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Varicella-Zoster Virus Vaccine DNA Differs from the Parental Virus DNA

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The DNAs of a varicella-zoster virus vaccine and its parental virus were compared by CsCl buoyant density centrifugation and restriction enzyme cleavage analysis. The varicella-zoster virus vaccine DNA showed a heterogeneous buoyant profile and altered restriction enzyme cleavage patterns. These changed properties are probably the result of the accumulation of virus containing defective varicella-zoster virus DNA during extensive cell culture passage of the vaccine virus.

Varicella-zoster virus (VZV) infection of humans causes an acute illness (varicella or chicken pox), occurs almost always in childhood, and is followed by a life-long, usually inapparent infection. For normal children, varicella is an annoying but not a dangerous disease (18). In sharp contrast, varicella in an immunocompromised child is a serious, often fatal, disease (7, 11). In response to the seriousness of varicella in immunocompromised children, Takahashi and his collaborators (24) have developed a live, attenuated VZV vaccine, which is now undergoing limited clinical trials. As the DNA of the VZV vaccine is uncharacterized, we undertook a side-by-side comparison of the vaccine VZV DNA with the parental VZV DNA.

VZV isolates were propagated at 37°C in whole human embryo cells (Flow 5000 cells; Flow Laboratories, Inc., Rockville, Md.) grown in minimal essential medium (MEM) containing 10% fetal calf serum, 10% tryptose phosphate broth, $NaHCO_3$, and antibiotics. The parental virus, VZV (parOka), at passage 5 in human embryo lung cells, was generously sent to us by Michiaki Takahashi. The derivation of the vaccine virus, VZV (vacOka), from VZV (parOka) has been described by Takahashi et al. (24). Briefly, the history of the vaccine before passage in our laboratory was as follows. VZV (Oka) was passed sequentially 11 times in human embryo lung cells at 34°C, 12 times in guinea pig embryo cells at 37°C, and 1 to 21 times in human diploid (WI-38) cells at 37°C (1, 9, 13). The vaccine, lots 3B and 77-10, was sent as frozen, lyophilized preparations. VZV-infected cells were mixed with uninfected cells at a ratio of 1:2 or 1:3 in 75-cm^2 plastic flasks (T-75; Falcon Plastics, Oxnard, Calif; 12, 17, 19). Eighteen hours after mixing, the medium was decanted, and 15 ml of MEM containing 2.5% dialyzed fetal calf serum and 20 μCi of [^{3}H]thymidine (60 to 80 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml was added. The infection was allowed to progress until the cytopathic effect was greater than 80%. Tritium-labeled VZV DNA was prepared from purified extracellular (J. R. Ecker and R. W. Hyman, Arch. Virol., in press) and intracellular virions (14) by treatment with sodium dodecyl sulfate (0.5%) and pronase (2 mg/ml). After deproteinization by rocking with phenol-chloroform-isoamyl alcohol (24:24:1), VZV DNA was precipitated. The final step in the purification of VZV DNA was CsCl buoyant density centrifugation.

In the first experiment, *Hin*dIII-cleaved VZV (parOka) DNA from each successive pool of fractions from the CsCl gradient was loaded into consecutive wells in an agarose gel for electrophoretic separation of the specific fragments. Figure 1A shows the results of one such experiment for VZV (parOka) DNA at in vitro passage 11. DNA was found only at or near density 1.705 g/cm^3 (16). The *Hin*dIII cleavage pattern of the VZV (parOka) DNA was indistinguishable from one lane to the next with the exception of two bands. There was an apparent change in the molarity of two fragments across density 1.705 g/cm^3 (Fig. 1A, lanes 2 to 4). Additional examples of this change are shown in Fig. 1B (lanes 3 to 5) and Fig. 1C (lanes 3 to 5). The significance of this last observation is not known at present and is being pursued.

The effect of in vitro passage on the buoyant behavior of VZV (parOka) DNA was investigated. When the same combined CsCl gradient fractionation and restriction enzyme digestion experiment carried out for passage 11 VZV (parOka) DNA (Fig. 1A) was performed for passage 22 VZV (parOka) DNA (Fig. 1B), the passage 22 VZV (parOka) DNA was much more

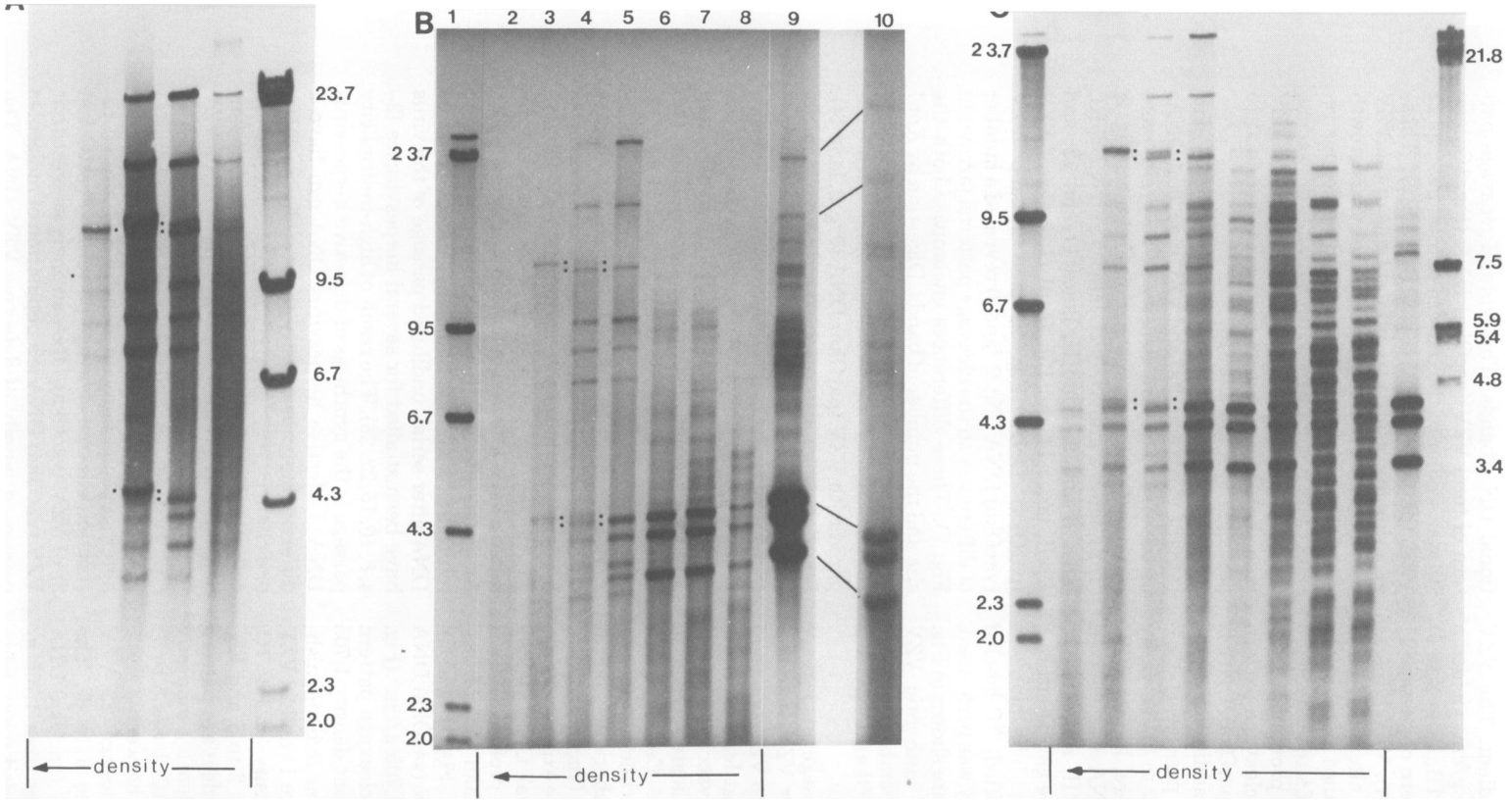

FIG. 1. *Fluorographs showing the HindIII cleavage patterns of VZV DNA from consecutive fractions of CsCl buoyant density gradients. Tritium-labeled VZV DNA was centrifuged to equilibrium in a 5-ml CsCl buoyant density gradient. The gradient was collected in fractions of 75 μl. Two to three fractions were pooled. The VZV DNA was dialyzed, concentrated by precipitation, resuspended in buffer, and cleaved by the restriction enzyme HindIII (Boehringer Mannheim Corp. Indianapolis, Ind.). Consecutive VZV DNA-containing fractions from the least dense to the most dense, as shown by the arrows at the bottom of the figures, were loaded into successive wells in a 0.5% agarose gel (19). After electrophoresis (19), the gel was dried, and fluorography was performed (5, 6). (A) VZV (parOka) DNA at passage 11 was found only in fractions centering at density 1.705 g/cm^3 (lanes 1 to 5). Lane 6 contained HindIII-cleaved λ DNA. The sizes (in kilobase pairs) of the specific fragments are given to the right. The dots between lanes 2 and 3 and 3 and 4 indicate VZV DNA fragments with relative molarities that change across density 1.705 g/cm^3. (B) The HindIII cleavage pattern of passage 22 VZV (parOka) DNA is shown (lanes 2 to 8). Lane 9 contained HindIII-cleaved total passage 22 VZV (parOka) DNA. Lane 10 is an autoradiograph (23) of a blot (21, 27) of total VZV (parOka) DNA hybridized (27) to nick-translated (20) ^{32}P-labeled VZV DNA from a fresh herpes zoster isolate. For orientation, the lines between lanes 9 and 10 point to some corresponding VZV DNA bands. Lane 1 contained HindIII-cleaved λ DNA as a standard. Sizes (in kilobase pairs) are given to the left. The dots between lanes 3 and 4 and 4 and 5 indicate VZV DNA fragments with relative molarities that change across density 1.705 g/cm^3 (corresponding to lanes 2 and 3 and 3 and 4 in [A]). (C) The HindIII cleavage pattern of passage 8 VZV (vacOka) DNA is shown (lanes 2 to 9). Lane 10 contained HindIII-cleaved total VZV (vacOka) DNA. Lanes 1 and 11 contained λ DNA cleaved with HindIII and EcoRI, respectively. Sizes (in kilobase pairs) are given on the outside of the figure. The dots between lanes 3 and 4 and 4 and 5 indicate VZV DNA fragments with relative molarities that change across density 1.705 g/cm^3 (corresponding to lanes 2 and 3 and 3 and 4 in [A]).*

broadly distributed in the gradient. The VZV (parOka) DNA of density 1.705 g/cm^3 yielded a *Hin*dIII cleavage pattern (Fig. 1B, lanes 3 to 5) indistinguishable from the pattern of passage 11 VZV (parOka) DNA (cf. Fig. 1A). VZV DNA at lighter densities gave rise to altered *Hin*dIII cleavage patterns (Fig. 1B, lanes 5 to 8). In particular, three DNA bands of sizes between 3 and 5 kilobase pairs were very prominent (Fig. 1B, lanes 6 to 8). The *Hin*dIII cleavage pattern of the total passage 22 VZV (parOka) DNA is shown in Fig. 1B, lane 9. Many additional bands were seen, in comparison with passage 11 VZV (parOka) DNA (Fig. 1A, lanes 2 to 5). To verify that these additional bands were, in fact, VZV DNA, total VZV (parOka) DNA was cleaved with restriction enzyme *Hin*dIII, subjected to electrophoresis in a 0.5% agarose gel, denatured, and blotted to prepared paper. The blot was then hybridized to highly purified, ^{32}P-labeled VZV DNA, and autoradiography was performed. The results of this experiment are shown in Fig. 1B, lane 10. Hybridization of authentic VZV DNA to the many additional bands of passage 22, *Hin*dIII-cleaved VZV (parOka) DNA was easily detected (Fig. 1B, lane 10).

After the effect of in vitro passage on cleavage pattern had been examined for VZV (parOka) DNA, we turned our attention to VZV (vacOka) DNA, which had been passed at least 24 times in culture before becoming the vaccine. Passage numbers for VZV (vacOka) indicate passage in our laboratory. The combined CsCl buoyant density-*Hin*dIII cleavage experiment was carried out for passage 8 (in our laboratory, but at least passage 32 overall) VZV (vacOka) DNA. The results of one representative experiment are shown in Fig. 1C. VZV (vacOka) DNA yielded a very disperse buoyant density profile in a CsCl gradient, even more disperse than that of passage 22 VZV (parOka) DNA (cf. Fig. 1B and C). A substantial portion of the VZV (vacOka) DNA banded at densities less than 1.705 g/cm^3 (Fig. 1C, lanes 6 to 9). The *Hin*dIII cleavage pattern of VZV (vacOka) DNA of buoyant density 1.705 g/cm^3 (Fig. 1C, lanes 2 to 4) was indistinguishable from the patterns of passage 11 and 22 VZV (parOka) DNA of the same density (cf. Fig. 1C, lanes 2 to 4 with Fig. 1A, lanes 2 to 5 and Fig. 1B, lanes 3 to 5). The *Hin*dIII cleavage pattern of VZV (vacOka) DNA of a density modestly lighter than 1.705 g/cm^3 (Fig. 1C, lanes 5 to 7) showed three prominent bands between 3 and 5 kilobase pairs, as seen for passage 22 VZV (parOka) DNA (Fig. 1B, lanes 6 to 8). The *Hin*dIII cleavage pattern of VZV (vacOka) DNA of a density significantly lighter than 1.705 g/cm^3 (Fig. 1C, lanes 7 to 9) yielded many additional DNA bands. The *Hin*dIII cleavage pattern of total VZV (vacOka) DNA (Fig. 1C, lane 10) was similar to the pattern of total passage 22 VZV (parOka) DNA (Fig. 1B, lane 9). These experiments, using two different lots of the VZV vaccine as the starting material for virus growth and DNA preparation, produced analogous results (data not shown). With both lots of vaccine, there were small changes in the total VZV (vacOka) DNA cleavage pattern at each in vitro passage (data not shown).

The restriction endonuclease cleavage patterns of VZV (parOka) and VZV (vacOka) DNAs of the CsCl buoyant density 1.705 g/cm^3 were compared, using six restriction enzymes (Fig. 2: *Hpa*I, lanes 2 and 3; *Bgl*II, lanes 5 and 6; *Eco*RI, lanes 8 and 9; *Hin*dIII, lanes 11 and 12; and *Kpn*I, lanes 14 and 15). *Hpa*I cleavage of VZV (parOka) DNA (Fig. 2, lane 2) and VZV (vacOka) DNA (Fig. 2, lane 3) revealed a number of differences in the cleavage patterns (arrows in Fig. 2). These differences encompass both the size and the number of bands. Digestion by *Kpn*I revealed that VZV (vacOka) DNA (Fig. 2, lane 15) gave rise to one additional band when compared with VZV (parOka) DNA (Fig. 2, lane 14). *Sma*I cleavage also revealed a difference in the size of one VZV (vacOka) DNA band compared with VZV (parOka) DNA (data not shown). Cleavage of VZV (vacOka) and (parOka) DNAs of density 1.705 g/cm^3 by *Bgl*II, *Eco*RI, and *Hin*dIII did not distinguish between the two (Fig. 2, lanes 5 and 6, 8 and 9, and 11 and 12, respectively).

VZV cannot be plaque-purified by current techniques. In addition, stable, truly cell-free VZV cannot be easily obtained, if at all (25). Therefore, we propagated VZV (parOka) by mixing VZV-infected cells with uninfected cells. As a consequence, there was little control of the multiplicity of infection. The effects on virus DNA after serial, undiluted passage of the virus have been studied for several herpesviruses (2–4, 8, 10, 15, 22, 26). The result of high-multiplicity passage is the synthesis of defective herpesvirus DNA. Defective herpesvirus DNA can have a different buoyant density than wild-type DNA or a changed restriction enzyme cleavage pattern or both. The observed changes in the buoyant density and *Hin*dIII cleavage pattern of VZV (parOka) DNA after 20 or more passages in culture (Fig. 1A and B) most probably reflect the accumulation of defective VZV DNA. The heterogeneous buoyant density and altered restriction enzyme cleavage patterns (Fig. 1C and 2) of VZV (vacOka) DNA strongly suggest that VZV (vacOka) DNA is a population of molecules composed largely of defective VZV DNA. The

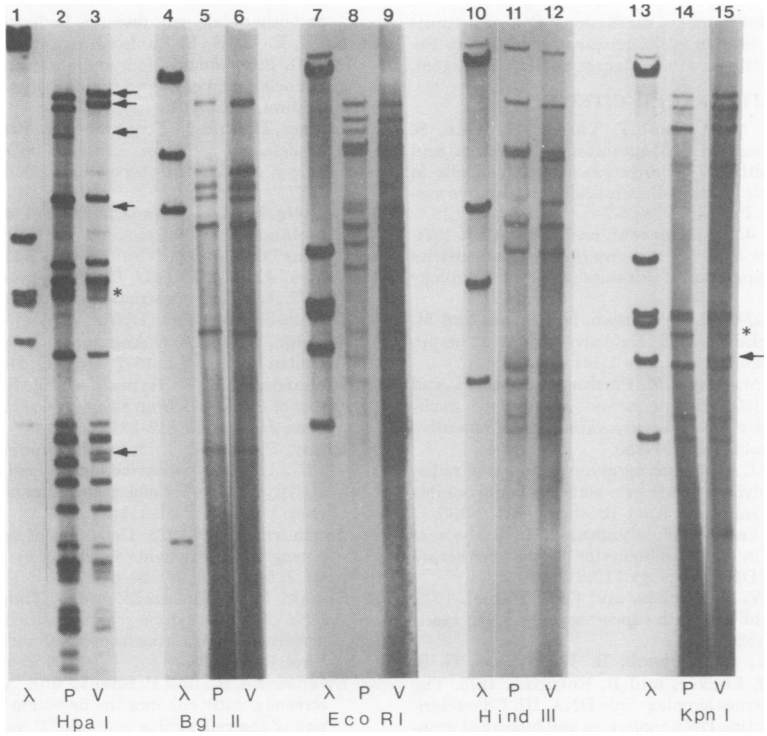

FIG. 2. *Fluorographs showing comparisons of the restriction endonuclease cleavage patterns of VZV (parOka) and (vacOka) DNAs of density 1.705 g/cm³. Tritium-labeled VZV (parOka) and VZV (vacOka) DNAs corresponding to density 1.705 g/cm³ were selected and digested by the specified restriction endonucleases. The resulting VZV DNA fragments were separated by agarose gel electrophoresis (19). The gels were dried, and fluorography was performed (5, 6). Lane 1 contained EcoRI-cleaved λ DNA. Lanes 2 and 3 contained HpaI-cleaved VZV (parOka) and (vacOka) DNAs, respectively. Lanes 4, 5, and 6 contained BglII-cleaved λ, VZV (parOka), and VZV (vacOka) DNAs, respectively. Lanes 7, 8, and 9 contained EcoRI-cleaved λ, VZV (parOka), and VZV (vacOka) DNAs, respectively. Lanes 10, 11, and 12 contained HindIII-cleaved λ, VZV (parOka), and VZV (vacOka) DNAs, respectively. Lane 13 contained EcoRI-cleaved λ DNA. Lanes 14 and 15 contained KpnI-cleaved VZV (parOka) and (vacOka) DNAs, respectively. Arrows point to differences between the VZV (parOka) and VZV (vacOka) DNAs. Asterisks mark regions of suspected differences.*

VZV (vacOka) DNA population contained DNA of more "defectiveness" than passage 22 VZV (parOka) DNA (Fig. 1B and C). This observation may be related to two facts: (i) VZV (vacOka) has been passed in vitro more times (at least 32 passages) than VZV (parOka); and (ii) VZV (vacOka) has been passed in guinea pig embryo cells between passages in human cells (24), whereas VZV (parOka) has been passed only in human cells.

VZV (vacOka) DNA of buoyant density 1.705 g/cm³ was isolated from a CsCl gradient and compared by restriction enzyme cleavage to VZV (parOka) DNA of the same density. Six restriction enzyme cleavage patterns were examined (Fig. 2). The *Eco*RI, *Hin*dIII, and *Bgl*II cleavage patterns did not distinguish VZV (vacOka) DNA from VZV (parOka) DNA, whereas the *Hpa*I, *Kpn*I, and *Sma*I cleavage patterns did show differences between the DNAs. These data demonstrate that VZV (vacOka) DNA has undergone some base sequence changes with respect to VZV (parOka) DNA. Our working hypotheses are that VZV (vacOka) DNA has accumulated point mutations and/or small deletions or insertions and that, in consonance with what is known of passage of herpesviruses in vitro at high multiplicities of infection, VZV (vacOka) DNA preparations are largely composed of defective VZV DNA.

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LITERATURE CITED

1. **Asano, Y., H. Nakayama, T. Yazaki, R. Kato, S. Hirose, K. Tsuzuki, I. Shigemitsu, S. Isomura, and M. Takahashi.** 1977. Protection against varicella in family contacts by immediate inoculation with live varicella vaccine. Pediatrics **59**:3–7.
2. **Ben-Porat, T., J. M. Demarchi, and A. Kaplan.** 1974. Characterization of defective interfering viral particles present in a population of pseudorabies virions. Virology **61**:29–37.
3. **Bronson, D. L., G. R. Dreesman, N. Biswal, and M. Benyesh-Melnick.** 1973. Defective virions of herpes simplex viruses. Intervirology **1**:141–153.
4. **Campbell, D., M. Kemp, M. Perdue, C. Randall, and G. Gentry.** 1976. Equine herpesvirus *in vivo*: cyclic production of a DNA density variant with repetitive sequence. Virology **69**:737–750.
5. **Chamberlain, J.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. **98**:132–135.
6. **Ecker, J. R., and R. W. Hyman.** 1981. Analysis of interruptions in the phosphodiester backbone of herpes simplex virus DNA. Virology **110**:213–216.
7. **Feldman, S., W. T. Hughes, and C. B. Daniel.** 1975. Varicella in children with cancer: seventy-seven cases. Pediatrics **56**:388–397.
8. **Frenkel, N. R., R. J. Jacob, R. W. Honess, G. S. Hayward, H. Locker, and B. Roizman.** 1975. The anatomy of herpes simplex virus DNA. III. Characterization of defective DNA molecules and biological properties of virus populations containing them. J. Virol. **16**: 153–167.
9. **Gershon, A.** 1980. Live attenuated varicella-zoster vaccine. Rev. Infect. Dis. **2**:393–407.
10. **Graham, B., Z. Bengali, and G. Vande Woude.** 1978. Physical map of the origin of defective DNA in herpes simplex virus type 1 DNA. J. Virol. **25**:878–887.
11. **Hattori, A., T. Ihara, T. Iwasa, H. Kamiya, M. Sakurai, and T. Izawa.** 1976. Use of varicella vaccine in children with acute leukemia or other malignancies. Lancet **ii**:210.
12. **Iltis, J. P., J. E. Oakes, R. W. Hyman, and F. Rapp.** 1977. Comparison of the DNAs of varicella-zoster viruses isolated from clinical cases of varicella and herpes zoster. Virology **82**:345–352.
13. **Izawa, T., T. Ihara, A. Hattori, T. Iwasa, H. Kamiya, M. Sakurai, and M. Takahashi.** 1977. Application of a live varicella vaccine in children with acute leukemia and other malignant diseases. Pediatrics **6**:805–809.
14. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. **8**:125–132.
15. **Locker, H., and N. Frenkel.** 1979. Structure and origin of defective genomes contained in serially passaged herpes simplex virus type 1 (Justin). J. Virol. **29**:1065–1077.
16. **Ludwig, H., H. G. Haines, N. Biswal, and M. Benyesh-Melnick.** 1972. The characterization of varicella-zoster virus DNA. J. Gen. Virol. **14**:111–114.
17. **Oakes, J. E., J. P. Iltis, R. W. Hyman, and F. Rapp.** 1977. Analysis by restriction enzyme cleavage of human varicella-zoster virus DNAs. Virology **82**:353–361.
18. **Preblud, S., and L. D'Angelo.** 1979. Chicken pox in the United States, 1972–1977. J. Infect. Dis. **140**:257–260.
19. **Richards, J., R. W. Hyman, and F. Rapp.** 1979. Analysis of the DNAs from seven varicella-zoster virus isolates. J. Virol. **32**:812–821.
20. **Rigby, P., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
22. **Stinski, M., E. Mocarski, and D. Thomsen.** 1979. The DNA of human cytomegalovirus: size heterogeneity and defectiveness resulting from serial undiluted passage. J. Virol. **31**:231–239.
23. **Swanstrom, R., and P. Shank.** 1978. X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotopes ^{32}P and ^{125}I. Anal. Biochem. **86**:184–192.
24. **Takahashi, M., Y. Okuno, T. Otsuka, J. Osame, A. Takamizawa, T. Sasada, and T. Kubo.** 1975. Development of a live attenuated varicella vaccine. Biken J. **18**:25–33.
25. **Taylor-Robinson, D., and A. E. Caunt.** 1972. The varicella virus, p. 1–88. Virology monographs, vol. 12. Springer-Verlag, Vienna.
26. **Wagner, M., J. Skare, and W. C. Summers.** 1975. Analysis of DNA of defective herpes simplex virus type 1 by restriction endonuclease cleavage and nucleic acid hybridization. Cold Spring Harbor Symp. Quant. Biol. **39**:683–686.
27. **Wahl, G., M. Stern, and G. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**: 3684–3688.