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Oncogenic herpesvirus DNA absence in kidney cell lines established from the northern leopard frog *Rana pipiens*

John W. Williams III[1,*], Kenyon S. Tweedell[2], Doreen Sterling[1], Nikki Marshall[1], Carolyn G. Christ[3], Debra L. Carlson[4], Robert G. McKinnell[3]

[1]Department of Biology, Tuskegee University, Tuskegee, Alabama 36088, USA
[2]Department of Biology, University of Notre Dame, Notre Dame, Indiana 46556, USA
[3]Department of Genetics and Cell Biology, University of Minnesota, Saint Paul, Minnesota 55108-1095, USA
[4]Department of Biology, Augustana College, Sioux Falls, South Dakota 57197, USA

ABSTRACT: The etiological agent of the Lucké renal adenocarcinoma of the northern leopard frog *Rana pipiens* is the Lucké tumor herpesvirus (LTV). LTV can be detected with the electron microscope in thin sections of spontaneous tumors from frogs that have been exposed to a cold environment. No viruses can be detected with the electron microscope in spontaneous tumors of frogs maintained under warm conditions even though the 'virus-free' (warm) tumors contain latent LTV. Because electron microscopy is an insufficient procedure to detect the oncogenic herpesvirus during its latent phase and because we sought to ascertain whether the virus is present in cells which do not manifest electron microscope-detectable viruses, we developed a polymerase chain reaction (PCR) procedure to amplify a *Hin*d III fragment of the viral DNA, JH12. While all warm, 'virus-free', spontaneous kidney tumors studied thus far contain this restriction enzyme fragment of LTV DNA, we report here that we failed to detect JH12 DNA in the tumor-derived cell line PNKT-4B. A normal frog cell line, WMPA, similarly failed to exhibit the restriction enzyme fragment of LTV DNA.

KEY WORDS: *Rana pipiens* · Lucké renal adenocarcinoma · PNKT-4B cell line WMPA cell line · Herpesvirus · Polymerase chain reaction

INTRODUCTION

The Lucké renal adenocarcinoma afflicts some populations of the northern leopard frog *Rana pipiens*. The neoplasm was the first tumor thought to be associated with a herpesvirus (Lucké 1938) and the Lucké tumor herpesvirus (LTV) was subsequently shown to be the etiological agent of the malignancy (Naegele et al. 1974). Viruses which infect eukaryotic cells often have a latent stage in which virions are not detectable (Griffiths 1992). The oncogenic LTV enters such a latent phase at warm temperatures (Zambernard & McKinnell 1969). We developed a PCR procedure to detect LTV DNA in tumors and other tissues (Carlson et al. 1994a, b, 1995, McKinnell et al. 1995). In the present study, we used the PCR procedure to ascertain if the Lucké pronephric tumor-derived cell line, PNKT-4B, and a normal frog pronephric cell line, WMPA, contain the oncogenic Lucké herpesvirus DNA. We report here that neither the tumor-derived cell line nor the normal cell line contains the LTV DNA *Hin*d III restriction fragment JH12.

MATERIALS AND METHODS

Cell lines. PNKT-4B is a variably aneuploid cell line (Williams et al. 1990) derived from a pronephric renal tumor of *Rana pipiens* (Tweedell 1978). Cells, grown in modified Leibovitz's L-15 (Leibovitz 1963), supplemented with fetal calf serum and antibiotics, were harvested at confluency and centrifuged to form a pellet. WMPA cells, derived from normal pronephroi (Wong &

*E-mail: jwilliams@acd.tusk.edu

Tweedell 1974), were similarly grown to confluency and harvested.

PCR analysis. DNA was isolated from the cell culture pellets by the method of Jackson et al. (1991). Two 22 bp oligonucleotide primers were used to detect the presence of a 1.2 kbp *Hind* III restriction fragment of LTV DNA, JH12 (Carlson et al. 1995). Between 1 and 4 µl of template DNA (200 to 400 ng μl^{-1}) was placed in a total reaction volume of 50 µl: 0.2 mM each of dATP, dCTP, dGTP and dTTP (Gibco/BRL, Grand Island, NY, USA); 1 µM of each primer; 1.25 units *Taq* in *Taq* polymerase buffer plus Mg^{2+} (Boehringer Mannheim, Indianapolis, IN, USA). The reaction was run through 40 cycles (1 min at 95°C, 2 min at 55°C, 3 min at 72°C) on a Coy Model 60 Tempcycler (Coy Laboratory Products, Ann Arbor, MI, USA). The initial denaturing step was extended to 3 min.

Analysis of PCR products. The PCR reaction products were subjected to electrophoresis on a 1% agarose gel in 0.5× Tris-borate-EDTA buffer. The DNA on the gel was transferred to a MagnaNT nylon membrane (Micron Separations, Westboro, MA, USA) for Southern hybridization and probed with the *Hind* III 1.2 kbp LTV restriction fragment labeled with digoxigenin. The membrane was washed and incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase. LumiPhos™ 530 was added to the membrane. Subsequent exposure to Kodak XAR film allowed for visualization at the sites of hybridization (The Genius™ System, Boehringer Mannheim).

RESULTS

PNKT-4B and WMPA cells were examined for the presence of the *Hind* III restriction fragment of LTV DNA by analysis of the PCR products and Southern hybridization. We did not detect the presence of the restriction enzyme fragment in either cell type (Fig. 1).

DISCUSSION

The PCR procedure used in this study was developed to detect the presence of LTV DNA in tissues lacking a productive viral infection. LTV-infected tissues which do not exhibit a productive infection and therefore lack virions detectable with electron microscopy (Zambernard & McKinnell 1969) retain viral DNA. We previously reported the presence of LTV DNA in all spontaneous mesonephric Lucké renal carcinomas (with both productive and latent LTV infections) (Carlson et al. 1994a, b, 1995, McKinnell et al. 1995).

PNKT-4B is a pronephric Lucké tumor-derived cell line which has been maintained in continuous culture for at least 17 yr (Tweedell 1978). Because the primary neoplasm was induced by LTV, it seemed reasonable to inquire if the etiological agent has persisted in the PNKT-4B cell line for this extended period. We report here that LTV DNA was not found by PCR amplification of the *Hind* III 1.2 kbp fragment in PNKT-4B.

Invasion is an essential step in the cascade of events leading to metastatic cancer. The Lucké renal adenocarcinoma, as expected, is invasive and metastatic as a primary tumor *in vivo* (McKinnell & Tarin 1984) and is invasive *in vitro* (Mareel et al. 1985, McKinnell et al. 1986). It is interesting to note that PNKT-4B retained this competence to invade *in vitro* (McKinnell et al. 1988). Primary Lucké renal adenocarcinoma cells, as well as normal frog cells, have a well developed typical cytoplasmic microtubule complex (CMTC) at 20 and 28°C. The CMTC is well organized in normal cells at

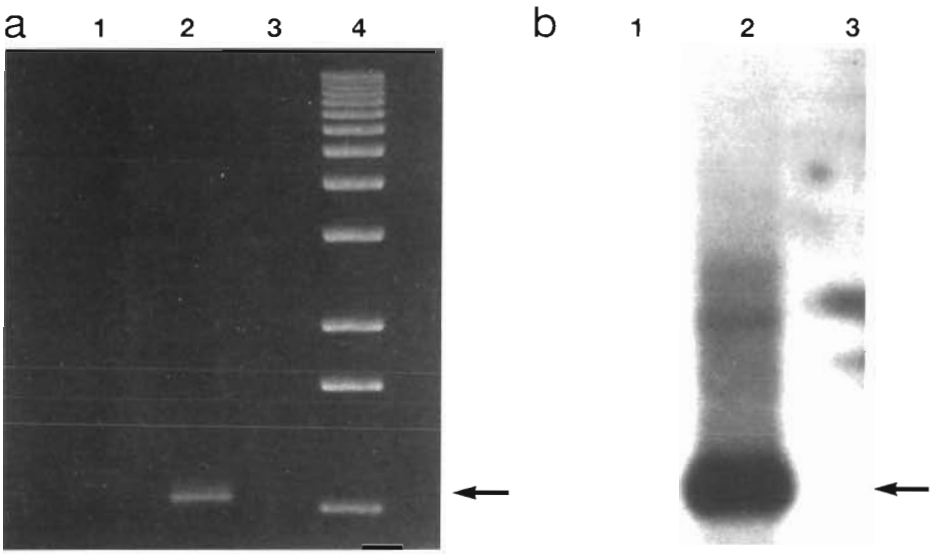

Fig. 1. Agarose gel electrophoresis and Southern hybridization of products resulting from PCR amplification of a 1.2 kbp *Hind* III restriction fragment of LTV DNA derived from *Rana pipiens* kidney cell lines. (a) Agarose gel of PCR products. Lane 1: PNKT-4B (negative for the 1.2 kbp fragment). Lane 2: Positive control (1.2 kbp *Hind* III fragment). Lane 3: Negative control (H_2O). Lane 4: 1 kbp DNA ladder. (b) Southern hybridization of agarose gel. Lane 1: PNKT-4B (negative for the 1.2 kbp fragment). Lane 2: Positive control (1.2 kbp *Hind* III fragment). Lane 3: Negative control (H_2O). Arrows indicate 1.2 kbp

7°C but it is disorganized in the frog renal adenocarcinoma at that temperature. The CMTC of PNKT-4B is intact at 20 and 28°C but is disorganized at 7°C. Thus, the CMTC of PNKT-4B resembles that of primary Lucké renal carcinoma, not normal frog cells (McKinnell et al. 1984). Current data indicate that sometime since its establishment as an immortal transformed cell line, PNKT-4B has apparently lost at least a fraction of the genome of the etiological agent responsible for transformation, LTV. At present, the nature of the genetic or epigenetic event in the PNKT-4B tumor-derived cells, which permits the escape of the etiological agent DNA, but allows for retention of neoplastic characteristics, is not known.

There is, at present, no evidence to indicate that LTV DNA is integrated into the genome of the host frog. The lack of integration may make LTV DNA vulnerable to loss from rapidly replicating cells such as PNKT-4B. There is another example of loss of LTV DNA from cells under rapid mitosis. Lucké tumor nuclei, when transplanted into enucleated ova, result in mitotic progeny which undergo significant embryonic differentiation (Lust et al. 1991, McKinnell 1994). Most (31 of 34) embryos produced by tumor nuclear transplantation lack the LTV *Hind* III 1.2 kbp restriction enzyme fragment (Carlson et al. 1994b). PNKT-4B similarly lacks the JH12 fragment as reported here.

The possibility of LTV DNA integration in the host genome, however, does not eliminate the potential for its loss as a cell cycle, rate dependent, event. It is possible that the replication origin, controlling synthesis of the JH12 fragment, is unable to adapt to the rapid cell cycles in the examples described above. Previous studies (DiBerardino & Hoffner 1970) have indicated that abnormal nuclear transplant embryos and larvae derived from late stage embryo nuclear donors are associated with chromosome abnormalities that appear to result from a failure of some chromosome regions to adapt to the rapid mitoses that follow nuclear transplantation. Observations of the karyotype of PNKT-4B, which displays significant chromosome rearrangements when compared to both normal frog and tumor karyotypes (Williams et al. 1990, 1993), suggest that alterations in replication patterns may result in immortalization of the cell line. Such alterations may be the mechanism by which JH12 is lost.

We did not expect WMPA cells, derived from normal *Rana pipiens* embryos, to contain JH12. WMPA met our expectations.

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