pseudoplasmodia, and then fruited. The salt solution beneath the dialysis membrane accumulated quantities of stable acrasin [as measured by the Konijn bioassay (*13*)] because of the inability of the inactivating enzyme to pass through the membrane to reach it (*14*).

The other procedure consisted of adding 2 g of dry Dowex 1 chloride (Bio-Rad AG 1-X8, 200 to 400 mesh) to a 500-ml cell suspension (2×10^6 cell/ml). The *p*H of the mixture was adjusted to 7.8 with NaOH, and the mixture was placed on a magnetic stirrer for several hours. These preparations bound acrasin to the resin beads and made it less susceptible to attack by the enzyme. With the Konijn bioassay for acrasin, it was possible to demonstrate an accumulation of elutable acrasin on the resin with time (*14*). The resin was washed free of amoebas with distilled water, and then eluted with 10 ml of 0.1*N* HCl. The eluate was neutralized with NaOH and placed in a boiling water bath for 5 minutes to eliminate any remaining phosphodiesterase. The eluate was then treated with 100 mg of Norit A, which was washed three times with water, and the acrasin was eluted twice with 10 ml of a solution of ethanol, ammonia, and water (50 : 2 : 48). The final product was stable and was concentrated by vacuum evaporation at 60°C and stored at 4°C.

Both of these procedures accumulated amounts of biological activity equivalent to 10^{-10} mole of commercial 3′,5′-AMP (Calbiochem). Acrasin collected by both of these methods was identified as 3′,5′-AMP by the following criteria. Because acrasin adsorbs on Dowex 1 chloride, it must be negatively charged. It is of sufficiently low molecular weight to be dialyzable. It can withstand 20 to 30 minutes of boiling at neutral *p*H without appreciable loss of activity. It can be adsorbed onto Norit. Paper chromatography on three different solvent systems shows that acrasin behaves identically to 3′,5′-AMP (Table 1). Activity completely disappeared after incubation for 45 minutes at 37°C with a cyclic phosphodiesterase purified from bovine heart (*15*). This enzyme preparation appears to be specific for 3′,5′-cyclic nucleotides—no other nucleotide or nucleotide phosphodiesterase activity has ever been detected (*15*).

Even with the protection afforded by the procedures described, only very low quantities have been collected. For this reason, other methods of characterizing the molecule (such as its predicted ultraviolet absorption spectrum) have not been possible. Nevertheless, the data presented here, together with the fact that 3′,5′-cyclic nucleotides are the only nucleotides of a large number tested which show acrasin activity (*16*), strongly suggests that 3′,5′-AMP is an acrasin of *D. discoideum*.

It is clear from previous work that the amoebas of *D. discoideum* respond chemotactically to 3′,5′-AMP (*3*). We have shown that it is also secreted by this species. From this it is apparent that 3′,5′-AMP is a natural acrasin of *D. discoideum*. It must be added, however, that the possibility remains that other acrasins exist—we have studied only those extracellular molecules which are either dialyzable or negatively charged.

DAVID S. BARKLEY*
Department of Biology,
Princeton University,
Princeton, New Jersey

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* Present address: Department of Neuropathology, Harvard Medical School, Boston, Massachusetts.

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Tumor Induction in Developing Frog Kidneys by a Zonal Centrifuge Purified Fraction of the Frog Herpes-Type Virus

Abstract. *Injection of frog embryos* (Rana pipiens) *with a zonal centrifuge purified fraction of herpes-type virus (prepared from virus-containing Lucké tumors) resulted in a high incidence of kidney tumors. This partially purified oncogenic fraction contained a high concentration of an enveloped form of the frog herpes-type virus (adjacent fractions lacked this particle and were not oncogenic), which suggests that this form of the virus plays a role in the genesis of the Lucké tumor.*

The association of herpes-type viruses (HTV) with malignancies of a variety of vertebrates has recently attracted a great deal of attention. In man, interest has focused upon the EB virus found in tissue culture cells derived from patients with Burkitt lymphoma (*1*). Evidence has also been accumulating to indicate that a herpes-type virus is involved in the etiology of Marek's disease, a lymphoproliferative disease of chickens (*2*).

The EB virus can be cultured and harvested in amounts sufficient for the preparation of cell-free, relatively clean virus inoculum; but, although this virus has been tested for tumorigenicity in a variety of experimental animals, tumors have never been produced, and an adequate assay system is unavailable. On the other hand, an assay system is available for Marek's disease; tissue culture cells containing herpes-type virus have produced typical Marek's disease lesions when injected into newly hatched chicks. Nevertheless, attempts to produce Marek's disease with *cell-free* virus preparations have been unsuccessful. Thus, although suspect as oncogenic agents, herpesviruses have failed to produce tumors in either of these two systems when prepared as cell-free extracts; and some investigators have interpreted this inability as a reflection of the unstable nature of herpes virions.

The most persuasive experimental evidence thus far uncovered for herpesvirus oncogenicity comes from investigations of the frog renal adenocarcinoma (Lucké tumor) and its associated herpes-type virus. In contrast to the above systems, in vitro cultivation is not a prerequisite for the demonstration of HTV in the frog system, since herpes-type virus is invariably present in tumors of hibernating frogs (*3*). Furthermore, in 1965 Tweedell (*4*) reported the induction of tumors from cell-free extracts prepared by differential centrifugation of herpesvirus-containing tumors. Infectivity was especially pronounced in the mitochondrial fraction.

After preliminary purification by differential centrifugation, we subjected mitochondrial fractions to sucrose-gradient, rate-zonal centrifugation. We now report oncogenic activity in a density gradient purified fraction of the frog herpes-type virus.

Two virus-containing tumors (*5*) (5.6 and 1.4 g) from freshly killed, cold-treated (*6*) frogs (*Rana pipiens*) were minced and homogenized for 4 minutes in a motor-driven, Teflon-glass homogenizer with 70 ml of 0.25*M* sucrose-TKM (*7*) as the suspending medium. The tumor homogenate was separated by the following procedures into nuclear, mitochondrial, and mitochondrial supernatant fractions. The homogenate was centrifuged at 600*g* for 15 minutes to deposit nuclei and intact cells, followed by a 3000*g* spin for 15 minutes to sediment mitochondria and subcellular debris. The final supernatant was designated the mitochondrial supernatant fraction. The nuclear and mitochondrial pellets were each gently aspirated in 40 ml of hypotonic buffer (*7*). After suspension in hypotonic buffer, the mitochondrial pellet was held at 0°C for 20 hours before zonal centrifugation. Although tumors were produced when other fractions were injected into embryos, this report will be limited to the results obtained with the mitochondrial fraction. All steps were carried out at 0° to 4°C.

The B-XV zonal centrifuge system (*8*), with 1-liter sucrose gradients ranging from 10 to 60 percent (by weight) sucrose in 0.002*M* tris and 0.002*M* ethylenediaminetetraacetate (EDTA) (*p*H 7), was utilized for further fractionation of the mitochondrial pellet. The gradient was cushioned with potassium citrate, density 1.35, and the samples were overlaid in each experiment with 190 ml of hypotonic buffer. Centrifugation was at 24,000 rev/min for 60 minutes ($\omega^2 t = 23 \times 10^9$ radian^2/sec, where ω is angular velocity and t is time). The 50-ml fractions were collected through the flow cell of a recording spectrophotometer set at 265 nm. Twenty-two fractions were collected and the sucrose content of each fraction was determined by refractometry. The fractions of interest were diluted to 70 ml with hypotonic buffer and subjected to high-speed centrifugation at 80,000*g* for 90 minutes; pellets from each fraction were suspended in 2 ml of phosphate-buffered saline (*7*) by aspiration.

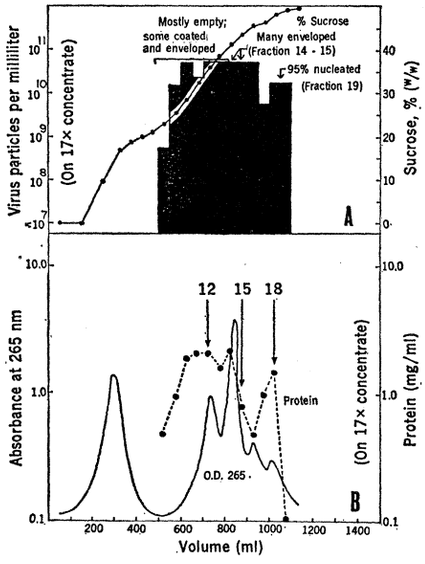

Fig. 1. Zonal centrifugation of mitochondrial fraction in sucrose gradient [10 to 60 percent (by weight)].

Each sample was examined by negative staining by applying 1:5 dilutions of the sample in 2 percent potassium phosphotungstate (*p*H 4.5) to 200-mesh ionized copper grids coated with carbon and Formvar, and viewing the preparations in the Siemens Elmiskop IA. Protein assays were carried out by the Lowry method (*9*), with crystalline bovine serum albumin as the assay standard.

Examination with the electron microscope by negative staining techniques indicated that the frog tumor homogenate contained the range of morphological forms of the herpes-type virus previously observed in thin sections of inclusion-containing frog tumors (*10*). Negative staining also revealed that all of the HTV forms found in cultures of human lymphoma (Burkitt) were present in the frog tumor (*11*). This included nucleated particles with and without outer envelopes. A small percentage of particles had an amorphous coat, presumably an antibody coating similar to that observed when EB virus from cultured Burkitt lymphoma reacts with Burkitt HTV antiserums (*12*). Empty, noncoated particles without outer envelopes predominated. All three differential centrifugation fractions—nuclear, mitochondrial, and mitochondrial supernatant—contained high concentrations of virus (10^{10} particles per milliliter); all the morphological forms were present in each of the three fractions, except for the apparent absence of coated virus or loosely enveloped particles (*13*) in the nuclear fraction.

After zonal centrifugation the fractions of interest were concentrated and examined immediately with the electron microscope by negative staining. The data from the zonal centrifuge experiment of the mitochondrial fraction is presented in chart form in Fig. 1. In Fig. 1A, total virus counts are given for each zonal fraction; the zones of particular interest with respect to virus morphology are indicated. Zonal centrifugation was partially effective in separating the various morphological forms of the frog HTV. Empty particles predominated in the lighter ranges of the gradient, whereas nucleated particles concentrated in the denser zones of the gradient [above 40 percent (by weight) sucrose]. For example, fraction 19 at 48 percent sucrose contained 95 percent nucleated particles. Enveloped and coated particles were found throughout the upper two-thirds of the gradient. The above information in conjunction with the data on absorption peaks and protein concentration (Fig. 1B) (plus our experience from earlier experiments) indicated that zonal fractions Nos. 12, 15, and 18 (percentage of sucrose, 36.3, 43.4, and 47, respectively) warranted in vivo testing for oncogenic activity.

Frog embryos were injected with virus fractions within 12 hours after zonal centrifugation. The standard injection procedure (*4*) was modified to permit the use of a repeating dispenser which delivered 0.2 μl of solution per injection. All injections were performed under a dissecting scope at a magnification of × 20. Stage 19 or 20 (Shumway) (*14*) embryos (5 to 6 mm in total length; 118 to 140 hours old) were injected in the region of the developing right pronephros. Animals injected with buffer solutions and uninjected animals served as controls for the experiment; all animals were reared in spring water at room temperature.

Periodically, animals from each injection group were killed and examined for tumor development. The first tumors were encountered 12 weeks after injection when 12 animals injected with the crude mitochondrial fraction were killed and five of these animals were found to have pronephric tumors. These tumors were extremely small; they measured 1 mm in the largest dimension and could be recognized only after the animals had been killed and autopsied under a dissecting scope. The earliest stage at which a definite tumor was encountered was Taylor-Kollros stage VII (*15*) (an early stage of metamorphosis). None of the animals injected with other fractions had developed tumors at this time. However, as metamorphosis continued, additional animals that had been injected with the crude mitochondrial fraction developed obvious bulges in the region of the differentiating kidneys, which at autopsy were verified to be tumors [19 of 33 animals (58 percent) that died or were killed during the later stages of metamorphosis]. A 1:1000 dilution of this fraction did not reduce its oncogenicity [five of seven late metamorphic animals (71 percent) that were killed had developed tumors].

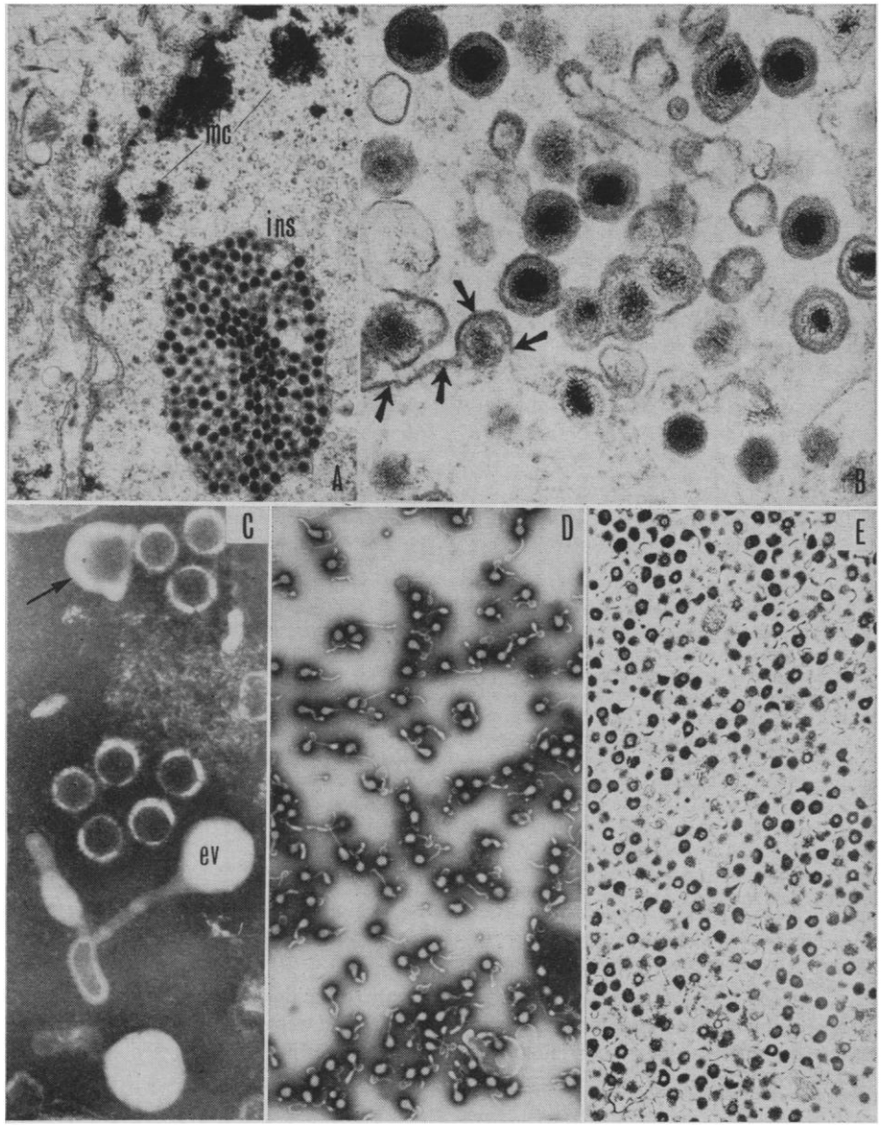

Fig. 2. (A) Electron micrograph of cold-treated primary tumor showing intranuclear sac (*ins*) containing a cluster of nucleated HTV. Note marginated chromatin (*mc*) along inner border of nuclear membrane (× 10,000). (B) High resolution of nuclear sac and enclosed virus. Note that most particles are invested with a thick envelope which is closely applied to the capsid. One virus is pictured as it is acquiring its envelope (arrows), and continuity between the sac unit membrane and the envelope is apparent (× 47,000). (C) Electron micrograph of negatively stained preparation of fraction 15, showing the enveloped viral (*ev*) form. Because the phosphotungstic acid does not penetrate the closely applied, thick envelope, the nuclear enveloped form of HTV appears as a lucent sphere. An empty, thick envelope that has ruptured can be seen in the upper portion of the photograph (arrow) (× 50,000). (D) Low-power view of negatively stained preparation of zonal fraction 14, soon after removal from the zonal centrifuge. The homogeneity and high concentration of the nuclear enveloped particle are evident (× 10,000). (E) Electron micrograph of sectioned pellet prepared from zonal fraction 14 after several freezings and thawings. Even after this harsh treatment it is still possible to recognize the nuclear enveloped form of the frog HTV with its thick envelope (× 10,000).

Pronephric tumors predominated in the tadpoles killed at early stages; in fact, during the course of the experiment, the majority of tumors were pronephric. However, as tumors developed in older animals, some mesonephric tumors were found. Since the pronephros was differentiating at the time of injection, the relatively high incidence of pronephric tumors indicates that the target cell of the oncogenic agent is, in all probability, the *differentiating* kidney cell.

Of the three zonal fractions injected, only zonal fraction 15 produced tumors (*16*). Tumors developed in 8 of 15, or 53 percent, of the animals that died or were killed during the later stages of metamorphosis. None of the uninjected controls or controls injected with buffer developed tumors.

When tumors developed in animals injected with fraction 15, but not in those injected with fractions 12 or 18, a careful evaluation of the electron microscopic photographs of these three fractions was undertaken. The difference between these fractions was the presence of a high concentration of a nuclear form of enveloped HTV particle in fraction 15 (Fig. 2, A, B, and C) and the lack of this tightly enveloped particle in fractions 12 and 18 (*17*). Figure 1B indicates that fraction 15 was located on the lower portion of the significant absorption peak and that the preceding fraction, fraction 14, was closer to the apex. Indeed, examination of the negative stain preparation of fraction 14 (Fig. 2D) and thin section of the pellet prepared from this zonal fraction (Fig. 2E) indicated that this zonal cut contained an unusually homogeneous, high concentration of this enveloped form of HTV.

The nuclear enveloped form was noted in Fawcett's original electron microscopic study of the Lucké tumor and later described by Lunger *et al.* (*10*). Tightly enveloped forms have also been reported for other herpesviruses

and the significance of these findings has recently been discussed by Nii *et al.* (*18*). When sucrose gradient rate-zonal centrifugation of the frog HTV was followed by isopycnic banding of the virus zones, the nuclear enveloped particles banded at density 1.20 to 1.21.

The results of our zonal fractionation–embryo injection study suggest that this enveloped form of the frog herpes-type virus plays a role in the genesis of the Lucké tumor.

MERLE MIZELL
Chapman H. Hyams III
Laboratory of Tumor Cell Biology,
Tulane University,
New Orleans, Louisiana 70118
IRV TOPLIN
John L. Smith Memorial for Cancer
Research, Chas. Pfizer Co., Inc.,
Maywood, New Jersey 07607
J. JOYCE ISAACS
Chapman H. Hyams III
Laboratory of Tumor Cell Biology

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17. The term "intranuclear sac" was used by Lunger *et al.* (*10*), who noted that the sac membrane was fused with the inner nuclear membrane. Virus particles within these intranuclear sacs probably lie between the inner and outer nuclear membranes. In this report the use of the term "nuclear sac" merely implies that in thin section these sacs of virus are surrounded by nucleoplasm rather than cytoplasm (see Fig. 2A, *ins*); and the term "nuclear enveloped form" refers to particles that are morphologically similar to the enveloped form found within these intranuclear sacs; that is, they are invested with a thick envelope that is closely applied to the capsid (see Fig. 2B).
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Interferon Induction Increased through Chemical Modification of a Synthetic Polyribonucleotide

Abstract. *The alternating copolymer riboadenylic-ribouridylic acid gained a significant increase in ability to stimulate interferon production (2- to 20-fold) and cellular resistance (100- to 10,000-fold) both in vitro and in vivo upon substitution of phosphate by thiophosphate groups. The resulting nucleotide analog was also 10 to 100 times less sensitive to degradation by pancreatic ribonuclease, as determined by residual antiviral activity.*

Various synthetic polyribonucleotides stimulate interferon production both in vitro and in vivo (*1–4*). A stable, highly ordered, hydrogen-bonded (*3*)—hence double- or multistranded (*1*)—secondary structure seems to be an essential requirement for their antiviral activity. The reason for the greater activity of stable, double- or multistranded complexes is not known but might be related to either increased inducer penetrability, better interaction with a specific receptor site, or resistance to premature enzymatic digestion. Chemical modification of the riboadenylic-ribouridylic acid copolymer [poly r(A-U)] to a nucleotide analog [poly r(AS-US)] in which each phosphate group was replaced by a thiophosphate group results in a significant decrease of the rate of breakdown by several enzymes (spleen phosphodiesterase, snake venom phosphodiesterase, micrococcal nuclease, and pancreatic ribonuclease) (*5*). This increased resistance to enzymatic degradation is associated with an increase of antiviral activity.

The antiviral activity of poly r(AS-US) was compared to poly r(A-U) and the homopolymer pairs polyriboadenylic

Table 1. Comparative study of antiviral activity of poly r(AS-US), poly r(A-U), (poly rA)•(poly rU), and (poly rI)•(poly rC) in human skin fibroblasts.

Polymer	Cellular resistance minimal inhibitory concentration* (μg/ml)		Interferon production† (unit/4 ml)		Thermal stability-T_m (°C)	
	Preparation 1	Preparation 2	Preparation 1 (40 μg/ml)	Preparation 2 (8 μg/ml)	In saline (0.15*M* Na^+)	In 0.01*M* citrate buffer (0.01*M* Na^+)
r(AS-US)	0.001	0.0001	62	24	N.T.‡	48
r(A-U)	0.1	1.0	26	⩽1	69.5§	48(*9*)
(poly rA)•(poly rU)	0.2	N.T.	⩽1	N.T.	57.5(*3*)	38(*10*)
(poly rI)•(poly rC)	0.002	0.01	72	18	62.5(*3*)	41.5(*11*)

* Concentration of polymer required to reduce formation of vesicular stomatitis virus plaques by 50 percent. Mean values for at least two observations. † From 3 to 24 hours after exposure of confluent cell monolayers in 60-mm petri dishes to 40 μg (preparation 1) or 8 μg (preparation 2) of the polymer in 1 ml of Eagle's minimal essential medium. Cells were freed from polymer after 3 hours, exhaustively washed with Eagle's medium, and further incubated with 4 ml of Eagle's medium per petri dish. Mean values for at least two observations. ‡ Not tested. § As observed in 0.01*M* tris(hydroxymethyl)aminomethane-hydrochloride, *p*H 8.1, 0.01*M* Na_2HPO_4, 0.5 m*M* $MgCl_2$ (*7*); coincides with T_m value obtained in sodium citrate buffer (0.15*M* Na^+) (*9*).