Zonal Centrifuge Applied to the Purification of Herpesvirus in the Lucké Frog Kidney Tumor

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A series of "winter" and "summer" Lucké kidney tumors of the frog (Rana pipiens) were homogenized and fractionated by differential centrifugation into nuclear, mitochondrial, and mitochondrial supernatant fractions. Winter tumors often contained high concentrations of herpesvirus, whereas no virus was observed in any of the summer tumors. The crude tumor fractions were further purified by rate-zonal sucrose gradient centrifugation in a B-XV zonal rotor. Gradient fractions rich in an enveloped, nucleated form of the herpesvirus from certain winter tumors have induced renal tumors when injected into developing frog embryos. Zonal centrifugation was followed by isopycnic banding of the virus zones for further purification of the different morphological forms of the virus.

It has been known for some years that a herpesvirus is associated with the Lucké renal adenocarcinoma of the frog Rana pipiens (4, 7). Herpesvirus synthesis is observed only when the tumor-bearing frogs are subjected to low-temperature incubation, either natural or in the laboratory ("winter" tumors, reference 10). Tweedell (16) obtained a high percentage of tumors in developing frog embryos by injection of winter tumor fractions separated by differential centrifugation. We have reported similar tumor induction with certain sucrose gradient zonal centrifuge fractions of winter tumors (9, 10). Only those fractions enriched in an enveloped nucleated form of the virus induced tumors, and it was suggested that this morphological form of the virus was a factor in the development of the Lucké tumor.

The original application of the zonal centrifuge to the purification of the Lucké herpesvirus was described earlier in a preliminary report (15). We have continued these studies, and this paper is a more detailed report of the results of this work.

MATERIALS AND METHODS

A number of tumors from cold-treated frogs were used in these studies. The histories, weights, and other pertinent data on these tumors are given in Table 1. Also, several "summer" tumors from frogs maintained at room temperature were studied, and the data on these samples are also given in Table 1.

The tumor-fractionation, zonal-centrifugation, isopycnic-banding, and electron-microscopy procedures used in our laboratory were described earlier (16). Briefly, the tumors were homogenized in isotonic sucrose and separated by differential centrifugation similar to methods of Tweedell (16) to yield tumor, nuclear, mitochondrial, and mitochondrial supernatant fractions. These crude fractions were subjected to rate-zonal sucrose gradient centrifugation employing a B-XV rotor (2). The virus in the zonal centrifuge fractions was concentrated by high-speed centrifugation and then banded isopycnically with sucrose density salt gradients with swinging-bucket rotors. All virus suspensions were examined in the electron microscope with the negative-staining method; solid tissue and selected virus pellets were studied by the thin-section technique.

The following chemical analyses were used in this work: total protein by the Lowry method (6), deoxyribonucleic acid (DNA) by the diphenylamine reaction, and ribonucleic acid (RNA) by the orcinol reaction as used by Ben-Porat and Kaplan (3) for other herpesviruses.

RESULTS AND DISCUSSION

Variation in size, appearance, and virus content of tumors. Winter tumor weights ranged from 1.4 to 9.0 g and, in gross appearance, from completely solid tumors to those with necrotic, fluid centers (Table 1). The tumors were generally 5 to 10% of the total frog body weight at sacrifice, body weights varying from 33 to 50 g. The low value was 3%; the high value was 18%. Certain of the kidney tumor-bearing frogs were observed to have involvements of other organs, i.e., liver metastases. The majority of winter tumors contained im-
pressive amounts of herpesvirus. Semiquantitative virus counts on tumor homogenates indicated a total virus content for most tumors in the range of $10^{11}$ to $5 \times 10^{11}$ particles per gram of tumor weight. On a weight basis, these frog tumors contained at least 100-fold more virus than the Burkitt lymphoma and other herpesvirus-positive human leukocytic cell cultures in our laboratory (14) and the herpesvirus-positive kidney cultures from chickens infected with Marek's disease (1).

Within this sample of winter tumors, there was no obvious correlation between the virus content of the tumors and the gross physical appearance of the tumor, time of hibernation, or weight of tumor.

The herpesvirus in the Lucké tumors exhibited the complete range of herpesvirus morphology as previously described (7, 12, 13). This included nucleated particles with and without outer envelopes, empty capsids, and a small percentage of particles with an amorphous coating, presumably an antibody coating as observed for human herpesvirus reacted with appropriate antisera (8). A striking feature of many of the winter Lucké tumors was the prominent viral inclusions in most of the tumor nuclei. Thin-section electron microscopy revealed that these inclusions consisted of membrane-bound sacs of closely packed nucleated virus with each par-

ticle in the sac individually contained within a tight outer envelope (Fig. 1). The same nuclei with sacs of enveloped virus also generally contained large numbers of nonenveloped empty capsids. Many virus-infected cells in the tumor were in a degenerated state suggestive of the cytopathic effect produced by other herpesviruses in susceptible cells. In addition to the morphological forms of herpesvirus just described, several tumors contained significant numbers of 55-nm particles and 65-nm tubular structures which are thought to be aberrant forms of viral protein (13).

No virus was observed by direct thin-section electron microscopy in any of the "summer" tumors from frogs held at room temperature.

**Tumor fractionation by homogenization and differential centrifugation.** Tweedell (16) obtained the highest tumor-inducing activity in tadpoles from the mitochondrial fraction of winter tumors. We have confirmed the finding of oncogenic activity in the mitochondrial fraction (10, 11). Electron microscopy of these infectious crude preparations always revealed the presence of large numbers of the membrane-bound viral sacs described above together with some dissociated, tightly enveloped nucleated particles. It was presumed that the "mitochondrial" fraction contained those viral inclusion sacs released from degenerating or damaged

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**Table 1. Frog kidney tumors used for fractionation studies**

<table>
<thead>
<tr>
<th>Frog no.</th>
<th>Sex</th>
<th>Source</th>
<th>Natural tumor</th>
<th>Tumor eye transplant</th>
<th>Time at room temp after tumor palpation (days)</th>
<th>Time at 7.5°C before sacrifice (days)</th>
<th>Wt of tumor (g)</th>
<th>Herpesvirus content of tumor</th>
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<tr>
<td>1</td>
<td>M</td>
<td>W</td>
<td>Yes</td>
<td>No</td>
<td>None</td>
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<td>1.6</td>
<td>High&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>F</td>
<td>W</td>
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<td>No</td>
<td>None</td>
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<td>1.9</td>
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<tr>
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<td>V</td>
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<tr>
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<td>V</td>
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<tr>
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<tr>
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<sup>a</sup> V = Vermont; W = Wisconsin.

<sup>b</sup> Frogs 1, 2, and 9 had natural "winter" tumors; frog 16 had a natural "summer" tumor.

<sup>c</sup> Showed many 55-nm particles and 65-nm tubular structures.
tumor nuclei during tumor homogenization in isotonic sucrose. Mitochondrial pellets were suspended in hypotonic buffer [2 mM tris (hydroxymethyl)aminomethane, 2 mM ethylenediaminetetraacetic acid, pH 7.5] and held at 0 to 4°C for 16 to 24 hr before zonal centrifugation. Electron microscopy of the suspensions after this treatment indicated an apparent release of virus from the sacs, a process most likely accelerated by the osmotic changes during the subsequent density gradient centrifugation.

The tumor nuclear pellets also were suspended in hypotonic buffer, but generally they were frozen at -70°C until thawed for zonal centrifugation. In several experiments, the thawed nuclear suspensions were briefly homogenized to promote thorough dispersal of virus.

Therefore, in tumors rich in nuclear sacs of enveloped virus, the mitochondrial and nuclear suspensions contained the majority of these particles, with some spillover of this form of the virus into the mitochondrial supernatant fraction from degenerating cells, enveloped nucleated virus in extranuclear spaces, and imperfect differential centrifugation.

Sucrose gradient rate-zonal centrifugation. Rate-zonal centrifugation (57,300 × g at R_{max} for 60 min), with 1-liter preformed 10 to 60% (w/w) linear sucrose gradients in the B-XV rotor, effected a significant separation of the various morphological forms of the Lucké herpesvirus present in the crude tumor fractions. The majority of nonenveloped nucleated particles were found in the 48 to 55% sucrose zone under the above centrifugation conditions; empty capsids predominated in the 33 to 40% sucrose zone. The tightly enveloped nucleated virus particles, seen in the nuclear sacs were observed in the 39 to 43% sucrose zone. The complete data from the rate-zonal centrifugation series on the tumor fractions from frog 11 are presented in chart form in Fig. 2 to illustrate these findings. This tumor contained high concentrations of nuclear inclusion sacs and the ultraviolet absorption profiles of the gradients at 265 nm were particularly interesting. The tightly enveloped particles from the nuclear sacs were observed at 40 to 42% sucrose in the gradients from the mitochondrial and nuclear suspensions and were clearly associated with sharp peaks of ultraviolet absorption (arrows in Fig. 2) that followed the usual zone of cellular microsomal membrane fragments at 35 to 40% sucrose. This distinctive peak of ultraviolet absorption was diminished or absent in experiments on preparations with low or negligible concentra-

![Figure 1](http://aem.asm.org/)
Frog Tumor Herpesvirus

**FIG. 2.** Rate-zonal centrifugation of various tumor fractions from frog 11. Centrifugation in a B-XV rotor was for 60 min at 57,300 X g at Rmax for each experiment. The virus quantitation was carried out on 17-fold concentrates of the 50-ml zonal fractions.

Note the morphological uniformity and purity of these selected viral suspensions as revealed by both negative staining and thin-section electron microscopy.

Zonal centrifuge concentrates rich in enveloped nucleated virus from the mitochondrial suspension of combined frog tumors 4 and 5 (Table 1) have induced high percentages of tumors when inoculated into tadpoles (9, 11). Tumor induction has been observed only with preparations containing high concentrations of the enveloped nucleated form of the Lucké herpesvirus. Figure 5 shows one of the typical tadpole tumors induced by a zonal centrifuge concentrate.

It should be noted that the high degree of separation and concentration achieved by the
Fig. 3. Rate-zonal centrifugation of the mitochondrial suspensions from various tumors. Centrifugation conditions were the same as in Fig. 2.

use of the zonal centrifuge failed to reveal any virus in any of the summer tumors studied.

Isopycnic banding of rate-zonal concentrates. Selected virus concentrates from the rate-zonal experiments were subjected to long-term centrifugation on preformed sucrose or salt gradients (potassium tartrate or cesium chloride) to effect a further separation based on particle density of the various virus forms and other particulates in the samples. This was an application of the S-ρ principle of Anderson (2): zonal separations based on sedimentation rate followed by isopycnic banding of the rate-zonal fractions. This procedure was effective in obtaining purified concentrates of the enveloped nucleated virus which banded isopycnically at density 1.20 in sucrose and potassium tartrate and density 1.22 in cesium chloride. Also, relatively purified concentrates of nonenveloped, nucleated particles could be obtained from the density 1.25 to 1.27 zone in sucrose and potassium tartrate and at density 1.27 to 1.29 in cesium chloride. The enveloped and nonenveloped nucleated virus concentrates after isopycnic banding showed significant levels of DNA (10 to 30 μg/ml), no detectable RNA (<5 μg/ml), and total protein contents of about 100 μg/ml.

The incomplete empty capsids banded isopycnically at density 1.18 to 1.19 in sucrose and potassium tartrate and density 1.19 to 1.21 in cesium chloride. These densities coincided with the banding densities of a major portion of the cellular membrane fragments present in the rate-zonal concentrates. Therefore, isopycnic gradient centrifugation was not particularly suc-
FIG. 4. Electron micrographs of purified virus from rate-zonal and isopycnic-banding experiments. (A) Negative stain of the rate-zonal concentrate obtained from the mitochondrial suspensions of frog tumor 14 rich in enveloped nucleated virus. Centrifugation conditions were the same as in Fig. 2. × 8,850. (B) Same as (A), but higher magnification. Note the distortion of many of the envelopes under the staining conditions revealing the virus capsids within the envelopes. × 35,500. (C) Thin section of the rate-zonal concentrate obtained from the mitochondrial suspension of frog tumor 8 rich in enveloped nucleated virus. × 14,000. (D) Negative stain of the isopycnically banded 55-nm particles from frog tumors 1 and 2 after rate-zonal centrifugation, fluorocarbon extraction, and potassium tartrate banding. × 35,000.
cessful in producing highly purified bands of empty capsids.

We have subjected several frog virus suspensions to fluorocarbon (trichlorotrifluoroethane) extraction (5, 7, 16) for further purification. Exposure of virus suspensions to fluorocarbon did not affect the virus morphologically, including the preservation of enveloped forms. A significant reduction of cellular membrane contamination was observed in fluorocarbon-extracted samples. For example, a zonal centrifuge fraction from combined frog tumors 1 and 2, rich in 55-nm particles (at 15.6% sucrose after 20 min at 22,000 rev/min), was fluoro-

**FIG. 5.** Typical renal tumor induced in tadpoles by rate-zonal concentrate from frog tumors 4 and 5 rich in enveloped nucleated virus. Details given by Mizell et al. (11).
carbon-extracted and banded isopycnically in potassium tartrate. The 55-nm particles banded at density 1.16 with relatively little cellular membrane debris (Fig. 4D).

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LITERATURE CITED