrfB proteins) induces some cells to proliferate abnormally, resulting in visible WDS or WEH by autumn. Lesions grow in size in the cold months,
but as spring approaches, the pattern and level of viral gene expression change to include high levels of all viral transcripts, and tumors begin to regress (10, 46). Tumor regression culminates in the spring, when WDS and WEH lesions are shed. These spring lesions contain an abundance of virions, which provide the inoculum to initiate another infectious cycle (9, 10).

In nature, neither WDS nor WEH has been found to develop into invasive or metastatic tumors, suggesting that WDSV, WEHV1, and WEHV2 are only weakly oncogenic. However, the observation that invasive tumors occur in experimentally inoculated walleye fingerlings indicates that WDSV does harbor a potent oncogene, possibly the rv-cyclin. Therefore, we suggest that in nature, tumor regression occurs before invasive or metastatic tumors develop into invasive or metastatic tumors, suggesting that transplantable tumors from naturally occurring walleye dermal sarcoma tissue (25). These spring lesions contain an abundance of virions, which provide the inoculum to initiate another infectious cycle (9, 10).

In summary, investigation into the processes of tumor induction and regression in this unique retroviral system will identify the viral, host, and environmental factors whose interactions and regulatory mechanisms determine tumor progression and tumor regression.