JOURNAL OF VIROLOGY, Jan. 1986, p. 335–339
0022-538X/86/010335-05$02.00/0

Vol. 57, No. 1

Detection of Pseudorabies Virus DNA in the Inner Ear of Intranasally Infected BALB/c Mice with Nucleic Acid Hybridization In Situ

NORBERT FALSER,[1] INGRID BANDTLOW,[1] MARGARETE HAUS,[2] AND HANS WOLF[2]*

Ear, Nose, and Throat Clinic, University of Innsbruck, 6021 Innsbruck, Austria,[1] and Max-von-Pettenkofer Institute, 8000 Munich 2, Federal Republic of Germany[2]

Received 21 February 1985/Accepted 9 May 1985

Evidence for the pathogenicity of pseudorabies virus for the auditory and vestibular organs of experimentally infected mice is presented. We demonstrate viral genomes in cells of the peripheral sensory organs, the nerve structures, and the affected areas of the brain in single sections from an entire cranium of an adult mouse. The data were obtained by an in situ hybridization technique adapted for use with fixed, plastic-embedded materials. In contrast to conventional methods which use frozen sections, we were able to analyze cartilaginous and bony materials with high resolution.

Viral infections of the inner ear and subsequent damage of the auditory and vestibular organs have been documented, especially after infections with mumps or rubella, in a series of clinical and morphological experiments (11, 13, 14, 23, 26). By immunofluorescence, Davis et al. (4, 5) demonstrated viral antigens in the cochlea and labyrinths of laboratory animals infected with cytomegalovirus and reovirus (2, 3). To date, however, viral DNA in the structure of the inner ear after viral infection has not been demonstrated.

To clarify this question, we intended to use in situ hybridization (16) on sections of mice intranasally infected with pseudorabies virus (PRV). However, adaptation of standard techniques was necessary because the head contains large proportions of bone, which do not permit frozen sectioning with standard procedures and equipment (24). In addition, the delicate structures are far better conserved in fixed and embedded materials, which allow high resolution and evaluation of histological detail, and the material is suitable for examination by electron microscopy.

MATERIALS AND METHODS

Cell lines. Suspensions of the Epstein-Barr virus (EBV)-carrying cell line P3HR-1 and an EBV-negative cell line, BJAB, were spread on slides and fixed and embedded as described in Table 1 (concentrations and incubation times are identical to those used for embedding tissue samples).

Preparation of the ^{125}I-labeled EBV DNA. The iodination of the single-stranded recombinant m13 phage DNA was performed as described by Han and Harding (10) and Gu et al. (8). Briefly, 10 μg of single-stranded DNA in 1 μl of water, 2 μl of 250 mM sodium acetate buffer (pH 4.65), 15 μl of ^{125}I (Amersham IMS 30; 100 mCi/ml), and 2 μl of 100 mM thallium chloride (dissolved in 250 mM sodium acetate buffer) were mixed, incubated for 30 min at 60°C, and transferred to ice water. The reaction was stopped by adding 150 μl of 100 mM Tris (pH 8.0)–10 mM EDTA–10 mM Na_2SO_3–100 μg of poly(A) per ml, separated over a Sephadex G-50 column (5-ml volume) in a 5-ml pipette, and eluted with 10 mM Tris (pH 8.0)–1 mM EDTA.

Preparation of ^{3}H-labeled EBV DNA. Charon 4A phage or plasmid DNA with virus-specific inserts was labeled with [^{3}H]dCTP with an Amersham nick translation kit (19). [^{3}H]dCTP (50 μl, 1 mCi/ml; Amersham) was lyophilized and dissolved in 20 μl of nucleotide buffer (100 μM dATP–100 μM dGTP–100 μM dTTP in Tris [pH 7.8]) containing 1 μg of DNA and 10 μl of enzyme (DNase and polymerase) in a total volume of 100 μl. The DNA was separated over a Sephadex G-50 column and eluted with 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–0.01% sodium dodecyl sulfate–10 mM NaCl.

In situ hybridization with ^{3}H- and ^{125}I-labeled EBV. The method of in situ hybridization used was based on procedures described elsewhere (27, 28) and was identical for [^{3}H]EBV DNA and [^{125}I]DNA. For hybridization, 10^5 cpm of [^{3}H]EBV DNA (specific activity, 2×10^7 cpm/μg) or 10^5 cpm of [^{125}I]EBV DNA (specific activity, 5×10^7 cpm/μg) was used per slide. Briefly, the slides with the fixed cells were incubated for 30 min at 70°C in 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate) and 15 min at 37°C in 20 mM Tris (pH 7.4)–2 mM $CaCl_2$–1 μg of proteinase K per ml, washed in distilled water, and dehydrated in alcohol. Immediately preceding the hybridization, the cells were denatured by immersion into 0.1× SSC for 30 s at 98°C and transferred to the same buffer at 0°C.

Portions of hybridization solution, containing the labeled EBV DNA in 50% formamide–10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–0.02% Ficoll–0.02% polyvinylpyrrolidone–100 μg of poly(A) per ml–1 mg of bovine serum albumin per ml–100 μg of calf thymus DNA per ml–1 mg of tRNA per ml, were denatured for 2 min at 98°C and rapidly cooled in an ice bath, and 5 M NaCl was added to a final concentration of 600 mM. Aliquots (10 μl) were placed under a siliconized cover slip and sealed with rubber cement. After hybridization for 24 h at 45°C, the cover slips were removed, and the slides were rinsed in 10 mM Tris (pH 7.4)–1 mM EDTA–50% formamide–600 mM NaCl, then washed two times for 5 min each time and one time overnight with the same buffer, two times for 5 min each time with 2× SSC, 1 min in 300 mM ammonium acetate in 70% ethanol, 1 min in 300 mM ammonium acetate in 90% ethanol, and air dried.

Autoradiography (used for all hybridizations). A 5-ml portion of 600 mM ammonium acetate was warmed to 45°C and gently mixed with 5 ml of Ilford nuclear emulsion gel G5. The slides were dipped in the emulsion mix for 1 to 2 s and

* Corresponding author.

TABLE 1. Combination of fixation and embedding protocols used

Fixative	Concn of fixative used	Time fixed (min)	Type of embedding
Glutaraldehyde	2%	20	SPURR (22), Epon (15), K4M (1)
Formaldehyde	4%	20	Paraffin (25)
Glutaraldehyde plus osmium tetroxide	2%:1% (glutaraldehyde to osmium tetroxide)	20 10	SPURR, Epon
Schaeffer solution[a]		20	Methyl methacrylate (17)
Ethanol plus acetic acid	3:1 (ethanol to acetic acid)	20	Frozen tissue section

[a] Schaeffer solution is formaldehyde (37%, 1 part) plus ethanol (80%, 2 parts).

allowed to dry in an upright position for 30 min at room temperature, placed in lightproof boxes containing silica gel desiccant, and exposed at 4°C for 1 week. The slides were soaked in distilled water and then developed for 10 min with Kodak D 19 X-ray developer, rinsed in 1% acetic acid, fixed for 20 min in Kodak Unifix, and stained with Ehrlich hematoxylin.

Infection experiment with BALB/c mice. Virus stock and determination of titer. For the infection of the test animals, we used a suspension of PRV (Aujeszky strain; ATCC VR-135) prepared from rabbit brain in 50% glycerol. For viral titer determination, the material was inoculated onto cultures of mouse adrenal tumor cells (Y-1; ATCC CCL 79) in Hams F-10 medium supplemented with 15% horse serum (not inactivated) and 2.5% fetal bovine serum (not inactivated) in the absence of antibiotics. After 2 days, the cells were transferred to baby hamster kidney cells for an endpoint dilution test, which yielded a titer of 2×10^7 PFU/ml for our stock virus.

Preparation of PRV DNA. The *Bam*HI DNA fragments of PRV cloned in pBR325 were kindly provided by T. Ben-Porat (12). Large-scale amplification and gradient centrifugation of recombinant DNA were performed by standard methods. Each plasmid DNA was identified by restriction enzyme digestion and hybridization. The physical map of the PRV *Bam*HI fragments was adopted from that of Ladin et al. (12) and adjusted to the PRV strain Phylaxia. All this was kindly performed in the laboratory of H. J. Rziha, Tübingen, Federal Republic of Germany.

Infection of experimental animals. Twenty inbred BALB/c mice were infected intranasally with 0.2 ml of virus suspension (4×10^6 PFU). After 4 to 5 days, when the animals showed clear symptoms of disease, they were decapitated under anesthesia, and the skull was prepared. The fixation of the cochlea or the total skull, dehydration, embedding in methyl methacrylate, and preparation of sections are described in detail elsewhere (N. Falser, I. Bandtlow, M. Haus, and H. Wolf, J. Microsc., in press).

In situ hybridization. [^{3}H]PRV DNA (5×10^4 cpm per section; specific activity, 1.5×10^7 cpm/μg) was used as in a procedure described above.

Quantitative evaluation of the autoradiograms. The hybridization efficiency was evaluated with a computer-controlled microspectrophotometer (Reichert Univar-R plus microspectrophotometer plus microcomputer Videoplan), establishing an intensity relation of the parameter grains of silver per optical density per 10 μm^2. As standards, we used 5-μm sections of a ^{3}H-labeled methyl methacrylate block (autoradiographic ^{3}H microscales, Amersham International RPA 501) which are composed of tritium-labeled polymer layers with increasing activity interspersed with layers of nonradioactive polymer. These sections were included in each series of autoradiograms with ^{3}H-labeled probes for the purpose of calibration.

RESULTS

The quantitative analysis of the autoradiograms of the P3HR1 cells treated with different fixation and embedding protocols is given in Table 1. From these results, we chose Schaffer solution for the fixation of the cochleae and the skull and embedding in methyl methacrylate. Osmium tetroxide, which fixes lipophilic substances, did not further reduce the hybridization signal; rather, it even seemed to reduce the unspecific background. This fixation was chosen because it allows both nucleic acid hybridization tests and morphological examination by light and electron microscopes.

In situ hybridization with ^{3}H-labeled PRV DNA revealed large amounts of viral DNA in the materials under study (Fig. 1 and 2). This was demonstrated particularly well in support and sensory cells of the vestibular organ, the organ of Corti, and the spinal ganglion and also in the ganglia cells (Fig. 1 and 2) of the eighth neuronal trunk of the brain (nucleus cochlearis). When DNA from an unrelated herpes-

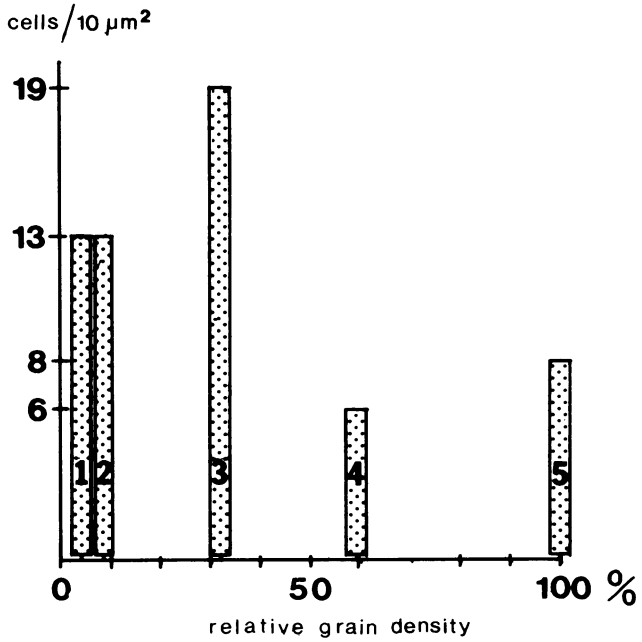

FIG. 1. The influence of different fixations on autoradiographic grain density. The effects are demonstrated after in situ hybridization with ^{3}H-labeled EBV DNA on EBV genome-bearing Burkitt's lymphoma cells after fixation in Schaffer solution (column 4; 41% decrease), additional fixation in Schaffer solution and embedding in methyl methacrylate (column 3; 68% decrease), fixation in glutaraldehyde (column 2; decrease 91.3%), and fixation in formaldehyde and paraffin embedding (column 1; 95.5% decrease). For comparison, ethanol-acetic acid fixation (column 5; 100% labeling rate) was included, simulating the protocol used for cryosections. The ordinate shows the number of cells bearing a label per 10 μm^2. The abscissa gives the mean density of the cells labeled relative to the result obtained with ethanol-acetic acid fixation.

virus (EBV, data not shown) was used on PRV-infected tissues or when PRV DNA was applied to uninfected tissues, the results were negative throughout. Chemography was excluded by control sections autoradiographed without radioactive traces (data not shown). Pigment granules have never been observed by us or others in the tissue and can also be excluded as a source of possible artifacts by the negative results with the control sections used (data not shown). All positive hybridization results were also confirmed by virus isolation (Falser et al, in press).

DISCUSSION

Pathogenic alterations and their questionable relationship to microorganisms or viruses and regulatory problems in connection with differentiation and regulation of cells and cell function are typical problems in which in situ hybridization has already proved to be a very valuable technique. Up to now, only frozen samples of tissue or paraffin-embedded materials could be used for thin sectioning, which limited the size of samples and the selection of materials, because rigid structures, like bone or cartilage, could not be handled by standard procedures and equipment (24). Furthermore, fixation and embedding allowed superior maintenance of the histology. During the preparation of this manuscript, modifications with "touch prints" of frozen sections onto nitrocellulose membranes (6) and thin sections from entire animals (21) were published. These methods resolve some of the problems but did not seem to allow high resolution and correlation with specific cell types. In our approach, embedding media which can be polymerized to give a solid support were investigated for their effects on the hybridization signal. We chose this procedure, which requires special but readily available equipment for cutting and polishing of thin slices, rather than partial or complete decalcification protocols of frozen tissue, as the latter led to dramatically reduced hybridization efficiency (data not shown).

The quantitative cytophotometric evaluation of autoradiograms of in situ-hybridized P3HR1 cell smears shows that caution is advised when the in situ hybridization technique is adapted to materials other than frozen tissue sections. Despite available literature on the influence of different fixation procedures on the hybridization signal (9, 20), fixation protocols had to be reassessed, especially with respect to their combination with embedding procedures.

The methanol-acetic acid-fixed cell suspensions treated like frozen tissue sections were defined as controls (100% labeling). In comparison, the brief fixation protocols with low concentrations of fixatives, especially aldehydes, show a definite reduction in the levels of hybridization. This effect is potentiated by embedding at higher temperatures (e.g., as used with paraffin). This procedure does not completely eliminate but considerably reduces the number of labeled cells by more than 50%. Osmium tetroxide seems to be an optimal structure-preserving lipophilic fixative without negative effects on the efficiency of annealing.

The best results were achieved with a combination of ethanol-formaldehyde fixation and methyl methacrylate-based embedding. This protocol was therefore used in all further experiments. A major advantage is that, in the in situ hybridization, the labeled DNA reacts exclusively with the viral nucleic acid of the infected cells. It does not (as often happens in the frozen tissue sections) show a nonspecific background.

With the restrictions discussed above, hybridization of fixed, embedded materials was of particular value for the identification of virus-related DNA sequences in tissues of the cochlea and labyrinth of infected animals and was a valuable tool for studying active replication of viruses in organs containing cartilage or bone which were previously unsuitable for these techniques. This should be of significant importance for many other systems involving nucleic acid hybridization on bony or cartilaginous materials. Our procedure also allows a coordination of ultrastructural and virological information on specific cell types. The protocols demonstrated, for the first time, PRV DNA in the support and sensory cells of the cochlea and the vestibular apparatus in experimentally infected animals.

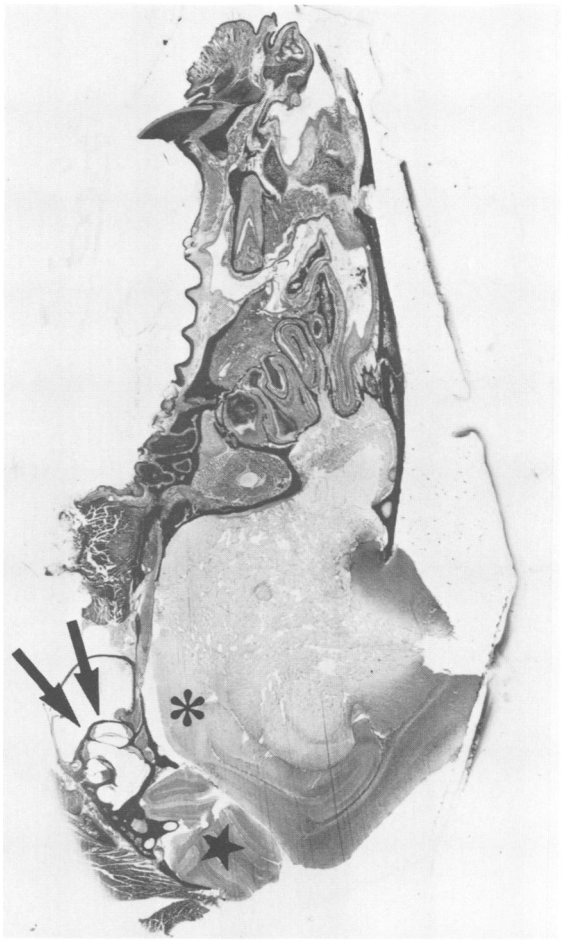

FIG. 2. Mid-sagittal section (sawing and polishing preparation) of the skull of a BALB/c mouse embedded in methyl methacrylate after intranasal infection with PRV. Localization of cochlear and vestibular area (arrows) corresponds with results shown in Fig. 3a, b, c, d, h, and g. The region of the cochlear nucleus (*), corresponding to Fig. 3f, and the region of the cerebellar nuclei (★), corresponding to Fig. 3e, are shown.

Although it is important to demonstrate viral effects directly at the location of the sensory organs of the inner ear to establish a causal relationship of virus infection and inner ear dysfunctions, we also suggest that the technique of nucleic acid hybridization can be used for diagnosis of disease. For this purpose other technical modifications of nucleic acid hybridization, such as spot hybridization on pellets of aspirated materials (18), can be used once a potential pathogen has been identified by the procedures described above. The membranes of the round and oval window do not seem to be barriers for microorganisms (7), hence sampling of material

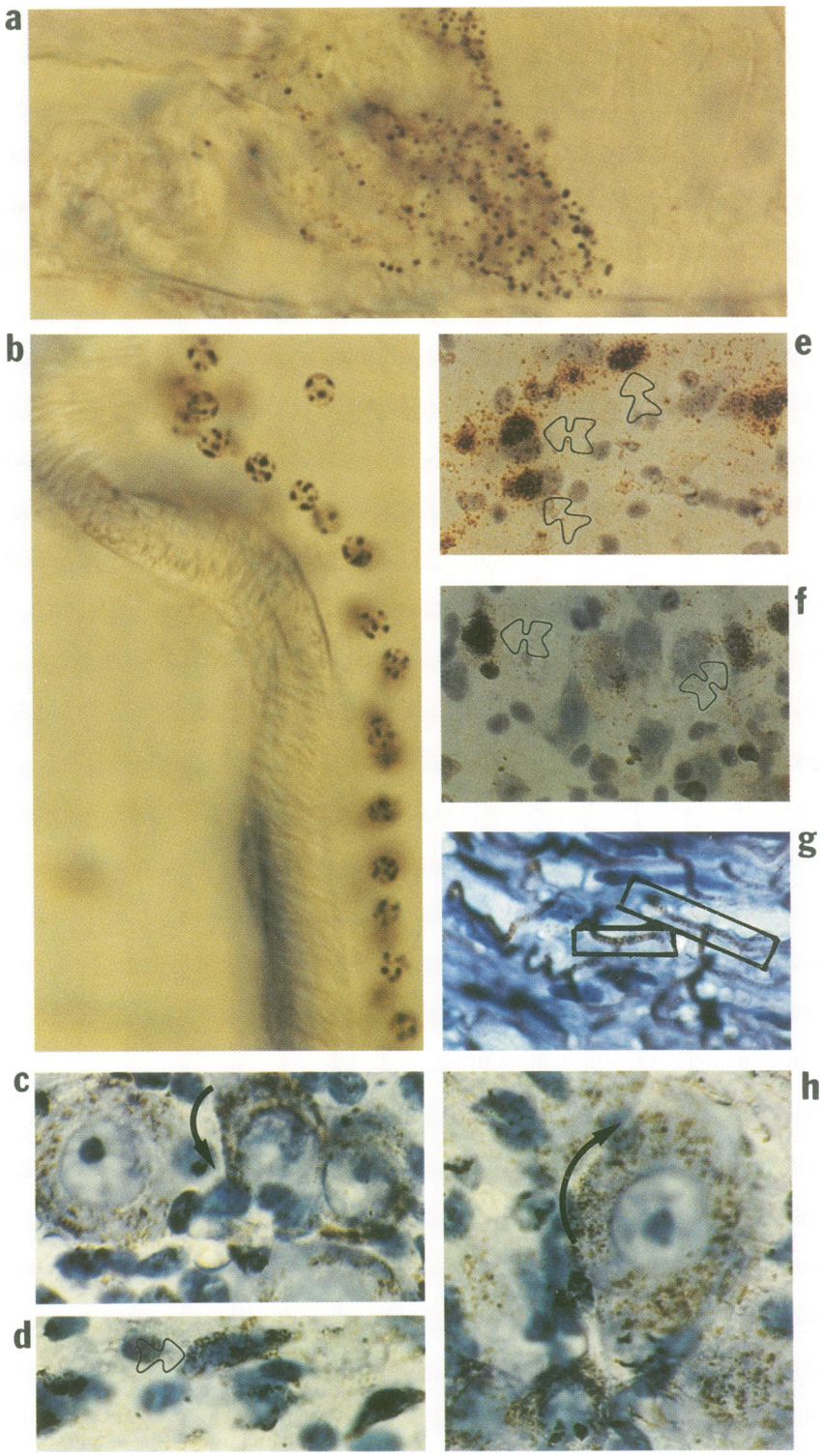

FIG. 3. Auditory and vestibular organs of a mouse infected intranasally with PRV with positive signal after in situ hybridization with ^{3}H-labeled PRV DNA. The following organs are shown: the organ of Corti (a), the crista region of the labyrinth (vestibular organ) (the label is located at the nuclei of the sensory cells) (b), the Schwann cells and possibly the cytoplasm of the ganglia cells of the spiral ganglion are labeled; the small nuclei of the ganglia cells are stained too darkly to allow detection of label. The arrows in panels c and h indicate that mainly the surrounding neural supporting tissue (Schwann cells) is labeled (c, d, and h). Also shown are the fibers of the eighth nerve; the heavy labeling is a consequence of the selective intraaxonal transport of viral DNA (g). The ganglion cells of nucleus cochlearis (f) and the cerebellar region (e) are also labeled.

from the middle ear via the tympanic membrane seems a promising approach for sample acquisition. Whereas Wetmore et al. (26) identified virus from material collected in the described way by culture methods, nucleic acid hybridization is potentially a much faster diagnostic procedure which works also for viruses which do not grow in culture, such as the human papillomaviruses, or grow slowly, such as varicella-zoster virus or cytomegalovirus. Fast nucleic acid hybridization should enable the clinician to adjust therapeutic protocols by the identified infectious agent.

LITERATURE CITED

1. **Carlemalm, E., W. Villiger, and J. D. Acetarin.** 1980. Advances in specimen preparation for electron microscopy. I. Novel low temperature embedding resins and a reformulated Vestopal. Abstr. Experientia **36**:740–742.
2. **Davis, L. E.** 1982. Experimental viral infection of the inner ear. III. Viremic spread of reovirus to hamster eighth nerve ganglion cells. Ann. Otol. Rhinol. & Laryngol. **91**:90–95.
3. **Davis, L. E., and L. G. Johnson.** 1983. Viral infections of the inner ear: clinical, virologic and pathologic studies in humans and animals. Am. J. Otol. **4**:347–362.
4. **Davis, L. E., and R. T. Johnson.** 1976. Experimental viral infections of the inner ear. I. Acute infections of the newborn hamster labyrinth. Lab. Invest. **34**:349–356.
5. **Davis, L. E., G. T. Nager, and R. T. Johnson.** 1979. Experimental viral infections of the inner ear. II. Simian virus 40-induced tumors of the temporal bone. Ann. Otol. Rhinol. & Laryngol. **88**:198–204.
6. **Dubensky, T. W., F. A. Murphy, and L. P. Villarreal.** 1983. Detection of DNA and RNA virus genomes in organ systems of whole mice: patterns of mouse organ infection by polyomavirus. J. Virol. **50**:779–783.
7. **Goycoolea, M., M. M. Paparella, and A. M. Carpenter.** 1978. Oval and round window changes in otitis media: an experimental study in the cat. Surg. Forum **29**:578–580.
8. **Gu, S., H. Wolf, and Z. Yi.** 1983. Cloning fragments of EBV-DNA in single-stranded phage m13 mp8. I. Preparation and identification of clone DNA. Chin. J. Virol. **5**:129–135.
9. **Haase, A., M. Brahic, L. Stowring, and H. Blum.** 1984. Detection of viral nucleic acids by in situ hybridization. Methods Virol. **7**:189–226.
10. **Han, J. H., and J. D. Harding.** 1983. Using iodinated single-stranded M13 probes to facilitate rapid DNA sequence analysis—nucleotide sequence of a mouse lysine tRNA gene. Nucleic Acids Res. **11**:2053–2064.
11. **Karmody, C. S.** 1983. Viral labyrinthitis: early pathology in the human. Laryngoscope **93**:1527–1533.
12. **Ladin, B. F., S. Ihara, H. Hampl, and T. Ben-Porat.** 1982. Pathway of assembly of herpes virus capsids: an analysis using DNA-temperature sensitive mutants of pseudorabies virus. Virology **116**:544–561.
13. **Lindsay, J., P. Davey, and R. Ward.** 1960. Inner ear pathology in deafness due to mumps. Ann. Otol. Rhinol. & Laryngol. **69**:918–927.
14. **Lindsay, J., and W. Hemenway.** 1954. Inner ear pathology due to measles. Ann. Otol. Rhinol. & Laryngol. **63**:754–763.
15. **Luft, J. H.** 1973. Improvements in epoxy-resin embedding methods. J. Biophys. Biochem. Cytol. **3**:409–414.
16. **Pardue, M. L., and J. G. Gall.** 1969. Molecular hybridization of radioactive DNA to the DNA of cytological preparations. Proc. Natl. Acad. Sci. USA **64**:600–604.
17. **Plenk, H., Jr.** 1976. Osteolothyrism. Quantitativ-Morphologische Untersuchurgen der experimentellen Skeletterkrankungen bei der Ratte, p. 5–6. S. Karger, Basel.
18. **Richter, W., S. Y. Gu, R. Seibl, and H. Wolf.** 1983. A new method for examination of carcinomas of the nasopharynx p. 25–32. *In* U. Prasad, D. V. Ablashi, P. Levine, and G. Pearson (ed.), Nasopharyngeal carcinoma: current concepts. University of Malaya, Kuala Lumpur. Plenum Publishing Corp., New York.
19. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1973. Labeling deoxyribonucleic acid by nick translation with DNA polymerase I. J. Mol. Biol. **133**:237–251.
20. **Singer, R. H., and D. C. Ward.** 1982. Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinated nucleotide analog. Proc. Natl. Acad. Sci. USA **79**:7331–7335.
21. **Southern, P. J., P. Blount, and M. B. A. Oldstone.** 1984. Analysis of persistent virus infections by in situ hybridization to whole-mouse sections. Nature (London) **312**:555–558.
22. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31–43.
23. **Swan, C., A. Tostevin, B. Moore, H. Mayo, and G. Black.** 1943. Congenital defects in infants following infectious diseases during pregnancy, with special reference to the relationship between German measles and cataract, deaf mutism, heart disease and microcephaly, and to period of pregnancy in which occurrence of rubella is followed by congenital abnormalities. Med. J. Austr. **2**:201–216.
24. **Ullberg, S.** 1977. The technique of whole body autoradiography. Cryosectioning of large specimens. Sci. Tools. Special issue, p. 2–29.
25. **Ward, P. H., and R. Gussen.** 1976. Preparation of the temporal bone for histopathologic study, p. 53–71. *In* C. A. Smith and J. A. Vernon (ed.), Handbook of auditory and vestibular research methods. Charles C Thomas, Publisher, Springfield, Ill.
26. **Wetmore, C. A., E. H. Pickard, and H. Stern.** 1979. Isolation of mumps virus from the inner ear after sudden deafness. Br. Med. J. **655**:14–15.
27. **Wolf, H., M. Haus, and E. Wilmes.** 1984. Persistence of Epstein-Barr virus in the parotid gland. J. Virol. **51**:795–798.
28. **Wolf, H., H. zurHausen, and V. Becker.** 1973. EB viral genomes in epithelial nasopharyngeal carcinoma cells. Nature (London) New Biol. **138**:245–247.