

## Antibodies specific for the carboxy- and amino-terminal regions of simian virus 40 large tumor antigen

(peptide synthesis/adenovirus 2-simian virus 40 hybrid viruses/small tumor antigen)

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**ABSTRACT** Antibodies specific for the amino- and carboxy-terminal portions of simian virus 40 large tumor (T) antigen were obtained by immunization of rabbits with synthetic peptides corresponding to these regions. The amino-terminal synthetic peptide has the sequence Ac-Met-Asp-Lys-Val-Leu-Asn-Arg-(Tyr). The tyrosine residue was introduced in order to couple the peptide to bovine serum albumin with bis-diazotized benzidine. The carboxy-terminal peptide has the sequence Lys-Pro-Pro-Thr-Pro-Pro-Glu-Pro-Glu-Thr. It was coupled to bovine serum albumin with glutaraldehyde. The antisera against both peptides reacted with large T antigen. The specificity of the immune reaction was demonstrated by inhibition experiments using excess synthetic peptides. Furthermore, fragments of T antigen encoded by the nondefective adenovirus 2-simian virus 40 hybrid viruses Ad2<sup>+</sup>ND2 and Ad2<sup>+</sup>ND4, which contain the carboxy terminus and lack the amino terminus of large T antigen, were precipitated only with antiserum to the carboxy-terminal peptide. Small T antigen was not precipitated with either serum, suggesting that the amino terminus of small T antigen has a conformation different from that of large T antigen or that it is sterically hindered by a host protein. The procedures used here are of general importance for identification and characterization of gene products.

The early region of the simian virus 40 (SV40) genome encodes two structurally related proteins, large tumor (T) antigen of apparent molecular weight 95,000 (1-5) and small T antigen of apparent molecular weight 17,000 (4). Large T antigen is encoded by two discontinuous sections from 0.65 to 0.59 and from 0.54 to 0.17 map units on the genome; small T antigen maps between 0.65 and 0.54 units (6, 7). Thus, both proteins share a common amino-terminal sequence (8-10), but they have unique carboxy-terminal regions. Both antigens have been identified and characterized with antisera from animals bearing SV40-induced tumors. These antisera are not specific in that they contain antibodies against both antigens. Antisera specific for either large or small T antigen have not been described. They would be useful, however, for analyzing the cellular localization of the antigens and for studies of structure-function relationships. One approach for obtaining such specific antibodies might be Köhler and Milstein's technique for the production of monoclonal antibodies by hybridomas (11). An alternative approach is to synthesize peptides corresponding to specific regions of large or small T antigen and to raise antibodies against these peptides. In principle, the latter approach became possible as a result of the recent determination of the entire DNA sequence of the SV40 genome (12, 13), from which the amino acid sequence of the antigens can be inferred. In this paper we describe the preparation of antibodies specific for the ends of large T antigen, with synthetic peptides as antigens.

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## MATERIALS AND METHODS

TC7 cells were used for infection with large-plaque SV40 and HeLa S3 cells for infection with adenovirus 2 (Ad2) and the nondefective Ad2-SV40 hybrid viruses Ad2<sup>+</sup>ND2 and Ad2<sup>+</sup>ND4. [<sup>35</sup>S]Methionine-labeled extracts were prepared and antibody-antigen complexes were precipitated by a *Staphylococcus aureus* preparation (Calbiochem) as described (5, 14). The NaDodSO<sub>4</sub>/polyacrylamide gel system of Laemmli (15) and Maizel (16) was used. Gels were prepared for fluorography as described by Laskey and Mills (17).

Solid-phase peptide synthesis was conducted by the Merrifield method (18). A complete description of the synthesis and isolation of the peptides will be published elsewhere. The amino acid analysis of the peptides is shown in Table 1. Peptide A, which corresponds to the amino-terminal segment of the T antigen sequence, was acetylated at the amino-terminal methionine with [<sup>14</sup>C]acetic acid. Peptide B was synthesized with [<sup>3</sup>H]proline adjacent to the amino-terminal lysine residue.

For immunization the peptides were coupled to bovine serum albumin as carrier. Peptide A was coupled with bis-diazotized benzidine as described by Bassiri *et al.* (19). Briefly, 50 mg of bovine serum albumin was dissolved in 10 ml of 0.16 M borate/0.13 M NaCl, pH 9.0, at 0°C; the peptide was added at a 40 times molar excess. To the bovine serum albumin/peptide solution was added 2 ml of bis-diazotized benzidine in 0.2 M HCl with stirring in an ice bath; the pH was adjusted by addition of 0.5 M NaOH and stirring was continued at 4°C for 2 hr. The conjugate was dialyzed against water at 4°C for 1 day and against 0.15 M NaCl for 1 day. Based on a binding efficiency

Table 1. Amino acid analysis of peptides A and B\*

Residue	Theoretical	Found†
Peptide A: Methionine	1	0.6
Aspartic acid	1	2.2‡
Asparagine	1	
Lysine	1	1.1
Valine	1	1.1
Leucine	1	1.1
Arginine	1	0.8
Tyrosine	1	1.2
Peptide B: Proline	6	6.4
Threonine	2	1.8
Glutamic acid	2	1.9
Lysine	1	0.9

\* See Fig. 1 for sequence.

† Molar ratio based on the average of 8 residues for peptide A and the average of 11 residues for peptide B.

‡ Combined value for aspartic acid and asparagine.

Abbreviations: T antigen, tumor antigen; SV40, simian virus 40; Ad2, adenovirus 2.

Peptide A: AcNH-Met-Asp-Lys-Val-Leu-Asn-Arg-Tyr-COOH  
 Peptide B: NH<sub>2</sub>-Lys-Pro-Pro-Thr-Pro-Pro-Glu-Pro-Glu-Thr-COOH

FIG. 1. Amino acid sequences of synthetic peptides. Peptide A: seven residues at the amino terminus of SV40 large and small T antigens. The eighth residue (tyrosine) was used for coupling the peptide to bovine serum albumin by diazotization. The same peptide was also synthesized with the glutamic acid residue that occurs naturally at position 8; in this case a water-soluble carbodiimide was used to couple the peptide to bovine serum albumin. In both cases the  $\alpha$ -amino group of methionine was acetylated. Peptide B: the undecapeptide corresponding to the 11 residues located at the carboxy terminus of SV40 large T antigen.

of 48% and a bovine serum albumin:peptide molar ratio of 1:40, it was calculated that, on the average, 19 peptide molecules were bound to each molecule of bovine serum albumin.

Peptide B was coupled with glutaraldehyde according to Kagan and Glick (20). Peptide was added at a 30 times molar excess to 26 mg of bovine serum albumin dissolved in 0.1 M sodium phosphate buffer (pH 7.5) to give a final volume of 2 ml. Glutaraldehyde (1 ml, 20 mM) was added dropwise with stirring, and the reaction was allowed to continue for 30 min at room temperature. The bovine serum albumin-peptide conjugate was separated on a Sephadex G-25 column. About 20% of the peptide was coupled (6 mol of peptide per mol of bovine serum albumin). The peptide-bovine serum albumin conjugates were analyzed by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis. The bovine serum albumin existed mainly in the monomeric form with an increased molecular weight due to the coupled peptides. In addition, higher molecular weight forms of bovine serum albumin were observed, indicating some covalent linkage between bovine serum albumin molecules.

Antisera were obtained by immunization of 5- to 6-month-old male rabbits with 1 mg of peptide-bovine serum albumin conjugate, emulsified in 2 ml of complete Freund's adjuvant, by intradermal injections at 20 different sites. The animals were boosted 4 and 8 weeks after the first immunization by combined intramuscular and subcutaneous injections, and antisera were drawn 2 weeks thereafter.

## RESULTS

**Antisera Against Two Synthetic Peptides Precipitate Large T Antigen.** Fig. 1 shows the two different peptides that were synthesized, coupled to BSA, and injected into rabbits. Peptide A represents the amino-terminal seven amino acids of large and small T antigens plus a tyrosine residue—not part of T antigen at this position—that was added for coupling the peptide to bovine serum albumin with bis-diazotized benzidine. The

peptide was also synthesized in its "native" form, with the glutamic acid residue that occurs naturally at position 8. In this case the peptide was coupled to bovine serum albumin with a water-soluble carbodiimide. The amino-terminal methionines of the peptides were acetylated. Peptide B represents the presumed carboxy-terminal 11 amino acids of large T antigen. The synthetic peptide was coupled to bovine serum albumin via the amino-terminal lysine residue with glutaraldehyde. All peptides were made in a radioactive form in order to estimate the efficiency of the coupling reaction. The antisera against the peptide-bovine serum albumin conjugates were assayed for their content of antibodies against T antigen by using [<sup>35</sup>S]methionine-labeled extracts of SV40-infected TC7 cells. The immunoprecipitates were analyzed on NaDodSO<sub>4</sub>/polyacrylamide gels. Preimmune sera and extracts of labeled uninfected cells were used as controls. As shown in Fig. 2, antisera to peptides A and B precipitated large T antigen, whereas neither serum precipitated small T antigen. Even after very long exposure times of the autoradiographs, small T antigen was still not found in immunoprecipitates with antisera to peptide A. It can be estimated that under these conditions 5% of the ordinary level (Fig. 2, track l) of small T antigen would have been detectable. Furthermore, small T antigen was not precipitable even when 10 times the amount of antisera to peptide A was used.

**Specificity of the Antisera.** The amino acid sequence in T antigen recognized by the antibodies is the same as that of the peptide used for immunization. This was demonstrated by competition experiments shown in Fig. 3. Increasing the amounts of peptide in the extract decreased precipitation of large T antigen by the corresponding antiserum.

In previous studies one of us (G. W.) analyzed the proteins in cells infected with nondefective Ad2-SV40 hybrid viruses containing pieces of the SV40 genome covalently inserted into the Ad2 genome (21, 22). The SV40 DNA segment in the hybrid virus Ad2<sup>+</sup>ND2 encodes two proteins of 42,000 and 56,000 molecular weight, and that in Ad2<sup>+</sup>ND4 encodes proteins ranging in size from 42,000 to 94,000 daltons. Both genetic analysis of the hybrid virus DNA as well as tryptic peptide analysis of the proteins suggested that the SV40 DNA segments encode a family of overlapping proteins with a common carboxy terminus of large T antigen (5, 23-26). Accordingly, these proteins seemed appropriate for testing the specificity of the peptide antisera. As shown in Fig. 4, both the 42,000- and 56,000-dalton proteins of Ad2<sup>+</sup>ND2, as well as all SV40-specific proteins encoded by Ad2<sup>+</sup>ND4, were precipitated with antiserum to the carboxy-terminal peptide but not with antiserum to the amino-terminal peptide. A polypeptide of apparent

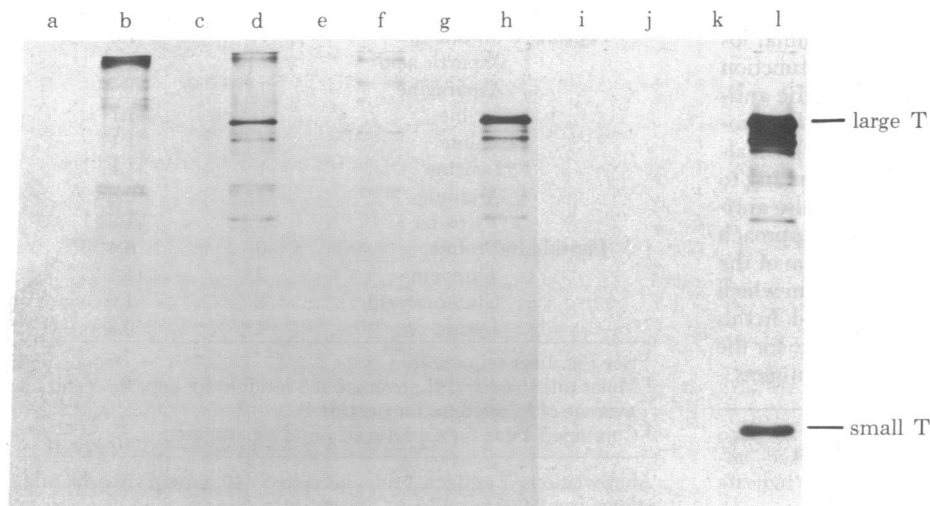


FIG. 2. Immunoprecipitation of large T antigen with antisera against synthetic peptides. [<sup>35</sup>S]Methionine-labeled extracts of mock-infected TC7 cells (tracks a, b, e, f, i, and j) or SV40-infected TC7 cells (tracks c, d, g, h, k, and l) were incubated with antiserum against peptide A (tracks b and d) or peptide B (tracks f and h) or with hamster SV40 tumor serum (tracks j and l). Tracks a, c, e, g, i, and k are from incubations with preimmune sera. Antigen-antibody complexes were precipitated with *Staphylococcus aureus* and analyzed on 12.5% polyacrylamide gels. All immunoprecipitations described in this paper were carried out with 25  $\mu$ l of extract from approximately  $5 \times 10^4$  cells and 10  $\mu$ l of undiluted serum.

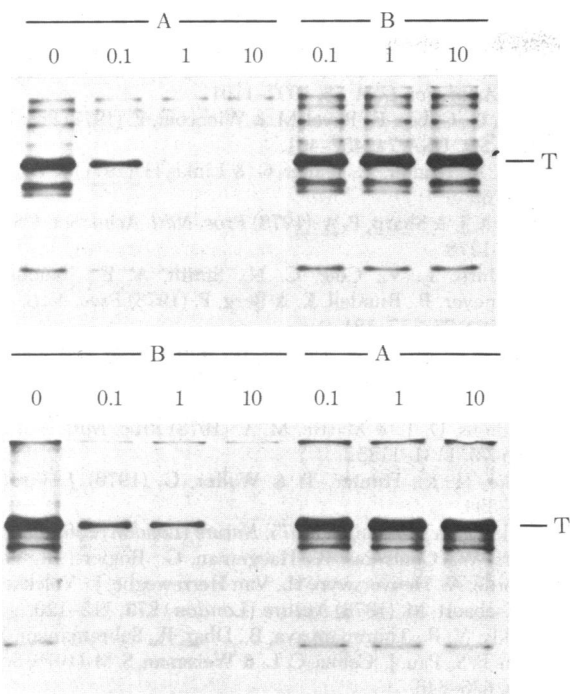


FIG. 3. Inhibition of immunoprecipitation of large T antigen with synthetic peptides. Large T antigen was immunoprecipitated from [<sup>35</sup>S]methionine-labeled extracts of SV40-infected TC7 cells with antiserum to peptide A (Upper) or to peptide B (Lower) in the presence of various amounts (0.1–10 μg) of peptide A or B. Precipitates were analyzed on a 12.5% polyacrylamide gel. The lower portions of the gels do not contain radioactive bands and are not shown. The fastest migrating protein seen has an apparent molecular weight of 45,000 and presumably represents VPI.

molecular weight 120,000 appearing nonspecifically in the immunoprecipitates from all infected cell extracts represents adenovirus hexon. The polypeptide larger than hexon appearing specifically in immunoprecipitates of Ad2+ND4-infected cell extracts with antiserum to peptide B and hamster tumor serum has not been further characterized.

### DISCUSSION

The goal of the present investigation was to produce antisera against SV40 large T antigen by using synthetic peptides corresponding to specific regions of the molecule as antigens. Previous studies have demonstrated that immunizations with peptides conjugated to protein carriers may induce antibodies capable of reacting not only with the peptides themselves but also with the proteins from which the peptides were derived (27–30). Indeed, studies on the antigenic nature of myoglobin and other proteins have demonstrated that antigenic regions usually consist of only a few amino acids (31). Often these involve polar residues that result in a high affinity between the antibody and the antigen. Moreover, polar residues are usually located on the surface of native molecules, a prerequisite for interaction with antibodies. Antigenic regions usually have a unique conformation in proteins (28, 32), whereas peptides, on the other hand, may assume various conformations. As a result, when using peptides as antigens, even though they are attached to protein carriers, one is likely to obtain antibodies with specificities for various conformational states of the peptide. Presumably only a fraction of the antibodies (namely, that directed against the same conformational state in the peptide and the protein) ought to recognize the protein.

We here report the production of antibodies to synthetic peptides corresponding to the amino-terminal heptapeptide and the carboxy-terminal undecapeptide of large T antigen.

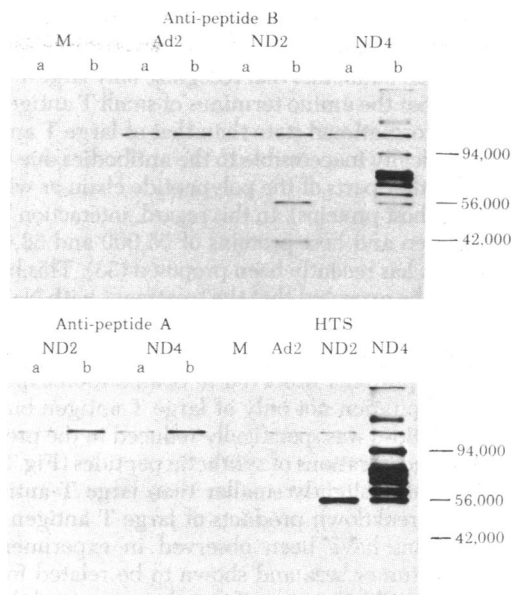


FIG. 4. NaDodSO<sub>4</sub>/polyacrylamide (12.5%) gel electrophoresis of immunoprecipitates of T antigen fragments encoded by hybrid viruses. [<sup>35</sup>S]Methionine-labeled extracts of mock-infected (M) and Ad2-, Ad2+ND2-, and Ad2+ND4-infected cells were immunoprecipitated with antisera to peptide A or peptide B, as well as with antisera from hamsters bearing SV40-induced tumors (HTS, hamster tumor serum). Tracks a represent precipitates from pre-immune sera; tracks b have the precipitates from the peptide antisera. The lower portions of the gels are not shown. Note that antibodies to peptide B (COOH-terminal) precipitate the same components in the hybrid virus system as do hamster tumor serum antibodies, but antibodies to peptide A (NH<sub>2</sub>-terminal) do not. Size in daltons is shown.

The antibodies produced were capable of reacting with the native protein. We chose small regions from the ends because they fulfill the above-mentioned criteria of known antigenic regions. In addition, they may be conformationally less restricted in the native protein than other parts of the molecule and, therefore, might be recognized by a larger fraction of the antibodies to the peptides. Also, it is possible to attach the peptides to carriers in a manner that best simulates the circumstance in the native protein. Thus, carboxy-terminal segments can be attached by their amino termini and amino-terminal peptides by their carboxy termini.

The common amino acid sequence of the amino termini of large and small T antigens, as predicted from the DNA sequence, has been confirmed by biochemical studies and determination of amino acid sequences (8). This was not the case, however, for the amino acid sequence of the carboxy terminus of large T antigen, which has only been predicted from the DNA sequence. The synthesis of the predicted carboxy-terminal peptide and the production of antibodies to it that are capable of recognizing the carboxy terminus of T antigen indirectly confirm that the DNA sequence and the presumed reading frame for this region are correct. Furthermore, precipitation of the SV40 proteins from hybrid virus-infected cells by antibodies against the carboxy-terminal peptide proves that these proteins do indeed share the carboxy-terminal sequence of large T antigen. These results also demonstrate that all T antigen fragments, despite their great variation in size, are folded in a way that leaves their carboxy termini exposed and accessible to antibodies. It will be of interest to discover if this unusual proline-rich sequence has any direct bearing on the function of large T antigen.

It is surprising that the antisera against the amino-terminal peptide from all immunized rabbits reacted with large T antigen, whereas none recognized small T antigen. Four other

rabbits, immunized with another form of this peptide (the naturally occurring glutamic acid instead of tyrosine at position 8), also produced antibodies that recognize only large T antigen. It is possible that the amino terminus of small T antigen is in a different conformational state than that of large T antigen or it may be sterically inaccessible to the antibodies due to interactions with other parts of the polypeptide chain or with other proteins (e.g., host proteins). In this regard, interaction between small T antigen and host proteins of 55,000 and 32,000 molecular weight has recently been proposed (33). This being the case, it might be expected that the treatment with NaDodSO<sub>4</sub> or reducing agents (or both) would render small T antigen accessible to the antibodies.

We have repeatedly observed in competition experiments that the precipitation not only of large T antigen but also of other polypeptides was specifically reduced in the presence of increasing concentrations of synthetic peptides (Fig. 3). Some of these proteins, slightly smaller than large T antigen, are presumably breakdown products of large T antigen because similar proteins have been observed in experiments with hamster anti-tumor sera and shown to be related to large T antigen by peptide mapping. If they have retained the appropriate end group, they should be precipitated by the antibodies to the peptides, but some proteins that may be structurally unrelated to large T antigen also appear to be coprecipitated with it as an association complex. This seems to be the case for a protein of molecular weight 45,000 that comigrates with the major capsid protein VP1 and most likely is VP1. Whether this association with large T antigen is fortuitous remains to be seen. It is possible that a fraction of large T antigen present in the extract is associated with a nucleoprotein complex containing SV40 DNA and VP1 (34, 35). It is interesting that the antisera to the peptides always precipitated less large T antigen than the hamster anti-tumor sera, even when used at higher concentrations than in the experiments described here. This result suggests that the end groups may be exposed in only a fraction of the large T antigen molecules, either because the protein is present in the extract in various conformations or because it is associated in part with cellular components such as proteins or nucleic acids. Antisera from hamsters with SV40-induced tumors are presumably less discriminating and recognize several antigenic determinants in various parts of the molecule.

The primary reason for generating the site-specific antibodies reported in this study derives from their potential use for analyzing the structure and function of the SV40 T antigens. Although antibodies to SV40 T antigen have recently been produced with hybridomas (36), the synthetic approach has the distinct advantage that the antibodies are directed against regions chosen by the investigator rather than the immune system. Moreover, it will not be an easy task to localize the antigenic determinants recognized by the monoclonal antibodies. Beyond that, the rationale of the experiments described in this paper has general applicability for the identification, isolation, and characterization of unknown proteins whose genes have been isolated and sequenced. Given the current speed of DNA sequencing, the number of such putative gene products needing direct characterization of this sort will rapidly increase.

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1. Tegtmeyer, P. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 9-15.
2. Carroll, R. B. & Smith, A. E. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2254-2258.
3. Ahmad-Zadeh, C., Allet, B., Greenblatt, J. & Weil, R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1097-1101.
4. Prives, C., Gilboa, E., Revel, M. & Winocour, E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 457-461.
5. Mann, K., Hunter, T., Walter, G. & Linke, H. (1977) *J. Virol.* **24**, 151-169.
6. Berk, A. J. & Sharp, P. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1274-1278.
7. Crawford, L. V., Cole, C. N., Smith, A. E., Paucha, E., Tegtmeyer, P., Rundell, K. & Berg, P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 117-121.
8. Paucha, E., Mellor, A., Harvey, R., Smith, A. E., Hewick, R. M. & Waterfield, M. D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2165-2169.
9. Simmons, D. T. & Martin, M. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1131-1135.
10. Linke, H. K., Hunter, T. & Walter, G. (1979) *J. Virol.* **29**, 390-394.
11. Köhler, G. & Milstein, C. (1975) *Nature (London)* **256**, 495-497.
12. Fiers, W., Contreras, R., Haegeman, G., Rogiers, R., Van de Voorde, A., Heuverswyn, H., Van Herreweghe, J., Volckaert, G. & Ysebaert, M. (1978) *Nature (London)* **273**, 113-120.
13. Reddy, V. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Celma, C. L. & Weissman, S. M. (1978) *Science* **200**, 636-646.
14. Walter, G. & Flory, P. J., Jr. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **44**, in press.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685.
16. Maizel, J. V., Jr. (1971) in *Methods in Virology*, eds. Maramorosch, K. & Koprowski, H. (Academic, New York), Vol. 5, pp. 179-246.
17. Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335-341.
18. Stewart, J. M. & Young, J. D. (1969) *Solid Phase Peptide Synthesis* (Freeman, San Francisco).
19. Bassiri, R. M., Dvorak, J. & Utiger, R. D. (1979) in *Methods of Hormone Radioimmunoassay*, eds. Jaffe, B. M. & Behrman, H. R. (Academic, New York), pp. 46-47.
20. Kagan, A. & Glick, M. (1979) in *Methods of Hormone Radioimmunoassay*, eds. Jaffe, B. M. & Behrman, H. R. (Academic, New York), pp. 328-329.
21. Walter, G. & Martin, H. (1975) *J. Virol.* **16**, 1236-1247.
22. Deppert, W., Walter, G. & Linke, H. (1977) *J. Virol.* **21**, 1170-1186.
23. Kelly, T. J., Jr. & Lewis, A. M. (1973) *J. Virol.* **12**, 643-652.
24. Lebowitz, P., Kelly, T. J., Jr., Nathans, D., Lee, T. N. H. & Lewis, A. M. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 441-445.
25. Morrow, J. F. & Berg, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3365-3369.
26. Morrow, J. F. & Berg, P., Kelly, T. J., Jr. & Lewis, A. M. (1973) *J. Virol.* **12**, 653-658.
27. Fearney, F. J., Leung, C., Young, J. D. & Benjamini, E. (1971) *Biochim. Biophys. Acta* **243**, 509-514.
28. Maron, E., Shiozawa, C., Arnon, R. & Sela, R. (1971) *Biochemistry* **10**, 763-771.
29. Furie, B., Schechter, A. N., Sachs, D. H. & Anfinsen, C. B. (1974) *Biochemistry* **13**, 1561-1566.
30. Young, N. S., Curd, J. G., Eastlake, A., Furie, B. & Schechter, A. N. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4759-4763.
31. Atassi, M. A. (1975) *Immunochemistry* **12**, 423.
32. Sachs, D. H., Schechter, A. N., Eastlake, A. & Anfinsen, C. B. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3790-3794.
33. Yang, Y.-C., Hearing, P. & Rundell, K. (1979) *J. Virol.* **32**, 147-154.
34. Mann, K. & Hunter, T. (1979) *J. Virol.* **29**, 232-241.
35. DePamphilis, M. L., Beard, P. & Berg, P. (1975) *J. Biol. Chem.* **250**, 4342-4347.
36. Martinis, J. & Croce, C. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2320-2323.