ATP by the mitochondria. It is not known whether this results from the loss of some chemical coupling or from the swelling of the mitochondria that prevents passage through the matrix of the axoplasm.

The use of AVEC-DIC microscopy permits direct analysis of all the moving, membranous organelles for the first time. To our knowledge, no other microscopy system is available that can detect the full range of membranous organelles in living preparations. Removal of the plasma membrane and other permeability barriers by mechanical extrusion makes biochemical, pharmacological, and mechanical manipulations possible without the extraction of components with detergents. The resulting preservation of polypeptide composition and organization in the native state results in a greatly extended life span for the model. Therefore, this model fulfills all the criteria for a study of the molecular mechanisms of intracellular transport of membranous organelles.

SCOTT T. BRADY
RAYMOND J. LASEK
Department of Anatomy,
Case Western Reserve Medical School,
Cleveland, Ohio 44106
ROBERT D. ALLEN
Department of Biological Sciences,
Dartmouth College,
Hanover, New Hampshire 03755

References and Notes

1. B. Grafstein and D. Forman, *Physiol. Rev.* **60**, 1167 (1980).
2. S. T. Brady and R. J. Lasek, *Methods Cell Biol.* **24B**, 365 (1982).
3. R. S. Smith, *J. Neurocytol.* **9**, 39 (1980); S. Tsukita and H. Ishikawa, *J. Cell Biol.* **84**, 513 (1980).
4. R. D. Allen, N. S. Allen, J. L. Travis, *Cell Motil.* **1**, 291 (1981).
5. R. D. Allen, J. Metuzals, I. Tasaki, S. T. Brady, S. P. Gilbert, *Science* **218**, 1127 (1982).
6. R. D. Allen, J. Metuzals, I. Tasaki, *Biol. Bull. Woods Hole* **161**, 303a (1981); A. C. Breuer, R. D. Allen, L. J. Lewis, *Neurology* **31**, 118a (1981).
7. R. S. Bear, F. O. Schmitt, J. Z. Young, *Proc. R. Soc. Ser. B* **123**, 520 (1938).
8. J. R. Morris and R. J. Lasek, *J. Cell Biol.* **92**, 192 (1982); J. R. Morris, thesis, Case Western Reserve University (1981).
9. J. Metuzals, A. J. Hodge, R. J. Lasek, I. Kaiserman-Abramov, *Cell Tissue Res.*, in press.
10. H. D. Lux, P. Schubert, G. W. Kreutzberg, A. Globus, *Exp. Brain Res.* **10**, 197 (1970); A. Dahlstrom, *Phil. Trans. R. Soc. London Ser. B* **261**, 325 (1971); S. Ochs, *Science* **176**, 252 (1972). See also Grafstein and Forman (*1*) for further examples.
11. See, for example, D. S. Forman, *J. Cell Biol.* **91**, 414a (1981); M. Stearns and D. P. Boggs, *ibid.*, p. 420a; W. Z. Cande, K. McDonald, R. L. Meeusen, *ibid.* **88**, 618 (1981).
12. The ATP requirement for particle movement has been shown by Ochs and his associates [see S. Ochs, in *Basic Neurochemistry*, G. J. Siegel and R. W. Albers, Eds. (Little, Brown, Boston, 1981), pp. 425] and R. J. Adams [*Nature (London)* **297**, 327 (1982)].
13. Support for these studies was provided by NIH grants NS15731 and NS07118 (R.J.L. and S.T.B.) and by the National Institute of General Medical Science, GM27284 (R.D.A.).

10 August 1982

In vivo Identification of the Transforming Gene Product of Simian Sarcoma Virus

Abstract. *Simian sarcoma virus (SSV) deletion mutants were constructed from a molecular clone containing the entire infectious provirus. Transfection analysis of these mutants localized the SSV transforming gene to a small region of the viral genome encompassing its cell-derived sequence (v-*sis*). Antiserum to a peptide synthesized on the basis of the predicted amino acid sequence of the SSV transforming gene detected a 28,000-dalton protein that was specifically expressed in SSV transformed cells and that corresponded in size to that predicted from the v-*sis *coding sequence. The v-*sis *gene product designated p28^{sis} was not a phosphoprotein, nor did it possess detectable protein kinase activity. These findings distinguish p28^{sis} from a number of other retroviral* onc *proteins.*

Acute transforming retroviruses have been isolated from a number of vertebrate species. These viruses cause sarcomas or hematopoietic tumors, but in some cases induce carcinomas as well (*1*). Simian sarcoma virus (SSV) is the only known acute transforming retrovirus of primate origin (*2*). The isolation of biologically active molecular clones containing the intact, integrated SSV genome has made it possible to approach an understanding of its transforming mechanism. Studies to date have shown that the 5.1–kilobase pair (kbp) SSV genome arose by recombination of simian sarcoma associated virus (SSAV) with a 1-kbp segment (v-*sis*) derived from a woolly monkey cellular gene (*3–5*). By analogy with information available concerning the cell derived sequences of other transforming retroviruses, v-*sis* might be expected to play an important role in transformation induced by SSV. Nucleotide sequence analysis of v-*sis* has revealed a long open reading frame that could code for the SSV transforming protein (*6*). Our studies were undertaken to establish directly the role of v-*sis* in SSV induced transformation and to use knowledge of the primary nucleotide sequence of v-*sis* to identify the SSV transforming protein.

To localize the region of SSV required for transformation, we constructed various deletion mutants from a molecular clone of SSV DNA and tested their ability to transform NIH/3T3 cells in a transfection assay (*7*, *8*). The intact viral genome (pSSV-11) exhibited a transforming activity of $10^{4.1}$ focus-forming units (FFU) per picomole of viral DNA (Fig. 1). A subgenomic clone, pSSV 3/1, from which the 3′ long terminal repeat (LTR) was deleted, showed no reduction

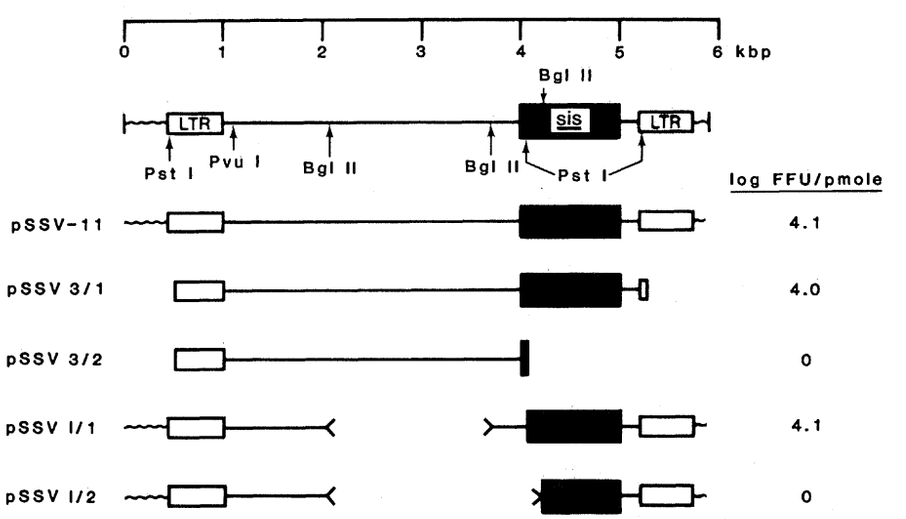

Fig. 1. Construction and biological analysis of SSV deletion mutants. The integrated form of SSV was excised from λ-SSV-11 Cl 1 (*3*) and purified by elution from a preparative agarose gel. Deletion mutants pSSV 3/1 and pSSV 3/2 were constructed by cloning products of a reaction in which purified SSV DNA was partially digested with Pst I. The intact viral genome, pSSV-11, was obtained by cloning the λ-SSV-11 Cl 1 insert at the Eco RI site of pBR322 (*30*). pSSV I/1 and pSSV I/2 were constructed by limited Bgl II digestion of pSSV-11 followed by religation. In each case, the structure of individual deletion mutants was determined by restriction enzyme and Southern blotting analysis. Transfection of NIH/3T3 cells with plasmids containing SSV wild-type or mutant DNA's was performed by the calcium phosphate precipitation technique (*7*) as modified by Wigler *et al.* (*8*). Transformed foci were scored at 14 to 21 days.

0036-8075/82/1210-1131$01.00/0

in biologic activity. In contrast, pSSV 3/2, a subclone that lacked the 3′ LTR as well as all but 82 base pairs (bp) of v-*sis*, demonstrated no detectable transforming activity. These results indicated that v-*sis* is essential in SSV transformation. The mutant pSSV I/1, which lacked an internal 1.8-kbp Bgl II fragment (Fig. 1), transformed NIH/3T3 cells with an efficiency of $10^{4.1}$ FFU per pmole of viral DNA. However, a subclone, pSSV I/2, which lacked an additional stretch of 345 bp of SSAV sequences as well as the first 250 bp of v-*sis* showed no transforming activity. These results localized the SSV transforming gene to a region encompassing v-*sis*, along with 345 and 305 bp of flanking SSAV sequences, to the left and right of v-*sis*, respectively.

Primary nucleotide sequence analysis of v-*sis* has revealed the presence of a long open reading frame starting 19 nucleotides upstream from the v-*sis*–SSAV junction and terminating within v-*sis* (Fig. 2). This potential coding sequence was included within the region shown to be required for SSV transformation. Peptides synthesized on the basis of a known genetic sequence can be used as haptens to elicit antibodies capable of recognizing its translational product (*9*, *10*). Thus, if the v-*sis* open reading frame is functional, antibodies prepared by this approach might be able to detect the v-*sis* gene product. We chose for synthesis a pentadecapeptide derived from the 5′ terminal region of v-*sis* (Fig. 2). Rabbits were immunized with 100 μg of the peptide, designated *sis*-N1, either alone or after it was conjugated with thyroglobulin (*11*). Thereafter, 100 μg of peptide was administered intraperitoneally at 14-day intervals. Animals were bled 1 week after each injection.

We monitored the effectiveness of the immune response by the ability of serum from sequential bleedings to precipitate the ^{125}I-labeled *sis*-N1 peptide. The titer for 20 percent precipitation increased to 1:2500 by the third immunization with the conjugated peptide, whereas the unconjugated peptide failed to elicit an immune response (titer < 1:20). Subsequent studies were therefore performed with antiserum to the conjugated peptide.

In an effort to detect an SSV translational product in SSV transformed cells, cultures were labeled for 3 hours with [^{35}S]methionine, and extracts were immunoprecipitated with preimmune serum or with antiserum to *sis*-N1. A protein with an apparent molecular size of 28,000 daltons (p28) was precipitated with the *sis*-N1 antiserum from SSV transformed marmoset cells (lane 2 in Fig. 3A) but not from uninfected cells (lane 1 in Fig. 3A). Thus, p28 seemed a suitable candidate for the SSV transforming protein.

An important test of the virus-coded nature of a protein is detection of the protein after virus infection of cells of distantly related species. We therefore examined the ability of our *sis*-N1 antise-

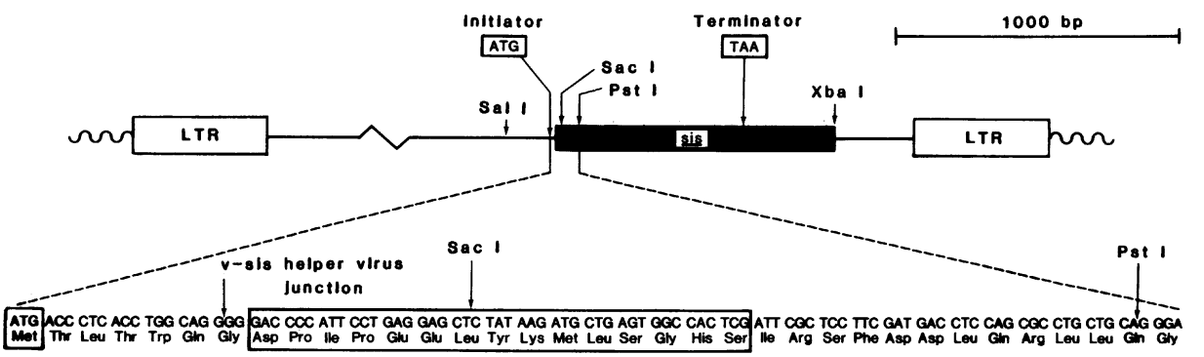

Fig. 2. Location of the *sis*-N1 peptide within the v-*sis* open reading frame. The amino acid sequence of the NH_2-terminal region of the putative v-*sis* gene product as well as the nucleotide sequence from which it was deduced are given in the bottom line. The amino acid sequence of the *sis*-N1 pentadecapeptide is also indicated. The procedure used for peptide synthesis has been described (*31*). Selected restriction enzyme cleavage sites are included for reference. *LTR* indicates long terminal repeat, and wavy lines indicate host DNA.

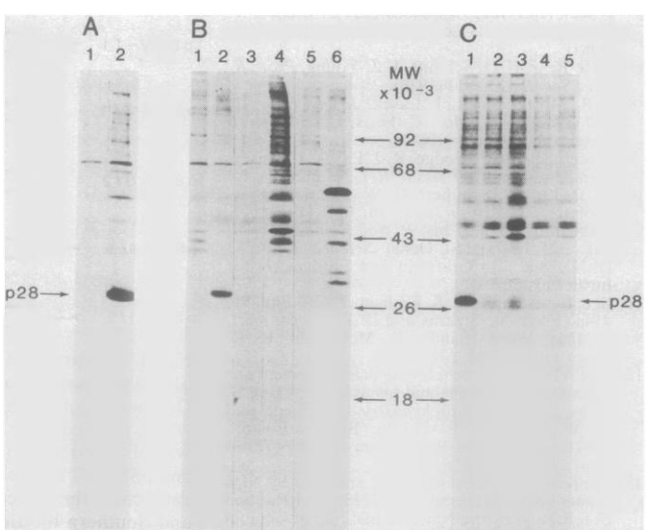

Fig. 3. Detection in vivo of a v-*sis* translational product by immunoprecipitation analysis. Subconfluent cultures (around 10^7 cells per 10-cm petri dish) were labeled for 3 hours at 37°C with 4 ml of methionine-free Dulbecco's modified Eagle's minimal essential medium containing 100 μCi of [^{35}S]methionine (1,200 Ci/nmole; Amersham) per milliliter. Labeled cells were lysed with 1 ml of a buffer containing 10 m*M* sodium phosphate, *p*H 7.5, 100 m*M* NaCl, 1 percent Triton X-100, 0.5 percent sodium deoxycholate and 0.1 m*M* phenylmethyl-sulfonyl fluoride per petri dish, clarified at 100,000*g* for 30 minutes and divided into four identical aliquots. Each aliquot was incubated with 4 μl of antiserum for 60 minutes at 4°C. Immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia) and analyzed by electrophoresis in sodium dodecyl sulfate–14 percent polyacrylamide gels (*32*). (A) Immunoprecipitation of labeled extracts of SSV(SSAV) transformed producer marmoset cells (lane 2) and uninfected (lane 1) marmoset cells with antiserum to *sis*-N1. (B) Immunoprecipitation of labeled extracts of SSV clone 11 transformed nonproducer NRK cells with preimmune rabbit serum (lane 1), with antiserum to *sis*-N1 (lane 2), and antiserum to SSAV (lane 6). Antiserum to *sis*-N1 was also used to immunoprecipitate uninfected (lane 3), Moloney murine sarcoma virus transformed nonproducer (lane 4), and SSAV infected (lane 5) NRK cell extracts. (C) Immunoprecipitation of extracts of SSV clone 11 transformed nonproducer NRK cells with antiserum to *sis*-N1 that had been incubated with 0, 0.1, 0.3, 1, or 10 μg (lanes 1 to 5, respectively) of *sis*-N1 peptide.

rum to detect p28 in normal rat kidney (NRK) cells transformed by SSV (Fig. 3B). When extracts of an SSV nonproducer NRK cell line were allowed to react with antiserum to *sis*-N1 (lane 2), we observed a 28,000-dalton protein that was not precipitable with preimmune serum (lane 1), nor detectable in uninfected NRK cells (lane 3). Analysis of NRK cells transformed by another retrovirus, Moloney-MSV, revealed no evidence of p28 expression (lane 4), a further indication that this protein was coded by SSV.

It is known that SSV clone 11 codes for the SSAV *gag* gene precursor polypeptide (*12*). To exclude the possibility that p28 was an SSAV related protein, we analyzed the ability of our *sis*-N1 antiserum to bind SSAV coded proteins. No SSAV gene products were precipitable with this antiserum, as shown by comparison of results with uninfected (lane 3) and SSAV infected NRK cells (lane 5). Moreover, although antiserum to SSAV detected SSAV *gag* related proteins in SSV nonproducer NRK cells (lane 6), none of these proteins had the apparent molecular weight of the p28 protein.

The above findings strongly implied that p28 was coded by the transforming region of SSV. As a final test of this conclusion, *sis*-N1 antiserum was incubated with varying amounts of the unlabeled *sis*-N1 peptide before immunoprecipitation of SSV nonproducer NRK cell extracts (Fig. 3C). Increasing amounts of *sis*-N1 peptide effectively competed for binding of p28, with complete inhibition observed with 10 μg of peptide (lanes 1 to 5 in Fig. 3C). In contrast, the peptide did not inhibit precipitation of other proteins nonspecifically observed both with control serum and antiserum to the peptide. All of these results identify p28 as the product of v-*sis*.

The transforming proteins of a number of acute transforming retroviruses are reported to be phosphoproteins with associated protein kinase activity (*13–22*). To test whether $p28^{sis}$ was a phosphoprotein, we labeled SSV transformed NRK cells with [^{32}P]orthophosphate and analyzed the cell lysate by immunoprecipitation. Under conditions in which the SSAV *gag* gene precursor was labeled, phosphorylation of $p28^{sis}$ could not be detected (data not shown). The fact that $p28^{sis}$ is not a phosphoprotein does not exclude the possibility that the molecule has protein kinase activity. Thus, immunoprecipitates containing $p28^{sis}$ were tested for their ability to catalyze the transfer of phosphate from adenosine triphosphate to associated immune globulins in a standard retrovirus protein kinase assay (*13*). Under conditions in which the transforming gene product of Snyder-Theilen feline sarcoma virus, a known protein kinase (*15*), readily scored positive, $p28^{sis}$ did not demonstrate protein kinase activity.

The 28,000-dalton protein identified as the translational product of the cell derived gene of SSV was specifically detected in SSV transformed cells and corresponded in size to the protein (27,000 daltons) predicted from the v-*sis* open reading frame (*6*). The absence of any other long open frame within v-*sis* (*6*) and localization of the SSV transforming gene to a small region of SSV encompassing v-*sis*, make it likely that $p28^{sis}$ is the SSV transforming protein.

Thiel *et al.* (*23, 24*), using antiserum to SSV transformed nonproducer cells, had earlier identified SSV transformation–specific proteins of 20,000 and 200,000 daltons. Neither of these proteins corresponds in size to $p28^{sis}$. Moreover, antiserum provided by Thiel did not detect $p28^{sis}$. Thus, these SSV transformation–specific proteins appear to be induced by SSV rather than coded by SSV. As such, their role in the induction and maintenance of SSV transformation remains to be determined.

The finding that $p28^{sis}$ is not phosphorylated indicates that $p28^{sis}$ differs from a number of other retrovirus transforming gene products that are known to be phosphoproteins (*13–22, 25*). In addition, $p28^{sis}$ did not demonstrate autophosphorylation or phosphorylation of the *sis*-N1 antibody. We cannot exclude the possibility that $p28^{sis}$ is a protein kinase with specificity for other substrates or that antibody to *sis*-N1 inhibits $p28^{sis}$ kinase activity by its specificity for a particular region of the protein. The generation of peptide antibodies directed against other regions of $p28^{sis}$ should aid in resolving this question.

Synthetic peptides prepared on the basis of known nucleotide sequences have been used in the analysis of several proteins coded by certain DNA (*9*) and RNA (*10, 26*) tumor viruses, as well as hepatitis virus (*27, 28*); these proteins had all been previously identified by other techniques. Our identification of $p28^{sis}$ with antibody to a predicted peptide sequence demonstrates the efficacy of this approach in the detection of an unknown gene product and thus its general applicability to the analysis of cloned genes.

The availability of antibodies capable of recognizing $p28^{sis}$ should be of use in locating this protein within transformed cells and in obtaining sufficient amounts of the protein for detailed biochemical analysis. Recent studies have revealed that certain human tumor cells specifically express v-*sis* related transcripts (*29*). It will be of interest to determine whether antibodies directed against the v-*sis* coded protein are also capable of detecting a v-*sis* related gene product in such tumor cells.

KEITH C. ROBBINS
SUSHILKUMAR G. DEVARE
E. PREMKUMAR REDDY
STUART A. AARONSON
Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland 20205

References and Notes

1. *Cold Spring Harbor Symp. Quant. Biol.* **44**, (1980), entire issue.
2. G. J. Thielen, D. Gould, M. Fowler, D. L. Dungworth, *J. Natl. Cancer Inst.* **47**, 881 (1971).
3. K. C. Robbins, S. G. Devare, S. A. Aaronson, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2918 (1981).
4. F. Wong-Staal *et al.*, *Nature (London)* **294**, 273 (1981).
5. K. C. Robbins, R. L. Hill, S. A. Aaronson, *J. Virol.* **41**, 721 (1982).
6. S. G. Devare, E. P. Reddy, K. C. Robbins, P. R. Andersen, S. R. Tronick, S. A. Aaronson, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3179 (1982).
7. F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).
8. M. Wigler, S. Silverstein, L. S. Lee, A. Pellicer, Y. Cheng, R. Azel, *Cell* **11**, 223 (1977).
9. G. Walter, K. Scheidtmann, A. Carbone, A. Laudano, R. F. Doolittle, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5197 (1980).
10. J. G. Sutcliffe, T. M. Shinnick, N. Green, F-T. Liu, H. L. Niman, R. A. Lerner, *Nature (London)* **287**, 801 (1980).
11. A. Kagan and M. Glick, in *Methods of Hormone Radioimmunoassay*, B. M. Jaffe and H. R. Behrman, Eds. (Academic Press, New York, 1979), pp. 328–329.
12. S. A. Aaronson, J. R. Stephenson, S. Hino, S. R. Tronick, *J. Virol.* **16**, 1117 (1975).
13. M. S. Collett and R. L. Erikson, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2020 (1978).
14. A. D. Levinson, H. Oppermann, L. Levintow, H. E. Varmus, J. M. Bishop, *Cell* **15**, 561 (1978).
15. M. Barbacid, K. Beemon, S. G. Devare, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5158 (1980).
16. R. A. Feldman, T. Hanafusa, H. Hanafusa, *Cell* **22**, 757 (1980).
17. T. Hunter and B. M. Sefton, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1311 (1980).
18. S. Kawai, M. Yoshida, K. Segawa, R. Sugiyama, R. Ishizaki, K. Toyoshima, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6199 (1980).
19. T. Pawson, J. Guyden, T. Kung, K. Radke, T. Gilmore, G. S. Martin, *Cell* **22**, 767 (1980).
20. W. J. M. Van de Ven, F. H. Reynolds, J. R. Stephenson, *Virology* **101**, 185 (1980).
21. O. M. Witte, A. Dasgupta, D. Baltimore, *Nature (London)* **283**, 826 (1980).
22. J. C. Niel, J. Ghysdael, P. K. Vogt, *Virology* **109**, 223 (1981).
23. H-J. Thiel, T. J. Matthews, E. M. Broughton, A. W. Butchko, D. P. Bolognesi, *ibid.* **112**, 642 (1981).
24. H-J. Thiel, T. J. Matthews, K. J. Weinhold, E. M. Broughton, *ibid.* **115**, 401 (1981).
25. T. Y. Shih, M. O. Weeks, H. A. Young, E. M. Scolnick, *ibid.* **96**, 64 (1979).
26. J. Papkoff, M. H-T. Lai, T. Hunter, I. M. Verma, *Cell* **27**, 109 (1981).
27. R. A. Lerner, N. Green, H. Alexander, F. Liu, J. G. Sutcliffe, T. M. Shinnick, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3403 (1981).
28. G. R. Dreesman *et al.*, *Nature (London)* **295**, 158 (1982).
29. A. Eva *et al.*, *ibid.*, p. 116.
30. F. Bolivar, R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, *Gene* **2**, 95 (1977).
31. A. Marglin and R. B. Merrifield, *Annu. Rev. Biochem.* **39**, 841 (1970).
32. M. Barbacid, A. V. Lauver, S. G. Devare, *J. Virol.* **33**, 196 (1980).
33. We thank M. Barbacid for many useful discussions and R. Mathews and M. Gordon for excellent technical assistance.

18 June 1982; revised 1 September 1982