Brief Definitive Report

Independence of Herpesvirus-induced T Cell Lymphoma from Viral Cyclin D Homologue

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Abstract

Cyclin D family members are cellular protooncogenes, and their viral homologues in the Kaposi's sarcoma-associated herpesvirus (KSHV, human herpesvirus type 8 [HHV-8]) and the closely related *Herpesvirus saimiri* have been implicated as putative cofactors of viral transformation and pathogenesis. KSHV is regularly found in Kaposi's sarcoma and in the primary effusion B cell lymphoma and Castleman's disease associated with immunosuppression and AIDS. *H. saimiri* strain C488 transforms human and marmoset T cells in vitro and causes polyclonal T cell lymphoma in New World monkeys. The viral cyclins stimulate cell cycle progression of quiescent fibroblasts, and they form active cyclin-dependent kinase (CDK)6 complexes of broad substrate specificity that can resist and downregulate cellular CDK inhibitors. This study shows that the viral cyclin of *H. saimiri* strain C488 is not required for viral replication, T cell transformation, and pathogenicity in New World primates.

Key words: cell cycle • gammaherpesviridae • herpesvirus, Kaposi sarcoma associated • oncogenic viruses • callitrichinae

Introduction

The rhadinoviruses *Herpesvirus saimiri* and Kaposi's sarcoma (KS)-associated herpesvirus (KSHV, human herpesvirus 8 [HHV-8]) both encode viral cyclin D homologues and several other candidate viral oncogenes. Cyclin D family members are known cellular protooncogenes (1, 2), and the viral cyclin of *H. saimiri* (V-cyclin) and KSHV (K-cyclin) have been implicated as putative cofactors of viral transformation and pathogenesis (3–5).

KSHV is almost invariably found in endemic and AIDS-associated Kaposi's sarcoma and in rare lymphoproliferative syndromes associated with immune suppression, like primary effusion B cell lymphoma (PEL) and multicentric Castleman's disease (MCD). Several candidate oncogenes and cofactors for viral transformation have been identified, among them the KSHV homologues to cyclin D (K-cyclin) and G protein–coupled receptors (GPCRs; reference 6).

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The viral cyclins stimulate cell cycle progression of quiescent fibroblasts, and they were shown to form active cyclin-dependent kinase (CDK)6 complexes resistant to inhibition by cellular CDK inhibitors (7). Interestingly, the K-cyclin is latently expressed in KS tissues (8) and PEL cells (9). The closely related *H. saimiri* transforms human and marmoset T cells in vitro and causes polyclonal T cell lymphoma in New World monkeys. The open reading frame (ORF)72 of *H. saimiri* encodes the viral cyclin D homologue (V-cyclin; reference 4), a 29-kD protein that is expressed in growth-transformed marmoset T cell lines (5). Both *H. saimiri* and KSHV cyclin D homologues bind and activate cellular CDKs 2, 4, and 6. The most stable and active complexes are formed with CDK6, and these complexes are resistant to cellular CDK inhibitors $p16^{Ink4a}$, $p21^{Cip1}$, and $p27^{Kip1}$, and K-cyclin expression has even been shown to result in $p27^{Kip1}$ degradation. In addition, they can stimulate S phase entry and cell growth of quiescent fibroblasts (3, 5, 7, 10, 11). The viral cyclin encoded by B lymphotropic murine gammaherpesvirus 68 (MHV-68 M-cyclin) is expressed as a lytic leaky-late or early-late transcript (12, 13). M-cyclin acts as an oncogene when overexpressed in transgenic mice

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which then develop T cell lymphoma (13). On the other hand, the M-cyclin has a restricted CDK preference, as it binds only to CDK2 in a similar way to cyclin A, and this binding is partially inhibited by $p27^{Kip1}$ (14). Although MHV-68 infection was reported to be associated with lymphoproliferative disease and lymphoma in aging mice (15), tumor cell lines established from such lesions typically do not harbor MHV-68 (16), and lymphocyte growth transforming properties are absent in vitro (17). Thus, there is an obvious contrast between an oncogenic phenotype in a transgenic system and uncertain tumorigenicity of MHV-68 infection in mice.

In view of the uncertain role of rhadinoviral cyclins, we studied the *H. saimiri* V-cyclin, as *H. saimiri* has a clearly defined growth transforming and oncogenic phenotype in New World primates. The V-cyclin gene was deleted from the genome of *H. saimiri* strain C488, resulting in a replication competent virus. This recombinant virus showed that the V-cyclin is not necessary for T cell transformation of human and common marmoset lymphocytes in vitro, and the virus remained oncogenic in *Saguinus oedipus* tamarins, indistinguishable from the wild-type strain.

Materials and Methods

Cell Culture and Virus Propagation. Owl monkey kidney (OMK) cells (American Type Culture Collection, CRL1556), cultivated in DMEM supplemented with 350 μg/ml glutamine, 100 μg/ml gentamycine, and 10% heat-inactivated FCS, were used for the propagation of *H. saimiri.* Virus stocks were generated by infection of confluent OMK cells seeded in 175-cm^2 tissue culture flasks at low multiplicity. When lysis was complete, supernatants were cleared from cellular debris by centrifugation at 2,000 *g* for 15 min and cell-free supernatants were stored at −80°C. For virus titration, OMK cells were grown in 48-well plates and infected with serial 10-fold dilutions (10^{-3}–10^{-7}) of *H. saimiri* C488 and C488Δcyclin in 400 μl DMEM with supplements. Single step growth curves for the analysis of virus replication kinetics were done by infection of OMK cells (3×10^5 cells seeded in a 25-cm^2 flask 2 d before infection) with 10^4 tissue culture infectious particles (TCIPs) in 10 ml medium, corresponding to 0.01 TCIPs/cell. Virus containing supernatant taken from subsequent days was titrated by limiting dilution until lysis was complete.

Construction of the Viral Deletion Mutant C488Δcyclin. The ORF72 was deleted from the *H. saimiri* strain C488 genome by a cosmid-based approach. All cloning procedures were performed by standard methods. A *KpnI-PinAI* fragment including ORFs 71, 72, and the 3′ portion of ORF73 was subcloned from cosmid Dc5 into pNEB193PIN (constructed by insertion of a PinAI adapter into the HindIII site of pNEB193 (New England Biolabs, Inc.). The complete ORF72 was deleted by PCR (Expand Long Template Kit™; Roche Diagnostics) using oligonucleotides 5′-GGCGCGCCTCGAAATTCTGTAAATGGAC-3′ and 5′-CGTACGTTTGATGATGTCATTCTATGGGC-3′. The modified *KpnI-PinAI* fragment lacking the ORF72 was verified by DNA sequencing, reinserted into cosmid Dc5, and finally the correct reinsertion was verified again by DNA sequencing. Nucleotide sequences were determined with an ABI 377A automated sequencer (Applied Biosystems) using the Dye-Deoxy Terminator Sequencing™ kit according to the manufacturer's instructions (PerkinElmer). Recombinant virus was generated by liposome-mediated cotransfection (Lipofectamine®; Life Technologies) of a set of overlapping cosmids including the altered cosmid Dc5Δcyclin into permissive OMK cells (see Fig. 1). The cosmids were linearized before transfection by restriction with NotI; this also removed the pWE15 cloning vector, as two NotI sites are flanking the BamHI site used to clone the viral DNA.

Detection of V-Cyclin Protein. Confluent OMK cells in 25-cm^2 flasks were infected with 10^6 TCIPs of C488 or C488Δcyclin virus or mock infected. Protein was extracted in lysis buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris, and 1% Triton X-100) when first signs of cytopathic effect (CPE) became visible. 20 μg of total protein were separated on 15% PAGE gel and blotted to polyvinylidine difluoride (PVDF) membrane. The V-cyclin was detected using a 1:500 dilution of specific rabbit polyclonal antiserum (a gift of J. Jung, New England Regional Primate Research Center, Southborough, MA and 1:5,000 diluted peroxidase-conjugated anti–rabbit immunoglobulin with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

In Vitro Transformation of Human and Marmoset Lymphocytes. Mononuclear cells from heparin-preserved human umbilical cord blood was purified by centrifugation in dextran separation medium, common marmoset blood with Histopaque™ solution (equal volumes of Histopaque™ 1.083 g/ml and 1.117 g/ml; Sigma-Aldrich). The mononuclear cells were recovered, washed, and resuspended in lymphocyte growth medium (LGM; RPMI medium with 10% FCS, 45% Panserin™ serum-free medium [Pansystems], 350 μg/ml L-glutamine, and 100 μg/ml gentamycin). For the transformation of human T cells, LGM was supplemented with recombinant IL-2 (100 U/ml Aldesleukin [Proleukin™; Chiron Corp.] or 20 U/ml human IL-2 [Roche Diagnostics]). Lymphocytes were expanded for 2–3 d after isolation by stimulation with 0.5–1 μg/ml phytohemagglutinin A. $3–5 \times 10^6$ cells were then infected with 1 ml of *H. saimiri* C488 or mutant virus C488Δcyclin (titer $>10^6$ TCIPs/ml) and cultivated as described above. The transformation of the resulting T cell lines was assessed microscopically and by the observation of accelerated growth compared with uninfected control cultures that stopped growing after 4–6 wk. None of the uninfected controls yielded a transformed T cell line.

Animal Experiments and Ex Vivo Culture. Two cottontop tamarins (*S. oedipus*; R224 and B214) were infected intravenously with 10^6 TCIPs of C488Δcyclin, and one control animal (B236) received the same dose of oncogenic C488 wild-type virus. As oncogenic *H. saimiri* strain C488 consistently showed a lethal phenotype in this species, larger numbers of animals were not considered due to ethical reasons. The study had been approved by the Institutional Animal Care and Use Committee and was performed according to governmental regulations with purpose bred, healthy adult cottontop tamarins at the Biomedical Primate Research Center (Rijswijk, The Netherlands). They were housed in separate cages and received a standard monkey diet and drinking water ad libitum. Blood samples were taken prior infection, at weekly intervals, and before necropsy, to expand T lymphoma cells and reisolate virus by cocultivation on OMK cells. The C488Δcyclin-infected animals died or were killed on days 14 and 16 after infection, the control animal died on day 15, and necropsy was performed. Single cell suspensions were prepared from fresh liver, spleen, kidney, thymus, and lymph node tissue and cultured in LGM without additional growth factors. In addition, tissues were fixed in formalin and stained with hematoxylin and eosin, and for immunohistochemistry additionally with antibodies specific for CD3 (Dako) and CD20 (Dako) and detected

with alkaline phosphatase–coupled avidin–biotin complex secondary reagents.

Detection of Viral DNA. Virus-containing supernatant from completely lysed cultures was harvested by centrifugation, and the pelleted virions were lysed in 100 μl of PCR buffer containing 100 μg/ml proteinase K (Roche Diagnostics) and 0.5% Tween 20 for 1 h at 56°C, then the proteinase K was heat-inactivated for 15 min at 95°C. Alternatively, 2×10^5 cells were lysed under the same conditions. An aliquot of 2–4 μl was used for PCR analysis.

The status of viral DNA in the transformed cell lines was analyzed by PCR and Southern blotting. PCR analysis was carried out in 25-μl reaction volumes, containing 2 μl of template DNA, 0.2 mM dNTP, 0.4 μM of each primer, and 2.5 U of AmpliTaq polymerase in 1× AmpliTaq buffer (PerkinElmer). PCR conditions were as follows: a 5-min denaturation at 95°C, 29 cycles of 30 s at 95°C, 30 s at 56°C, and 1 min at 70°C; a 4-min extension at 70°C; and a 4°C hold. The following primer pairs specific for the respective ORFs were used for the analysis: StpC/Tip, 5′-GTAGTAAACTAAGAGCAAAGCAAGC-3′ and 5′-GTACAAGCTGTTCAAGTTTGTTAGC-3′; ORF3, 5′-CACAACACTGGTATGTACCAATG-3′ and 5′-CTGTGGAGGTAATGCAGATAC-3′; ORF75, 5′-TGGCTGCTAACAGGCATGG-3′ and 5′-AGCACGTTGCCCGAGATTG-3′; V-cyclin a61=5′-CTAAAAATGCAGCATCGTCACC-3′ and 5′-TGCGTTAGACAAATATCCC-3′.

For Southern blotting, 20 μg of total DNA were digested with SstI or PstI and electrophoresed on 1% agarose gels. The DNA was transferred to a nylon membrane (Hybond N; Amersham Pharmacia Biotech) and hybridized with a DNA probe that was random-labeled with [α-^{32}P]dATP. This 1.9-kb probe specific for ORFs 71 and 72 was amplified by PCR from cosmid 40 (primers: 5′-TGCGTTAGACAAATATCCC-3′ and 5′-CTAAAAATGCAGCATCGTCACC-3′, conditions as above) and purified from a 1% agarose gel.

RNA and cDNA Analysis. Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction. 5 μg RNA were treated with RNase-free DNaseI (Roche-Diagnostics) in 1× DNaseI buffer for 30 min, followed by heat inactivation at 70°C for 10 min. Then first strand cDNA was synthesized with random hexamer and oligo-dT primers and Superscript II reverse transcriptase (GIBCO BRL). RNA complementary to the cDNA was then removed by addition of 2 U RNaseH (MBI Fermentas) and incubation for 20 min at 37°C. An identical sample was prepared in parallel where the reverse transcriptase was omitted from the reaction mixture (−RT control sample). 2 μl of the reactions were used for reverse transcription (RT)-PCR analysis (as above except that 40 cycles of 95°C, 52°C, and 70°C were done). Primers were specific for the following ORFs: NH_2-terminal ORF73, 73RT5, 5′-CACACTCTGCGTTCTGAGTGC-3′ and 73RT3, 5′-AACGTTGGGCACTTGGTCCTG-3′, amplification product 962 bp; COOH-terminal ORF73, 73ns, 5′-CTATGGGCAAGCTTTTGC-3′ and A61, 5′-CTAAAAATGCAGCATCGTCACC-3′, amplification product 396 bp; V-cyclin 73RC3, 5′-CCCTAGATCTGCTAAATTACAAGC-3′ and 71RT, 5′-TGCGTTAGACAAATATCCC-3′ amplified products of 1,060 and 265 bp, respectively; ORF71, 5′-TCGAAATTCTGTAAATGGAC-3′ and 71RT, 5′-TGCGTTAGACAAATATCCC-3′, amplification product size 190 bp; β-Actin ACT5, 5′-CGGGAAATCGTGCGTGACAT-3′ and ACT3, 5′-GAACTTTGGGGGATGCTCGC-3′, amplification product size 587 bp.

Flow Cytometry and Cell Cycle Analysis. Transformed human and simian T cells were analyzed by flow cytometry (FACSCalibur™; Becton Dickinson) with directly labeled monoclonal antibodies specific against B and T cell surface epitopes (Cy-Chrome or phycoerythrin conjugated): CD2 (RPA-2.10; BD PharMingen), CD3e (SP34; BD PharMingen), CD3 (Leu-4; Becton Dickinson), CD4 (Leu-3a SK3; Becton Dickinson) CD8 (RPA-T8; BD PharMingen), CD20 (Leu16 L27; Becton Dickinson), HLA-DR (L243; Becton Dickinson), isotype-matched control antibodies (Becton Dickinson/BD PharMingen).

Results and Discussion

The complete V-cyclin gene was precisely deleted from cosmid-cloned *H. saimiri* strain C488 DNA by subcloning and PCR. Recombinant *H. saimiri* C488Δcyclin was generated by homologous recombination after transfection of overlapping cosmids into permissive OMKs cells (Fig. 1 A). Absence of the V-cyclin gene was verified by PCR and Southern blotting, and absence of protein expression by Western blotting from infected OMK cells (Fig. 1 B). Lytic viral replication in OMK cells was comparable to wild-type virus even when infection started at a low multiplicity of 0.01 TCIPs per cell, and endpoint titers of both wild-type and recombinant virus were between 10^6 and 10^7 TCIPs/ml in three independent experiments.

H. saimiri C488 and related strains are distinguished by their ability to transform human primary T cells to permanent antigen-independent growth (18, 19). In vitro transformation assays were performed with 3–5 × 10^6 primary human cord blood lymphocytes (CBLs) and *Callithrix jacchus* PBMCs from independent donors. Limiting dilution of lymphocytes for transformation assays is not feasible due to the specific growth requirements of *H. saimiri*–

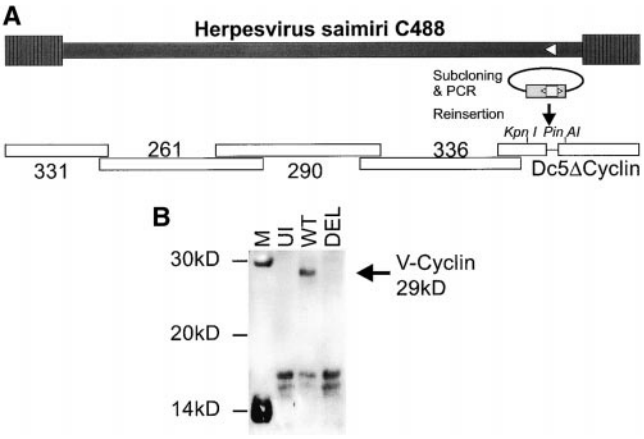

Figure 1. V-cyclin transcript mapping and recombinant virus. (A) Construction of recombinant virus C488Δcyclin from five overlapping cosmids 331, 261, 290, 336, and Dc5Δcyclin. (A) *KpnI-PinAI* fragment from cosmid Dc5 was subcloned into pNEB193PIN, the ORF72 deleted by PCR, and the fragment reinserted into the cosmid. Recombinant virus was generated by cotransfection of the overlapping linearized cosmids into permissive OMK cells. (C) Absence of 29 kD V-cyclin protein expression in OMK cells infected with recombinant virus compared with parent strain C488. M, marker; UI, uninfected; WT C488, wild-type infected; DEL, C488Δcyclin infected (polyclonal antibody was a gift of J. Jung).

Table I. *Transformation by H. saimiri C488Δcyclin In Vitro and Pathogenesis in Infected Cottontop Tamarins*

Virus	Species	In vitro transformation experiments (pos./total)	Pathogenesis (survival time in days)
C488	Human CBL	8/8	na
	C. jacchus	9/10	nd
	S. oedipus	nd	15
C488Δcyclin	Human CBL	8/8	na
	C. jacchus	5/5	nd
	S. oedipus	nd	15, 16

Transformation by recombinant viruses in vitro and pathogenesis in infected cottontop tamarins. Results from in vitro transformation experiments performed with human CBLs (eight different donors), and PBMCs from *C. jacchus* (five donors). Cells were infected in parallel with *H. saimiri* C488 or C488Δcyclin; the number of successful transformation experiments is shown. Pathogenesis was studied in cottontop tamarins (*S. oedipus*). Two *S. oedipus* tamarins were infected with C488Δcyclin (animals R224 and B214), and one animal (B236) received the wild-type positive control strain C488. pos., positive; na, not applicable; nd, not done.

transformed lymphocytes (19, 20). In these assays, the C488Δcyclin mutant-transformed human and simian lymphocytes to T cell lines like the C488 wild-type virus (Table I). Thus, the V-cyclin is not required for in vitro transformation of human and common marmosets lymphocytes by *H. saimiri* strain C488.

However, in vitro transformation not always parallels in vivo pathogenicity (21; for a review, see reference 22). Two *S. oedipus* tamarins (R224 and B214) were therefore infected intravenously with 10^6 TCIPs of C488Δcyclin, and a control animal (B236) received the equivalent dose of oncogenic C488 wild-type virus. Larger numbers of animals were considered unnecessary, as oncogenic *H. saimiri* strains consistently have a lethal phenotype in this species. The C488Δcyclin infected animals became symptomatic and died or were killed on days 15 and 16 after infection, and the control animal died on day 15. Necropsy was performed and macroscopic examination of tissues revealed enlarged hyperemic lymph nodes, and white infiltrations in kidneys. Histological examination of various tissues showed infiltration of blast-like lymphoid cells that was consistent with acute disseminated $CD3^+$ T cell lymphoma (Fig. 2 A). Single cell suspensions were prepared from liver, spleen, kidney, thymus, and lymph nodes, and at least four independent lymphocytic cell lines growing without IL-2 were established from different tissues of each animal. Flow cytometry analysis of the tumor-derived cell lines revealed a phenotype of CD3 and CD4 and/or CD8-positive T

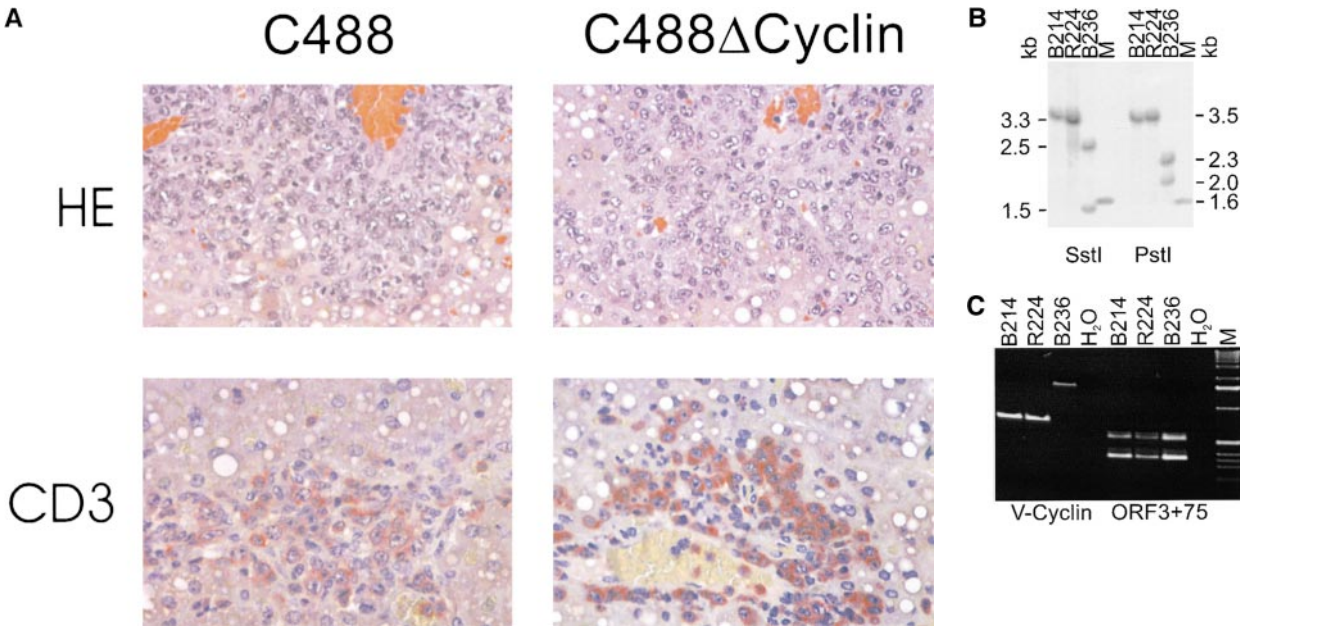

Figure 2. Animal experiments and ex vivo culture. (A) Histology from C488Δcyclin (R224) and C488 (B236) infected monkeys. Both show infiltration of liver tissue with large numbers of blast-like cells. Immunohistochemistry revealed that almost all the infiltrating cells stained positive for CD3. HE, hematoxylin and eosin stain. (B) Analysis of viral genome in cell lines established from diseased cottontop tamarins. Southern blot with a ^{32}P-labeled probe spanning viral ORFs 71 and 72 shows the expected fragment pattern and absence of wild-type C488 virus in C488Δcyclin mutant. Cell lines established from animal B214, R224, and B236, respectively. (C) Analysis of cell lines established from diseased cottontop tamarins. V-cyclin: PCR performed with oligonucleotide primers flanking the V-cyclin deletion show expected amplification products of 629 or 1413 bp in animals infected with C488Δcyclin (B214, R224) or C488 (B236), respectively. ORF3+75: multiplexed PCR for the detection of two reading frames from the left and right end of the *H. saimiri* genome. H_2O, negative control; M, marker.

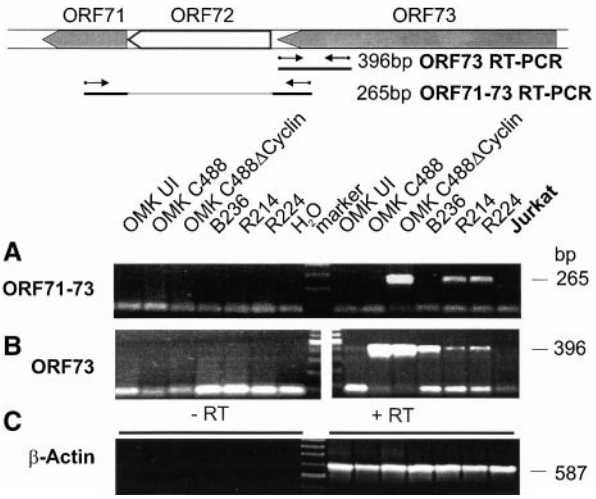

Figure 3. Transcription of the V-cyclin neighboring genes. RT-PCR analysis from total RNA of infected OMK-cells and tumor-derived *S. oedipus* T cell lines: ORF73/ORF71- (A) and ORF73- (B) derived transcripts. (C) Shows amplification of cellular β-actin transcripts. OMK UI, uninfected OMK cells; Jurkat, cDNA from cell line Jurkat are shown as additional controls. +RT and −RT: parallel reactions performed with template generated by first strand cDNA synthesis with and without Superscript™ reverse transcriptase, respectively, to control for genomic DNA contamination.

cells, along with high expression of MHC class II, CD25, CD80, and CD86 (not shown). All cell lines were harboring the respective viral genome as shown by Southern blotting (Fig. 2 B). The absence of the deleted V-cyclin gene and of wild-type virus was also shown by PCR (Fig. 2 C). In summary, the *H. saimiri* V-cyclin homologue is also not contributing to the pathogenicity by this oncogenic rhadinovirus. The transcription of the *H. saimiri* V-cyclin in simian and latently infected human T cells was investigated by Northern blot analysis, but no transcripts were found. As there are no available specific and sensitive reagents, protein expression of the ORF73 and ORF71 could not be studied. However, a specific spliced mRNA that can encode ORFs 73, 72, and 71 was detectable by RT-PCR and ribonuclease protection assays (RPA; unpublished data). Sensitive RT-PCR found no evidence for expression of additional spliced mRNAs that would encode ORF72 and 71 only, and which had been found in KSHV-infected cells (23–25). As the V-cyclin is encoded by a tricistronic transcript, RT-PCR for the flanking ORFs 71 and 73 was performed, demonstrating that transcription of these genes still occurs in the deletion virus infected or transformed cells (Fig. 3).

Although it was shown that the MHV-68 M-cyclin is dispensable for viral replication and pathogenicity in immunodeficient mice and that it is necessary for efficient reactivation from latency (12, 26), this model does not allow to address the question of oncogenesis and transformation. Pathogenesis of this recombinant virus was assessed in immune-deficient SCID mice not having lymphocytes, thus lymphoma induction can not be studied (12, 26). Most likely, though not defined precisely, death results of overwhelming lytic viral replication, and associated cell destruction. Even from lymphomas occasionally observed in long-term infected mice (15), tumor cells harboring MHV-68 are only infrequently cultivated (16). However, after induction of B- or T cell lymphoma in immune competent cottontop tamarins by Epstein-Barr virus or *H. saimiri*, respectively, growth-transformed tumor-derived cell lines harboring the transforming virus can be regularly expanded.

In conclusion, the data show that the V-cyclin of *H. saimiri* is dispensable for viral replication, transformation of simian or human lymphocytes in vitro, and pathogenesis in cottontop tamarins in vivo. Remarkably, experiments in vitro had lead to speculations on the viral cyclins as putative oncogenes or cofactors for transformation (3, 5, 7, 13). The finding that the V-cyclin of *H. saimiri* has no oncogenic properties in the background of the viral genome stresses the importance of studying viral genes in their original genetic context and shows the limitations when isolated viral genes are overexpressed. Nevertheless, the viral cyclin genes may be relevant in the persistently infected natural host, where they could contribute to improved survival and spread of latently infected cells. It does not exclude implications on the AIDS-associated malignancies associated with the closely related KSHV, where analogous studies are not possible due to a lack of permissive systems and animal models for pathogenesis.

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