the prevalence of intranuclear inclusion bodies in osteoclasts, mirrored in degree the lead concentration in bone. Thus, they increased with increasing chronicity and were less pronounced in pigs with higher levels of dietary calcium.

Our interpretation of the sequence of events in lead intoxication is based on an application of Belanger's postulates on bone metabolism—namely, that osteocytic osteolysis is the primary mechanism in bone resorption in both physiological and pathological situations and that osteoclasts is a late phenomenon, concerned with the removal of already altered bone (*10*). During osteolysis, the osteocyte is metabolically very active and responds quickly to lead intoxication, and with greater chronicity it dies and the dead bone tissue is resorbed by osteoclasts. Lead is ingested by these cells of known phagocytic capacity. The pathogenesis of the lead-containing inclusion bodies is yet to be elucidated.

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Virus-Free Adenocarcinoma of the Frog (Summer Phase Tumor) Transcribes Lucké Tumor Herpesvirus-Specific RNA

Abstract. *[^{3}H]RNA isolated from "virus-free," summer phase, renal adenocarcinomas of* Rana pipiens *labeled with [^{3}H]uridine hybridizes with DNA of herpesvirus particles isolated from the winter phase Lucké tumor. Transcription of the herpesvirus genome in virus-free tumors provides additional evidence for the viral etiology of this tumor.*

Herpesviruses are associated with several vertebrate neoplasms including Burkitt's lymphoma (human), Marek's disease (chicken), the Lucké renal adenocarcinoma (frog), and possibly human nasopharyngeal carcinoma and cervical carcinoma (*1*, *2*). However, evidence for the herpesvirus etiology of human cancer, although compelling in some instances, is mainly circumstantial particularly in view of the widespread occurrence of herpesviruses in nature and their possible occurrence as passenger viruses. The Lucké renal adenocarcinoma is a naturally occurring neoplasm of the leopard frog which exists in nature in two temperature-related states; winter phase tumors contain herpesvirus particles whereas summer phase tumors are free of detectable virus (*2*). Prolonged exposure to lowered temperature results in the change from the "virus-free" summer state to the overtly virus-containing winter state. These tumors provide a favorable system for determining unequivocally the relation between a herpes-type virus and cancer. If viral genetic material was transcribed in the "virus-free" summer tumor, the herpesvirus etiology of the Lucké adenocarcinoma would be established more firmly. We show in this report that RNA from the summer adenocarcinoma hybridizes with viral DNA isolated from Lucké tumor herpesvirus (LTHV).

LTHV was isolated and purified from winter tumors by a modification of the method of Wagner *et al.* (*3*). The mitochondrial supernatant was concentrated by centrifugation onto a cushion of 65 percent sucrose at 100,000*g* for 45 minutes in the Spinco SW41 rotor. Viral DNA was extracted (*4*) from purified virions banding at a density of 1.20 to 1.26 g/cm^3 in su-

Table 1. Detection by molecular hybridization of virus-specific RNA in the virus-free Lucké renal adenocarcinoma (summer phase tumor) labeled with [^{3}H]uridine. Hybridization was performed with duplicate 6.5-mm DNA-containing nitrocellulose filters incubated with 200 μl of [^{3}H]RNA in 4 × SSC (SSC is 0.15*M* sodium chloride and 0.015*M* sodium citrate) plus 0.1 percent sodium dodecyl sulfate for 18 to 24 hours at 66°C. Filters were washed in 2 × SSC before and after incubation at 25°C for 1 hour with pancreatic ribonuclease (20 μg/ml), then dried and counted by liquid scintillation. Background counts bound to an empty filter were not subtracted.

Experiment	[^{3}H]RNA (count/min)	Source of DNA (1 μg per filter)	RNA bound (count/min)	
			Filter 1	Filter 2
		Lucké renal adenocarcinoma		
1 (VR11-69M)	5×10^5	Lucké virus	1613	1614
		Normal frog	422	414
		Empty filter	34	31
2 (VR10-71K)	1.2×10^5	Lucké virus	1515	1451
		Normal frog	324	318
		Empty filter	31	31
3 (VR10-71F)	1.1×10^5	Lucké virus	1600	1608
		Normal frog	896	896
		Empty filter	30	31
4 (VR11-70F)*	6.8×10^5	Lucké virus	1637	1632
		Normal frog	405	406
		Empty filter	33	38
Controls 1–4		Adenovirus 2	67 to 68	
		E. coli	28 to 34	
		Normal frog kidney		
5	8.4×10^5	Lucké virus	121	132
		Normal kidney	428	452
		Empty filter	29	35
6	7.5×10^5	Lucké virus	39	49
		Empty filter	0	0

* Additional experiments in which 5 μg of Lucké virus DNA was used did not increase the fraction of RNA bound.

crose density gradients (*2*). Labeled tumor RNA was prepared by intraperitoneal inoculation of the frogs bearing "spontaneous" virus-free summer tumors with 10 mc of [5-^{3}H]uridine (20 c/mmole) in New Orleans. Tumors were removed after 7 hours, frozen at −65°C, and shipped to St. Louis for further analysis. Electron microscopy, routinely performed on all tumors, showed that they were virus-free. Labeled RNA from normal kidneys was prepared in a similar manner from nontumored frogs. Renal tumors or normal frog kidneys were minced in 15 volumes of ice-cold 0.05*M* sodium acetate buffer, *p*H 5.1, and homogenized for 1 minute at full speed in the Polytron PT-10-00 homogenizer. The homogenate was treated with Pronase (500 μg/ml) and 1 percent sodium dodecyl sulfate (SDS) for 12 hours at 37°C. Labeled RNA was extracted by the hot phenol, chloroform-isoamyl alcohol SDS procedure (*5*). Unlabeled DNA was prepared from normal frogs or frog kidneys by homogenization in 15 volumes of acetate buffer, digestion with Pronase and SDS, and extraction with phenol (*6*).

Purified LTHV DNA and frog cellular DNA preparations were subjected to analytical equilibrium centrifugation in CsCl density gradients (Fig. 1). The buoyant density of cell DNA is 1.705 g/cm^3 by comparison with that of *Pseudomonas aeruginosa* DNA ($\rho = 1.727$) and *Clostridium perfringens* DNA markers ($\rho = 1.691$) (*7*), in agreement with previous determinations (*7*). LTHV DNA bands at a buoyant density of 1.703 in agreement with Wagner *et al.* (*3*).

[^{3}H]RNA isolated from summer tumors was annealed with viral DNA immobilized on nitrocellulose filters to detect virus-specific RNA (*8*). The LTHV DNA (1 μg) bound [^{3}H]RNA (1400 to 1600 count/min) from four different tumors while control DNA's from *Escherichia coli* and human adenovirus type 2 bound only 30 to 70 count/min (Table 1, controls for experiments 1 to 4). Frog cellular DNA bound [^{3}H]-RNA (300 to 900 count/min) from different summer tumors (Table 1), as expected, since both cell-specific and virus-specific RNA are present in tumors. Similar amounts of [^{3}H]RNA from normal frog kidneys bound to frog kidney DNA (Table 1, experiment 5). Most significant is the lack of appreciable hybridization of normal frog kidney RNA to viral DNA (Table 1, experiments 5 and 6).

Table 2. Purification of virus-specific RNA from renal adenocarcinoma by sequential hybridization to and elution from Lucké tumor herpesvirus DNA.

RNA purification*	Input [^{3}H]RNA (count/min)	Bound radioactivity†	
		Count/min	Percent
1	1.9×10^6	8.1×10^4	4
2	3×10^3	4.9×10^2	16
3	3.7×10^2	9.3×10^1	24

* Number of purification steps by hybrid formation with 5 μg of LTHV DNA and elution with 0.1 × SSC containing 0.1 percent sodium dodecyl sulfate at 90°C for 10 minutes. † Average of duplicate hybridization reactions corrected fo nonspecific binding to DNA-free filters.

Labeled RNA from the summer tumors which hybridized with Lucké virus DNA was purified by three cycles of hybridization with viral DNA and elution (Table 2). Four percent of the RNA hybridized in the first hybridization, 16 percent in the second, and 24 percent in the third. Virus-specific RNA purified in this manner did not hybridize to cellular DNA.

Thus Lucké tumor herpesvirus-specific RNA is transcribed in relatively large quantities (4 percent for a 7-hour labeling period) in the "virus-free" phase of the renal adenocarcinoma, but not in appreciable quantities in normal frog kidneys. These viral RNA sequences reveal the presence of herpesvirus genetic information in the summer phase tumor cells. The continued transcription of viral information in the "virus-free" tumor cell may be necessary to maintain the transformed state of the Lucké carcinoma cell. These findings and the previous demonstration by Mizell and co-workers of tumor induction in frog embryos by inoculation with a partially purified fraction of LTHV (*9*) provide convincing evidence for the viral etiology of this tumor.

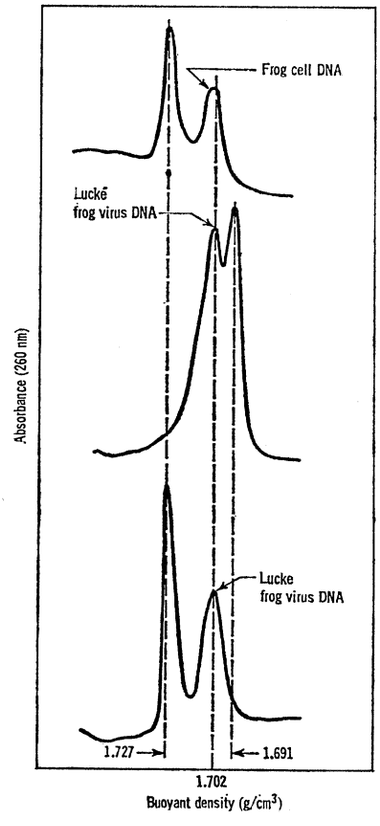

Fig. 1. Densitometer tracing of CsCl density gradients containing Lucké tumor herpesvirus DNA and normal frog cellular DNA after centrifugation to equilibrium in the Spinco model E ultracentrifuge. Lucké tumor herpesvirus DNA and frog cellular DNA (1 to 3 μg) in CsCl (initial density, 1.710 g/cm^3) were centrifuged for 20 hours at 44,000 rev/min at 25°C in the Spinco AnF rotor. Ultraviolet photographs were taken and traced with a Joyce-Loebl densitometer. The equilibrium density of each DNA was calculated relative to *C. perfringens* (1.691 g/cm^3) and *P. aeruginosa* (1.727 g/cm^3) DNA's added as markers (*10*).

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