JOURNAL OF BACTERIOLOGY, Oct., 1966
Vol. 92, No. 4
Copyright © 1966 American Society for Microbiology
Printed in U.S.A

Synthesis of Virus Deoxyribonucleic Acid During Abortive Infection of Simian Cells by Human Adenoviruses

FRED RAPP,[1] LAWRENCE A. FELDMAN, AND MANLEY MANDEL

Department of Virology and Epidemiology, Baylor University College of Medicine, and Department of Biology, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas

Received for publication 1 July 1966

ABSTRACT

RAPP, FRED (Baylor University College of Medicine, Houston, Tex.), LAWRENCE A. FELDMAN, AND MANLEY MANDEL. Synthesis of virus deoxyribonucleic acid during abortive infection of simian cells by human adenoviruses. J. Bacteriol. **92**:931–936. 1966.—Inoculation of green monkey kidney cells (GMK) with adenovirus types 2 or 12, under conditions where neither infectious virus was synthesized, resulted in an increase in the uptake of H^3-thymidine into deoxyribonucleic acid (DNA). Extraction of the DNA from infected cells, followed by identification by isopycnic analysis in CsCl gradients, revealed the presence of virus DNA. Cells infected with adenovirus type 2 yielded DNA giving bands with peak densities of 1.699 g/ml [GMK DNA with 40 moles % guanine + cytosine (GC)] and 1.714 g/ml (adenovirus type 2 DNA with 55 moles % GC). Cells infected with adenovirus type 12 also yielded the GMK DNA and a band at 1.706 g/ml (adenovirus type 12 DNA with 47 moles % GC). The rate of synthesis of adenovirus type 2 DNA in KB cells (productive cycle) and in GMK cells infected only with adenovirus (nonproductive cycle) or with adenovirus and simian virus 40 (adeno-productive cycle) was not significantly different.

It has been previously documented that simian papovavirus 40 (SV40) potentiates the replication of human adenovirus types 2, 4, 5, 7, and 12 in African green monkey kidney (GMK) cell cultures (1, 7, 14, 18, 20, 23). These adenoviruses, in the absence of SV40, induce an abortive cycle of replication in GMK cells. The adenoviruses adsorb and penetrate into the GMK cell and induce the synthesis of adenovirus tumor antigens (7, 14). However, by use of immunofluorescent techniques, adenovirus capsid antigens were not detected during the abortive infection (7, 14). Joint infection of GMK cells with adenovirus and with SV40 resulted in the synthesis of both SV40 and adenovirus tumor and viral antigens (7, 14), the development of both types of virions in the same cell (18), and an increase in the infectious titer of both viruses (7, 8).

To explore the interaction of SV40 and adenoviruses in GMK cells, it seemed logical to carry out studies designed to determine whether SV40 was required for early or late steps in the potentiation of adenoviruses in the simian cells. Since several of the early events noted in the abortive cycle of adenoviruses in GMK cells are not dependent on DNA synthesis, it became necessary to investigate whether adenovirus DNA was produced during the abortive process and, if so, whether coinfection of GMK cells with SV40 markedly changed the rate of synthesis of adenovirus DNA. The present communication describes the results of this study.

[1] American Cancer Society Professor of Virology.

MATERIALS AND METHODS

Cultures. Primary GMK cultures were grown in 16-oz (453.6 ml) bottles as previousy described (17) in Melnick-Hanks' lactalbumin hydrolysate (M-H) medium containing 2% calf serum. Maintenance medium consisted of M-H with no serum added. For pulse-labeling experiments, primary GMK cells were grown in 60-mm plastic petri dishes with M-H containing 2% calf serum. Maintenance medium for these cultures consisted of Melnick-Earle's lactalbumin hydrolysate with no serum added. KB cells were grown in 16-oz

(453.6-ml) bottles in Eagle's minimal medium supplemented with 10% fetal calf serum; maintenance fluid contained 2% fetal calf serum.

Viruses. The adenovirus types 2 and 12 used in these experiments have been described (7). These viruses had never been passaged in simian cells, and the stocks were devoid of adeno-satellite viruses (15). The Baylor reference strain of SV40 has also been previously described (16, 21).

Radioactive labeling of deoxyribonucleic acid (DNA). Tritiated thymidine (2 c/mmole, New England Nuclear Corp., Boston, Mass.) was used to pulse-label newly synthesized DNA. A 10-μc amount of tritiated thymidine was added to 60-mm plastic petri dishes containing 5 ml of maintenance medium, and the period of pulse-labeling was extended for 1 hr. The fluid was then removed, and the cells were washed five times with cold tris(hydroxymethyl)aminomethane (Tris) saline (0.025 M, *p*H 7.6). The cells were then scraped into 1 ml of cold 0.3 N perchloric acid (PCA), centrifuged at 2,000 $\times$ *g* in the cold, and washed three times in cold 0.3 N PCA. After the final wash, 0.5 ml of 0.5 N PCA was added, and the pellet was hydrolyzed at 70 C for 1 hr. The supernatant fluid was used for determining radioactivity and DNA. DNA concentrations were determined by the Burton method (4).

Measurement of radioactivity. Tritium content of a known volume of material was assayed with a liquid scintillation spectrometer (CPM 200, Beckman Instruments, Inc., Fullerton, Calif.). Aqueous samples were counted in a dioxane-phosphor mixture (3). The samples were corrected for quenching with the aid of an external standard and a quench curve prepared by adding a known number of disintegrations per minute (dpm) of tritium to the dioxane mixture. In the experimental procedures, counts per minute were converted to dpm with the aid of such a quench curve, and the data were presented as dpm/μg of DNA.

Lysate production and isopycnic analysis of extracted DNA. Culture fluids were decanted from tissue culture bottles, and the tissue layer was rinsed with saline-EDTA solution (0.15 M NaCl plus 0.1 M Na_2 ethylenediaminetetraacetate, *p*H 8.0). The cultures were incubated with 1 ml of pronase (Calbiochem; 300 μg/ml in saline-EDTA) at 37 C for 10 min to release the cells from the glass. The suspended cells were then lysed by the addition of sodium dodecyl sulfate to a final concentration of 2%. The viscous solution was deproteinized by shaking with an equal volume of phenol saturated with saline-EDTA adjusted to *p*H 8 with concentrated NaOH just prior to use. The top aqueous phase was removed after centrifugation at 10,000 $\times$ *g* for 10 min at 5 C, and the nucleic acids were precipitated with 2 volumes of 95% ethyl alcohol. The spooled fibers were washed twice with 70% ethyl alcohol, drained of excess ethyl alcohol, and dissolved in 0.15 M NaCl in 0.015 M Na_3 citrate at *p*H 7.0. Nucleic acid concentrations of the partially purified lysates were estimated from the ultraviolet absorbancies between 230 and 280 mμ (2), and portions estimated to contain from 2 to 4 μg of DNA were taken for analytical CsCl density gradient ultracentrifugation (24). Centrifugation was carried out for at least 23 hr at 25.0 C at 42,040 rev/min in a Spinco model E analytical ultracentrifuge fitted with monochromator and direct ultraviolet photoelectric scanner. Cells were scanned at a wavelength of 265.4 mμ. Four-cell operation was employed, with use of appropriate wedge windows and rotating mask to separate the images of each cell. DNA of *Bacillus subtilis* bacteriophage SP8 of a density of 1.742 g/ml was employed as a reference, and the density [and consequent guanine plus cytosine (GC) content] of each peak of DNA at equilibrium in the gradient was calculated as described by Schildkraut, Marmur, and Doty (24). The relative amount of DNA in each molecular distribution in the sample was estimated from the planimetric measurements of the area under the absorbance curves. The measurements of area were reproducible within 5%.

RESULTS

Initial experiments were carried out to determine the effect of adenovirus infection on DNA synthesis in GMK cells. Primary GMK cell cultures (10 days old) in 60-mm plastic petri dishes were used. Growth medium was replaced by maintenance medium 24 hr before inoculation of the virus. At the time of inoculation, the maintenance fluid was removed, and 0.5 ml of an adenovirus type 2 stock was added to the petri dishes. The multiplicity of infection in all experiments was 10 plaque-forming units (PFU) per cell; the titers had been obtained in human embryonic kidney cells (8). The virus was allowed to adsorb at 37 C for 1 hr, after which the excess virus was removed by washing the cultures with warm Tris-saline buffer (*p*H 7.6). Duplicate plates were sham-infected with 0.5 ml of Tris-saline buffer. At various times after a 1-hr pulse with H^3-thymidine, the virus-infected cultures and the sham-infected cultures were harvested as described in Materials and Methods.

The results of one experiment are presented in Fig. 1. A slight decrease in uptake of H^3-thymidine was observed 5 to 6 hr after inoculation, at which time the virus-infected cultures were compared with uninoculated control cultures. From 24 to 48 hr after inoculation, the virus-infected cultures showed a higher rate of incorporation of H^3-thymidine than the uninfected cultures; 72 to 73 hr after inoculation, the rate of uptake of label was the same in infected and in control cultures. Duplicate samples of virus-infected GMK cells were harvested at 6, 24, 30, 48, and 72 hr after inoculation by scraping the cells into the 5.0 ml of culture fluid. The cells were frozen and thawed three times, and titrations were performed on the supernatant fluids after removal of the cell debris by centrifugation. No increase in infectious virus was observed throughout the course of the experiment. Similar results have been obtained in three separate experiments; the rate of thymidine uptake into DNA by the infected cultures

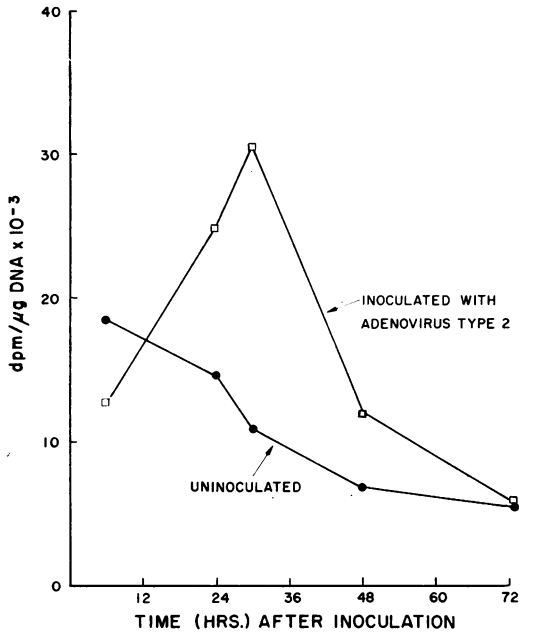

FIG. 1. *Effect of adenovirus type 2 on the rate of synthesis of DNA in green monkey kidney cells given in disintegrations per minute (dpm). Multiplicity of infection was 10 plaque-forming units per cell. The cultures were pulsed with tritiated thymidine for 1 hr and were harvested as described in Materials and Methods.*

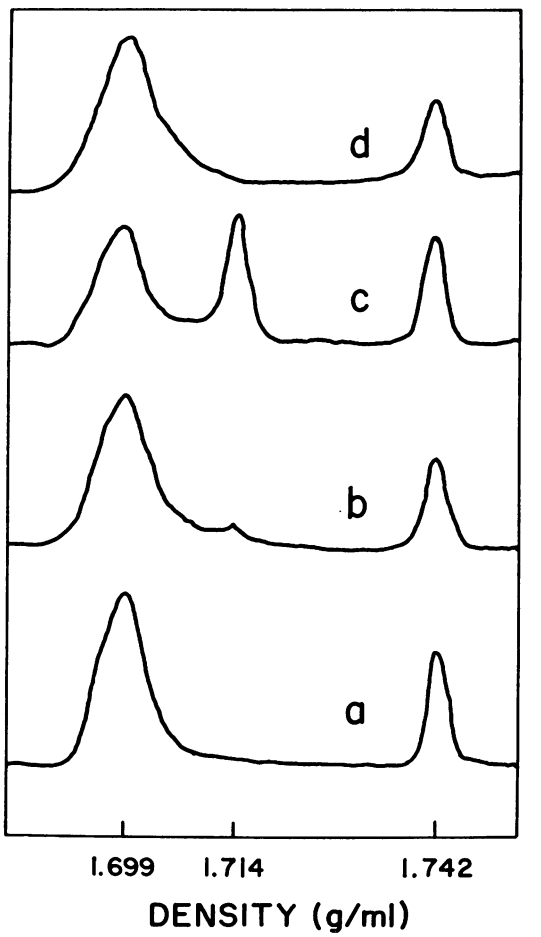

FIG. 2. *Tracings of direct photoelectric scannings of extracted DNA from green monkeys kidney cells infected with adenovirus type 2 at equilibrium in CsCl gradients. (a) One hour after inoculation, (b) 24 hr after inoculation, (c) 41 hr after inoculation, and (d) 66 hr after inoculation with neutralized virus. Reference DNA of Bacillus subtilis bacteriophage SP8 (density, 1.742 g/ml) is at right.*

was always two- to threefold greater than in the uninoculated controls at 18 to 48 hr after inoculation.

Since adenovirus type 2-infected GMK cell cultures showed an increased rate of H^3-thymidine incorporation into DNA, we asked whether viral DNA was being synthesized in the abortive cycle of infection. A series of 16-oz (453.6 ml) bottles containing primary GMK cells were inoculated with adenovirus type 2 at a multiplicity of 40 PFU per cell. DNA was extracted from these cells at 1, 24, and 41 hr after inoculation. This material was banded in CsCl, and the direct tracings are shown in Fig. 2. At 1 hr after inoculation (curve a), a single band of DNA appeared at a density of 1.699 g/ml; such DNA had a GC content of 40 moles %, was identical to DNA extracted from uninoculated control cultures, and was therefore identified as green monkey cell DNA. At 24 hr after inoculation, a small band appeared at a density of 1.714 g/ml (curve b); the height of this band increased significantly by 41 hr after inoculation (curve c). The average of 13 buoyant densities of DNA extracted from adenovirus type 2-infected GMK cells 24 to 66 hr after inoculation was 1.7138 g/ml ± 0.001, a figure in good agreement with the buoyant density of 1.716 g/ml (GC = 57 moles %) previously reported by Piña and Green (19) for adenovirus type 2 DNA extracted from the virion. GMK cells inoculated with adenovirus type 2 previously neutralized with type-specific antiserum failed to synthesize detectable adenovirus type 2 DNA within 66 hr after inoculation (curve d).

The position of the absorbance peak of the adenovirus type 2 DNA was not altered when the GMK cells were simultaneously co-infected with SV40 (Fig. 3a, b). A peak for SV40 DNA was not detected (Fig. 3b) nor should one be expected, inasmuch as the GC content of SV40

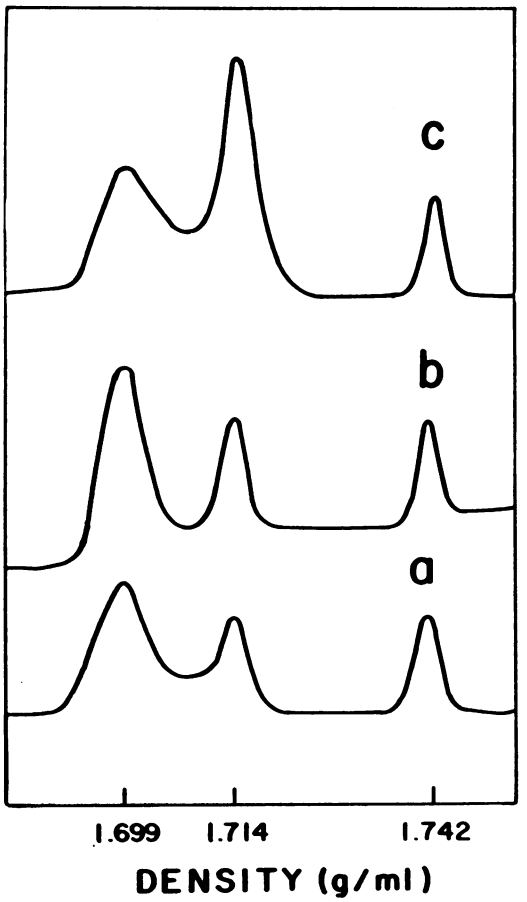

FIG. 3. *DNA extracted from cell cultures 60 hr after inoculation with adenovirus type 2 or with adenovirus type 2 and SV40. Tracings as in Fig. 2. (a) GMK cells inoculated with adenovirus type 2, (b) GMK cells inoculated simultaneously with adenovirus type 2 and with SV40, and (c) KB cells inoculated with adenovirus type 2. Multiplicity of adenovirus was 25 plaque-forming units (PFU) per cell and that of SV40 was about 1 PFU per cell.*

DNA is 41 moles % (6), and would therefore be masked by the GMK DNA. The same peak of viral DNA appeared in lysates of infected KB cells (Fig. 3c).

A similar series of experiments were performed with adenovirus type 12 (Fig. 4). An input multiplicity of about 4 PFU per cell was used, and the adenovirus DNA peak appeared 41 hr after inoculation at a density of 1.706 g/ml (curve b). This value, and the corresponding 47 moles % of GC, was again in good agreement with previously published figures for adenovirus type 12 DNA (19). Co-infection of the cells with SV40 did not alter the DNA results described.

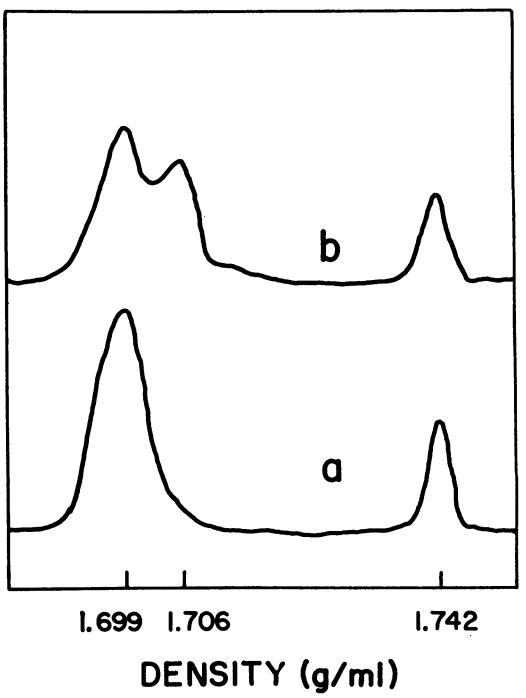

FIG. 4. *DNA extracted from green monkey kidney cells infected with adenovirus type 12. Tracings as in Fig. 2. (a) One hour after inoculation; (b) 41 hr after inoculation.*

The data thus far presented indicated that adenovirus DNA is synthesized in the abortive infection of GMK cells. We next investigated the rate of synthesis of adenovirus DNA during the abortive cycle in GMK cells, during the replicative cycle in KB cells, and in GMK cells co-infected with SV40. A series of 16-oz (453.6 ml) bottles of KB cell cultures were inoculated with adenovirus type 2. At the same time, a series of 16-oz (453.6 ml) bottles of GMK cell cultures were inoculated with adenovirus type 2, and another series with adenovirus type 2 and SV40. The bottles were harvested at 6-hr intervals from 18 to 66 hr after inoculation, and the DNA was extracted and analyzed at equilibrium in CsCl gradients. The areas under the curves were measured as described and the results are presented in Fig. 5. In all systems, a significant rate of adenovirus DNA synthesis was observed, as seen from the slopes of lines a, b, and c. The rate of adenovirus DNA synthesis in GMK cells inoculated only with adenovirus (curve a) appeared to be very similar to that obtained in cultures infected with both adenovirus type 2 and SV40 (curve b). Neither of these curves was significantly different from the rate of synthesis of adenovirus type 2

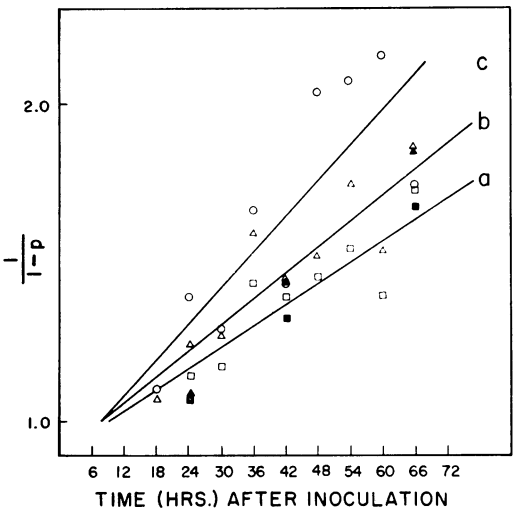

FIG. 5. *Relative increase of viral DNA in inoculated cell cultures. The value p is the fraction of viral DNA in the total primate plus viral DNA measured as areas under the curve of absorbance at 265.4 mμ at equilibrium in CsCl gradients. Lines fitted by method of least squares. (a) GMK cells inoculated with adenovirus type 2, □; (b) GMK cells inoculated with adenovirus type 2 and SV40, △; (c) KB cells inoculated with adenovirus type 2,○. Filled squares and triangles indicate values obtained the same as were those with open squares and triangles, but these data are from a separate experiment and were not included in calculation of best fit. Multiplicity of adenovirus was 25 plaque-forming units (PFU) per cell and that of SV40 was about 1 PFU per cell.*

DNA in KB cells (curve c). The probability that the positive slopes represent a real increase in DNA is significant at the 99.95% level.

DISCUSSION

The results of these experiments indicate that adenovirus DNA is synthesized during the abortive infection of GMK cell cultures. The rate of adenovirus DNA synthesis does not appear to be enhanced by co-infection of the cells with SV40. Reich et al. (22) reached a similar conclusion based on homology studies. Pulse-labeling experiments in GMK cells indicate an increase in DNA synthesis from 18 to 48 hr after inoculation. The rate of increase and the suggestion that host cell DNA synthesis is inhibited after infection by adenovirus type 2 is similar to results obtained in KB and HeLa cell cultures (11, 12).

Arabinofuranosylcytosine and 5-fluorodeoxyuridine, which inhibit the synthesis of adenovirus and SV40 DNA (10, 13), also prevent the synthesis of capsid antigens in infected human (9, 10) and simian (5, 21) cell cultures. It has therefore been suggested that the formation of progeny viral DNA is necessary for the production of viral capsid antigens. Immunofluorescent studies have failed to reveal the induction of viral capsid proteins in GMK cells abortively infected with adenovirus types 2, 7, and 12 (7, 14). The present study does not reveal whether the adenovirus DNA synthesized during the abortive infection of GMK cells is competent to code for messenger RNA molecules and their subsequent translation into viral capsid proteins. Obviously, competent adenovirus DNA can be formed in GMK cells, since co-infection of the cells with SV40 allows the production of infectious adenovirions (1, 7, 8, 20, 23). An alternate hypothesis for the failure to detect the formation of adenovirus capsid material during the abortive infection is that the DNA is transcribed but that faulty translation results in aberrant protein which is antigenically different from the normal capsid protein and does not react with the immunoreagents employed. Should this prove to be the case, potentiation by SV40 might involve a step in the replicative cycle of the adenovirus in the simian cells subsequent to synthesis of virus DNA.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grants CA-04600 and CA-10036 from the National Cancer Institute, grants AI-05382 and 5 T1 AI 74 from the National Institute of Allergy and Infectious Diseases, and grant GB 2262 from the National Science Foundation. The authors acknowledge the expert technical assistance of Janet Bergendahl, and thank Reimut Wette (Department of Biomathematics, University of Texas, M. D. Anderson Hospital and Tumor Institute) for his valued advice on statistical analyses.

LITERATURE CITED

1. BEARDMORE, W. B., M. J. HAVLICK, A. SERAFINI, AND I. W. MCLEAN, JR. 1965. Interrelationship of adenovirus (type 4) and papovavirus (SV40) in monkey kidney cell cultures. J. Immunol. **95**:422–435.
2. BEAVEN, G. H., E. R. HOLIDAY, AND E. A. JOHNSON. 1955. Optical properties of nucleic acids and their components, p. 493–553. *In* E. Chargaff and J. N. Davidson [ed.], The nucleic acids, vol. 1. Academic Press, Inc., New York.
3. BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279–285.
4. BURTON, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
5. BUTEL, J. S., AND F. RAPP. 1965. The effect of arabinofuranosylcytosine on the growth cycle of simian virus 40. Virology **27**:490–495.

6. CRAWFORD, L. V., AND P. H. BLACK. 1964. The nucleic acid of simian virus 40. Virology **24**:388–392.
7. FELDMAN, L. A., J. S. BUTEL, AND F. RAPP. 1966. Interaction of a simian papovavirus and adenoviruses. I. Induction of adenovirus tumor antigen during abortive infection of simian cells. J. Bacteriol. **91**:813–818.
8. FELDMAN, L. A., J. L. MELNICK, AND F. RAPP. 1965. Influence of SV40 genome on the replication of an adenovirus-SV40 "hybrid" population. J. Bacteriol. **90**:778–782.
9. FELDMAN, L. A., AND F. RAPP. 1966. Inhibition of adenovirus replication by 1-β-D-arabinofuranosylcytosine. Proc. Soc. Exptl. Biol. Med. **122**: 243–247.
10. GILEAD, Z., AND H. S. GINSBERG. 1965. Characterization of a tumorlike antigen in type 12 and type 18 adenovirus-infected cells. J. Bacteriol. **90**:120–125.
11. GINSBERG, H. S., AND M. K. DIXON. 1961. Nucleic acid synthesis in types 4 and 5 adenovirus-infected HeLa cells. J. Exptl. Med. **113**:283–299.
12. GREEN, M. 1959. Biochemical studies on adenovirus multiplication. I. Stimulation of phosphorus incorporation into deoxyribonucleic acid and ribonucleic acid. Virology **9**:343–358.
13. KIT, S., D. R. DUBBS, P. M. FREARSON, AND J. L. MELNICK. 1966. Enzyme induction in SV40-ininfected green monkey kidney cultures. Virology **29**:69–83.
14. MALMGREN, R. A., A. S. RABSON, P. G. CARNEY, AND F. J. PAUL. 1966. Immunofluorescence of green monkey kidney cells infected with adenovirus 12 and with adenovirus 12 plus simian virus 40. J. Bacteriol. **91**:262–265.
15. MELNICK, J. L., H. D. MAYOR, K. O. SMITH, AND F. RAPP. 1965. Association of 20-millimicron particles with adenoviruses. J. Bacteriol. **90**:271–274.
16. MELNICK, J. L., S. E. STINEBAUGH, AND F. RAPP. 1964. Incomplete simian papovavirus SV40. Formation of non-infectious viral antigen in the presence of fluorouracil. J. Exptl. Med. **119**: 313–326.
17. MELNICK, J. L., H. A. WENNER, AND L. ROSEN. 1964. The enteroviruses, p. 194–242. *In* E. H. Lennette and N. J. Schmidt [ed.], Diagnostic procedures for viral and rickettsial diseases, 3rd ed. American Public Health Association, Inc., New York.
18. O'CONOR, G. T., A. S. RABSON, I. K. BEREZESKY, AND F. J. PAUL. 1963. Mixed infection with simian virus 40 and adenovirus 12. J. Natl. Cancer Inst. **31**:903–917.
19. PIÑA, M., AND M. GREEN. 1965. Biochemical studies on adenovirus multiplication. IX. Chemical and base composition analysis of 28 human adenoviruses. Proc. Natl. Acad. Sci. U.S. **54**: 547–551.
20. RABSON, A. S., G. T. O'CONOR, I. K. BEREZESKY, AND F. J. PAUL. 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. Proc. Soc. Exptl. Biol. Med. **116**:187–190.
21. RAPP, F., J. S. BUTEL, L. A. FELDMAN, T. KITAHARA, AND J. L. MELNICK. 1965. Differential effects of inhibitors on the steps leading to the formation of SV40 tumor and virus antigens. J. Exptl. Med. **121**:935–944.
22. REICH, P. R., S. G. BAUM, J. A. ROSE, W. P. ROWE, AND S. M. WEISSMAN. 1966. Nucleic acid homology studies of adenovirus type 7-SV40 interactions. Proc. Natl. Acad. Sci. U.S. **55**:336–341.
23. ROWE, W. P., AND S. G. BAUM. 1965. Studies of adenovirus SV40 hybrid viruses. II. Defectiveness of the hybrid particles. J. Exptl. Med. **122**: 955–966.
24. SCHILDKRAUT, C. L., J. MARMUR, AND P. DOTY. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. **4**:430–443.