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Antibodies specific for the polyoma virus middle-size tumor antigen

(transformation/protein kinase/immunoprecipitation/antipeptide serum)

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ABSTRACT **We have obtained antibodies specific for the polyoma virus middle-size tumor antigen (middle T antigen) by immunizing rabbits with a synthetic peptide, Lys-Arg-Ser-Arg-His-Phe, corresponding to the six carboxy-terminal amino acids of the middle T antigen predicted from the nucleotide sequence of polyoma DNA. The antipeptide serum precipitates the polyoma middle T antigen but not the small or large tumor antigens, and precipitation is inhibited in the presence of the peptide. Two cellular proteins, 30,000 and 26,000 daltons, are also precipitated specifically by the antipeptide serum and may have amino acid sequences related to the peptide. Two other cellular proteins, 33,000 and 25,000 daltons, are precipitated only in the presence of the peptide and may associate with it in cell extracts. Antisera directed against synthetic peptides are likely to be important in various ways, including the production of antibodies directed against particular determinants and the recognition of unknown proteins whose genes have been analyzed.**

The early region of the polyoma virus genome encodes three proteins, called the large, middle, and small tumor antigens (T antigens) with M_rs of approximately 89,000, 48,000, and 22,000, respectively (1, 2). The coding regions for the three proteins overlap: the three proteins have common NH_2-terminal regions encoded between 74 and 79 map units, and the small and middle T antigens have common sequences encoded between 79 and 85 map units that are not present in the large T antigen. The large and middle T antigens have unique sequences encoded between 86 and 99 map units, translated in different reading frames from the viral DNA (1, 2). In addition, the large T antigen has unique sequences encoded between 99 and 26 map units.

Mutations affecting the T antigens suggest that the T antigens are involved in cell transformation. The temperature-sensitive tsA mutations, which affect the large T antigen, block the ability of the mutant virus to transform at the nonpermissive temperature (3, 4). The host range nontransforming (hr-t) mutations, which affect the small and middle T antigens coordinately, render the mutant virus unable to transform infected cells (5–7). Viable deletion mutations have been located in the common coding region for the large and middle T antigens between 86 and 99 map units. These mutations in some cases affect the frequency and expression of transformation (8, 9).

The role of the T antigens in cell transformation is difficult to define precisely because the proteins may have more than a single function and because the transformed phenotype is complex. Nevertheless, several lines of evidence indicate that the small T antigen alone is insufficient to cause transformation and that an intact large T antigen, although required for some initial event during transformation, is not required for continuous expression of the transformed phenotype (7, 10). Therefore, attention has focused recently on the middle T antigen as the viral protein potentially most directly involved in altered cell growth control (11).

One approach to understanding the role of the individual T antigens in cell transformation is to prepare antisera that are specific for the individual proteins. We have used a synthetic peptide, corresponding to the six amino acid residues at the carboxy terminus of the middle T antigen, to prepare an antiserum specific for this antigen. A similar approach was used previously (12) to prepare antisera specific for the amino- and carboxy-terminal regions of the simian virus 40 large T antigen (12). The nucleotide sequence of polyoma DNA has been used to deduce the amino acid sequence of the virus-encoded proteins (13, 14). The middle T antigen has a number of interesting features, including a sequence of hydrophobic amino acids near the carboxy terminus similar to sequences involved in the association of other proteins with membranes. We chose to use the carboxy-terminal hexapeptide Lys-Arg-Ser-Arg-His-Phe, which lies just beyond the hydrophobic region because it has the characteristics of a good antigen (it is hydrophilic and located at the end of the polypeptide chain) and because it might provide a means of studying the portion of the protein adjacent to the membrane.

In the present report we describe the preparation of the antiserum and its ability to precipitate viral and cellular proteins.

MATERIALS AND METHODS

Cell Culture and Virus Infection. Mouse 3T6 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. For infection by polyoma virus, the cells were seeded on 5-cm tissue culture dishes at 4×10^5 cells per dish. The cultures were infected on the following day with polyoma at a multiplicity of approximately 20 plaque-forming units/cell. After infection, the cells were covered with fresh medium and incubated at 37°C prior to radiolabeling or harvesting.

T Antigen Analysis. Approximately 24–27 hr after infection, infected cell cultures were radiolabeled with [^{35}S]methionine (≈1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; Amersham) at 100 μCi/ml in medium lacking methionine but supplemented with 5% dialyzed calf serum, for 3 hr. Cell extracts were prepared, immunoprecipitated, and analyzed by polyacrylamide gel electrophoresis as described (4). Assays of nonradioactive T antigen immunoprecipitates for the presence of an activity capable of transferring a phosphate residue from [^{32}P]ATP to protein were also performed as described (15).

Preparation of Antiserum. The peptide Lys-Arg-Ser-Arg-His-Phe, corresponding to the six carboxy-terminal residues of the polyoma middle T antigen, were purchased from Bachem (Bubendorf, Switzerland). According to the manufacturer, the

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Abbreviation: T antigen, tumor antigen.

peptide was 95% pure as judged by high-pressure liquid chromatography analysis. The peptide was coupled (with glutaraldehyde) to bovine serum albumin, as described (12). The conjugate was emulsified in complete Freund's adjuvant and injected intradermally into a rabbit (1 mg per rabbit). An intramuscular injection was given 4 weeks after the first immunization, and serum was collected for analysis 2 weeks after that (12). The serum was fractionated by affinity chromatography on a 0.5 × 6 cm column containing 20 mg of peptide coupled to 2 g of CH-Sepharose 4B by the carbodiimide coupling procedure recommended by the manufacturer (Pharmacia). Ten milliliters of serum was adsorbed to bovine serum albumin-linked Sepharose, dialyzed against 0.02 M Tris·HCl, pH 8/0.029 M NaCl, and applied to a DEAE Affi-Gel Blue column (Bio-Rad). The excluded IgG fraction was concentrated in a Millipore immersible CX ultrafiltration unit. The IgG fraction was then added slowly to the affinity column (2–3 ml/hr). The flow-through volume (10 ml, containing 9.6 A_{280} units) was saved for use as a control; the bound material was eluted with 0.1 M glycine (pH 2.5) for use as the specific serum. The eluted fractions were neutralized immediately by addition of crystals of Tris base. The bound IgG fraction was eluted in approximately 3 ml and contained about 2.5 A_{280} units.

RESULTS

Immunoprecipitation of Proteins by Antipeptide Serum. We prepared extracts of mouse 3T6 cells infected with wild-type polyoma virus or with a viable deletion mutant, dl 8. [The dl 8 mutation removes approximately 90 base pairs of DNA at about 90 map units on the polyoma genome and shortens the large and middle T antigens without affecting the small T antigen (8, 11).] The altered mobility of the middle T antigen synthesized by the dl 8 mutant makes it easier to identify the protein because the shortened T antigen migrates in a region of the gel where there are fewer background bands. The infected cells were radiolabeled with [^{35}S]methionine during the late phase of infection. Cell extracts were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis. Fig. 1 shows the comparison of rat antitumor antiserum with the antipeptide serum. The rat antitumor serum (4, 15) precipitated all three T antigens. The antipeptide serum precipitated the middle T antigen but not the large or small T antigen. Proteins corresponding to the T antigens were not precipitated from extracts of infected cells by control sera or from mock-infected cell extracts. The specificity of the interaction of the antipeptide serum with the middle T antigen is shown by the ability of added peptide to compete with the middle T antigen in the precipitation. Furthermore, two-dimensional tryptic peptide maps of the [^{35}S]methionine-labeled middle T antigens precipitated by the antitumor serum and by the antipeptide serum were the same (not shown).

Two other proteins in the extracts appeared to be precipitated specifically by the antipeptide serum. These are designated 30 and 26 kilodaltons (for their apparent sizes) in Fig. 1. These proteins were precipitated by the antipeptide serum from both mock-infected and virus-infected cell extracts but not by the rat antitumor serum or by either of the control sera. The precipitation of these proteins, like that of the middle T antigen, was inhibited by added peptide, whereas the precipitation of other proteins (considered to be nonspecific background) was not affected.

Fig. 1 also shows that two proteins, M_r 33,000 and 25,000, were precipitated from infected cell extracts by the antipeptide serum only in the presence of added peptide. These proteins also were precipitated from mock-infected cell extracts in the presence of added peptide but were not precipitated from either

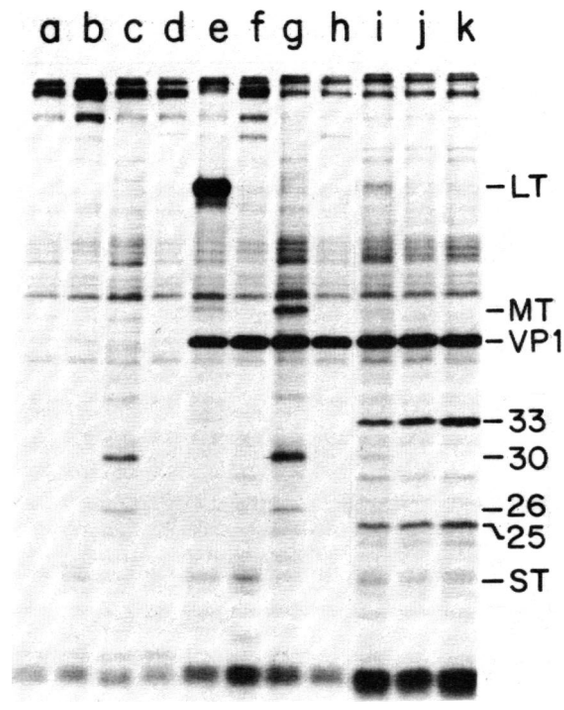

FIG. 1. Immunoprecipitation of polyoma T antigen and cellular proteins. Mouse 3T6 cells were mock-infected (lanes a–d) or infected with the polyoma deletion mutant dl 8 (lanes e–k) and radiolabeled 24–27 hr later. Extracts (10^6 cells per ml of 0.15 M NaCl/10 mM sodium phosphate, pH 7.0/1% sodium deoxycholate/ 1% Nonidet P-40/ 0.1% $NaDodSO_4$/1% Trasylol) were precipitated with: 10 μl of rat antitumor serum (lanes a and e); 10 μl of preimmune rat serum (lanes b and f); 4 μl of antipeptide serum (c and g); 4 μl of control serum (the flow-through from the affinity column) (lanes d and h); or 4 μl of affinity-purified antipeptide serum in the presence of 0.1, 1, and 10 μg of peptide (lanes i, j, and k). LT, large T antigen; MT, middle T antigen; ST, small T antigen. Numbers are molecular mass in kilodaltons.

extract by antitumor or control sera (Fig. 2; other data not shown). These results suggest that the M_r 33,000 and 25,000 proteins may associate with the added peptide and be precipitated with it.

Similar results were obtained when extracts of cells infected with wild-type polyoma were used (Fig. 2). The antipeptide serum precipitated the wild-type middle T antigen as well as the M_r 30,000 and 26,000 proteins and this was competitively inhibited by added peptide. The M_r 33,000 and 25,000 proteins which were precipitated only in the presence of added peptide also were present in mock-infected cell extracts, but were not precipitated by control serum.

Precipitation of an Activity Phosphorylating the Middle T Antigen *in Vitro*. An intriguing property of the polyoma middle T antigen is its association with a protein kinase-like activity (15–17) which phosphorylates a tyrosine residue on the middle T antigen in immunoprecipitates (15). Whether this activity is a property of the middle T antigen or is a cellular protein associated with the middle T antigen is not clear. We tested whether the phosphorylating activity associated with the middle T antigen is found in immunoprecipitates formed with the antipeptide serum.

When incubated with [γ-^{32}P]ATP, immunoprecipitates made with antitumor serum and extracts of cells infected by wild-type polyoma or by the deletion mutant dl 8 showed phosphorylation of the middle T antigen (Fig. 3) as reported (15). Also evident were a number of other phosphorylated proteins, including large T antigen, that were lacking from immunoprecipitates

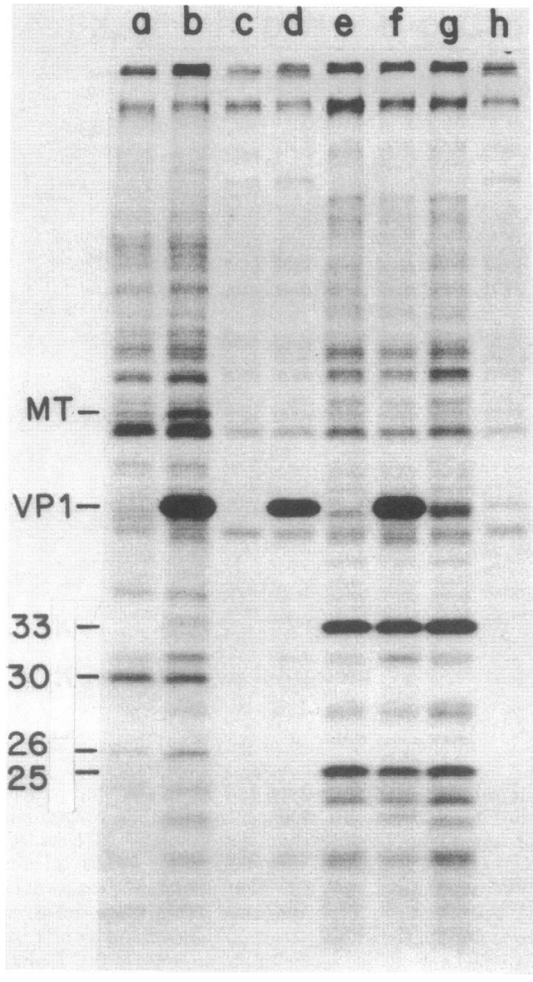

FIG. 2. Immunoprecipitation of polyoma wild-type or dl 8-infected cell extracts by antipeptide serum. Mouse 3T6 cells were infected, radiolabeled, and extracted as in Fig. 1. Extracts (200 μl) of mock-infected cells (lanes a, c, and e), wild-type infected cells (lanes b, d, and f), or dl 8-infected cells (lanes g and h) were precipitated with 4 μl of antipeptide serum (lanes a and b), 4 μl of control serum (lanes c and d), 4 μl of antipeptide serum in the presence of 10 μg of added peptide (lanes e, f, and g), or 4 μl of control serum in the presence of 10 μg of added peptide (lane h). MT, middle T antigen.

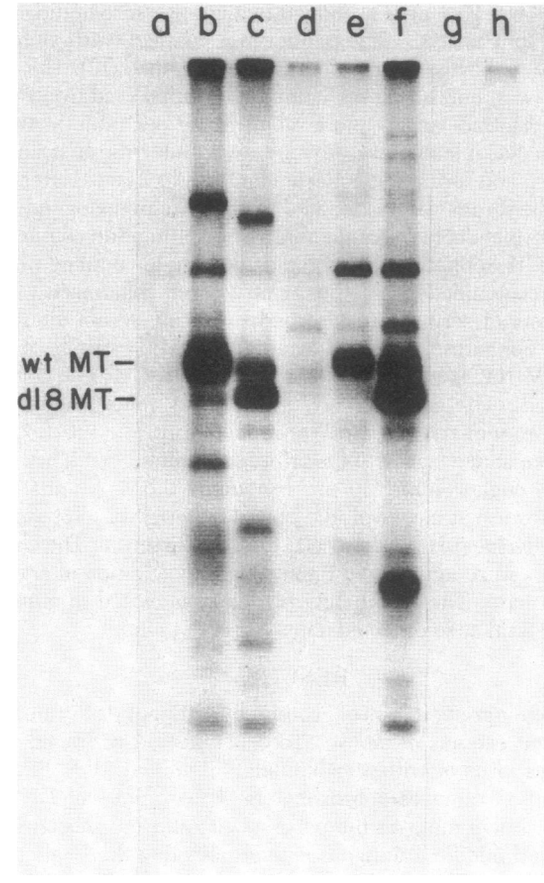

FIG. 3. Immunoprecipitation of protein kinase activity by antipeptide serum. Extracts of mouse 3T6 cells mock-infected (lanes a, d, and h), wild-type polyoma-infected (lanes b and e), or dl 8-infected (lanes c, f, g, and i) were prepared 27 hr after infection and precipitated with 10 μl of rat antitumor serum (lanes a, b, and c), 4 μl of control serum (lanes h and i), 4 μl of antipeptide serum (lanes d, e, and f), or 4 μl of antipeptide serum in the presence of 10 μg of added peptide (lane g). The immunoprecipitates were incubated with [γ-^{32}P]ATP before gel electrophoresis.

made from mock-infected cells. The large T antigen bands were readily identifiable because the large T antigen of the dl 8 deletion mutant migrates slightly faster than that of wild-type polyoma. Immunoprecipitates made with antipeptide serum from parallel infected cell extracts also showed phosphorylation of the middle T antigen when incubated with [γ-^{32}P]ATP. As with antitumor serum, other phosphorylated proteins were present; some were seen at lower levels in immunoprecipitates of mock-infected cell extracts. We assume that most of these proteins are precipitated nonspecifically and can act as substrates for the protein kinase associated with the middle T antigen. The immunoprecipitate made with antipeptide serum from dl 8-infected cells contained a unique phosphorylated band of M_r about 30,000. On the basis of preliminary peptide mapping studies, this M_r 30,000 band appears to be a fragment of the dl 8 middle T antigen.

Phosphorylation of the middle T antigen was abolished when peptide was present during the immunoprecipitation (Fig. 3). The phosphorylation of the other proteins also was diminished, even though the nonspecifically precipitated bands, as monitored by [^{35}S]methionine labeling, still were present in the immunoprecipitates made in the presence of peptide (see Fig. 1). This suggests that the major protein kinase activity in immunoprecipitates of polyoma T antigens is associated with the middle T antigen.

The amino acid phosphorylated in the middle T antigen in immunoprecipitates made with the antipeptide serum is tyrosine (data not shown), as we previously found for immunoprecipitates made with antitumor serum (15). Furthermore, the same phosphotyrosine-containing tryptic peptide was present in the two preparations of middle T antigen.

DISCUSSION

The results presented here show that an antiserum directed against a synthetic peptide corresponding to the six carboxy-terminal amino acid residues of the polyoma middle T antigen will immunoprecipitate the middle T antigen but not the large or small T antigens. The serum should prove useful for the affinity purification of the middle T antigen. For this purpose, an advantage of an antipeptide serum over a normal monospecific serum is that, as is the case for monoclonal antibodies, only a single antigenic site per molecule is recognized. The antipeptide serum has an additional advantage over monoclonal antibodies, however, because in principle the antigen can be dissociated from the antibody–antigen complex by competition

with soluble peptide. Preliminary experiments indicate that at least partial release of the middle T antigen from immunoprecipitates can be achieved in this manner. Such an elution procedure is likely to be considerably less denaturing than the conventional methods which use chaotropic agents or extremes of pH.

The antipeptide serum should also be useful for localizing the middle T antigen in infected or transformed cells by immunofluorescence, provided that cellular proteins reactive with the antipeptide serum do not interfere with the assay. The specificity of the immunofluorescence can be ascertained by competition with soluble peptide. We have localized the transforming protein of Rous sarcoma virus in transformed cells by immunofluorescence with another antipeptide serum prepared against the carboxy-terminal hexapeptide of the transforming protein of the virus (unpublished data). Finally, because the antipeptide serum is directed against an amino acid sequence located adjacent to a stretch of hydrophobic residues potentially involved in the membrane association of the middle T antigen, it may be possible to use the serum to study the orientation of the protein in the cell membrane.

The present study was conducted with antibodies purified by affinity chromatography rather than with unfractionated serum. This has several advantages. First, the background of the immunoprecipitates is lower. Second, the unbound IgG fraction of the affinity column (being derived from the same immunized animal as the specific fraction) is an ideal control for the immunoprecipitation experiments. Third, the isolation procedure presumably selects for antibodies of high affinity and specificity. The serum used in the present study was obtained from a single immunized rabbit. In this serum, 20% of the total IgG was bound and eluted from the peptide affinity column. This binding appeared to be specific and not simply an ionic interaction with the peptide because the amount bound was not decreased by a 0.5 M salt wash. The high proportion of specific IgG indicates that the peptide elicits a strong immune response. The serum from a second rabbit immunized with the same peptide-conjugate also showed a high level of specific IgG. This second affinity-purified antipeptide serum also precipitated middle T antigen in a specific fashion.

The middle T antigen of polyoma is associated with a protein kinase activity in immunoprecipitates of the polyoma T antigens made with antitumor serum. The results presented here show that a similar activity is present in immunoprecipitates made with a serum directed against the carboxy terminus of the middle T antigen. Such immunoprecipitates do not contain the large or small T antigens, which shows that neither of these proteins is necessary for the phosphorylating activity, confirming previous results (15–17). The protein kinase activity observed may be an intrinsic property of the middle T antigen or may reflect a tightly associated cellular enzyme. Our results show that antibody bound to the carboxy terminus of the middle T antigen does not affect the activity. In principle, however, use of antisera directed against peptides corresponding to other parts of the middle T antigen might allow one to distinguish between the two possibilities.

Two cellular proteins, M_r 30,000 and 26,000, are specifically recognized by the antipeptide serum. The most likely reason for the recognition is that one or both of these proteins contains a sequence related to that of the carboxy-terminal hexapeptide of the middle T antigen. We do not know whether the antibodies present in the antipeptide serum recognize every residue in the hexapeptide or whether the antigenic determinants are provided by only some of the amino acids. If the latter were true, then a related but not identical sequence might be present in the M_r 30,000 and 26,000 proteins. If the hexapeptide were the only region of homology among the three proteins it would be hard to determine this by comparative structural analysis. However, it is possible that the M_r 30,000 and 26,000 proteins might possess more extensive regions of homology with the middle T antigen. Therefore, we have begun analysis of these proteins by tryptic peptide mapping to see whether any obvious homology exists. The difficulties inherent in analyzing sequence homology by peptide mapping are well exemplified by comparison of the large T antigens of simian virus 40 and polyoma. There are no methionine-containing tryptic peptides in common between the two proteins, yet it is clear from the nucleotide sequence of the two viruses that there is in fact extensive homology between the two antigens. Preliminary results with tryptic peptide mapping indicate that the M_r 30,000 and 26,000 proteins are not closely related to one another or to the middle T antigen. More complete analysis is clearly desirable because of the possibility that normal cells may contain proteins related to the viral transforming proteins.

Nucleic acid hybridization experiments have not revealed any relatedness between the polyoma genome and cellular DNA, yet homology might exist at the protein level. The region corresponding to the carboxy-terminal domain of the middle T antigen of polyoma has no counterpart in simian virus 40, although the rest of their genomes are closely related (13, 14). This region may have been acquired more recently in evolutionary terms and might be more closely related to cellular sequences.

Two other cellular proteins, M_r 33,000 and 25,000, are immunoprecipitated in the presence of peptide. The nature of this association should be explored further because it might suggest ways in which the carboxy-terminal region of the middle T antigen could be associated with other proteins. The specific associations of cellular proteins with the large and small T antigens of cellular proteins with the large and small T antigens of simian virus 40 provide a precedent for this type of interaction (18, 19).

The experiments described here were based on previous experiments with simian virus 40 in which synthetic peptides were used to prepare antisera specific for the amino- and carboxy-terminal regions of its large T antigen (12). We have also used synthetic peptides to prepare antibodies that will precipitate $pp60^{src}$, the transforming protein of Rous sarcoma virus (unpublished data). Synthetic peptides have also been used to prepare antisera directed against putative retroviral proteins (20) and to immunize animals against a region of the diptheria toxin molecule (21). Therefore, the method appears likely to be applicable in many cases in which the amino acid sequence of a protein is either known or predictable, and it may be especially useful for immunizing animals against specific portions of proteins. Synthetic peptides also may be useful as immunogens for the production of vaccines against viruses.

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1. Ito, Y. (1980) in *Viral Oncology*, ed. Klein, G. (Raven, New York), pp. 447–480.
2. Türler, H. (1980) *Mol. Cell. Biochem.* **32**, 63–93.
3. Fried, M. (1965) *Proc. Natl. Acad. Sci. USA* **53**, 486–491.
4. Hutchinson, M. A., Hunter, T. & Eckhart, W. (1978) *Cell* **15**, 65–77.
5. Benjamin, T. (1970) *Proc. Natl. Acad. Sci. USA* **73**, 394–399.
6. Staneloni, R., Fluck, M. & Benjamin, T. (1977) *Virology* **77**, 598–609.
7. Fluck, M. M. & Benjamin, T. L. (1979) *Virology* **96**, 205–228.
8. Griffin, B. E. & Maddock, C. (1979) *J. Virol.* **31**, 645–656.
9. Magnusson, G. & Berg, P. (1979) *J. Virol.* **32**, 523–529.

10. Lania, F., Gandini-Attardi, D., Griffiths, M., Cooke, B., DeCiccu, D. & Fried, M. (1980) *Virology* **101,** 217–232.
11. Ito, Y., Spurr, N. & Griffin, B. E. (1980) *J. Virol.* **35,** 219–232.
12. Walter, G., Scheidtmann, K. -H., Carbone, A., Laudano, A. P. & Doolittle, R. F. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5197–5200.
13. Deininger, P., Esty, A., LaPorte, P., Hsu, H. & Friedmann, T. (1980) *Nucleic Acids Res.* **8,** 856–860.
14. Soeda, E., Arrand, J. R., Smoler, N., Walsh, J. E. & Griffin, B. E. (1980) *Nature (London)* **283,** 445–453.
15. Eckhart, W., Hutchinson, M. A. & Hunter, T. (1979) *Cell)* **18,** 925–933.
16. Smith, A. E., Smith, R., Griffin, B. & Fried, M. (1979) *Cell* **18,** 915–924.
17. Schaffhausen, B. & Benjamin, T. (1979) *Cell* **18,** 935–946.
18. Lane, D. P. & Crawford, L. V. (1979) *Nature (London)* **278,** 261–263.
19. Yang, Y. -C., Hearing, P. & Rundell, K. (1979) *J. Virol.* **32,** 147–154.
20. Sutcliffe, J. G., Shinnick, T. M., Green, N., Liut, F. -T., Himan, H. L. & Lerner, R. A. (1980) *Nature (London)* **287,** 801–805.
21. Audibert, F., Jolivet, M., Chedid, L., Alouf, J. E., Boquet, P., Riveille, P. & Siffert, O. (1981) *Nature (London)* **289,** 593–594.