processed and transported to the plasma membrane without expression of other retrovirus genes. Thus, recombinant vaccinia viruses should prove useful for further analysis of the processing of the *env* protein, to prepare native antigens free of other HTLV-III proteins, make target cells for studying cell-mediated immunity, and to obtain polyclonal and monoclonal antibodies to the *env* protein. Further work is needed to determine whether antibody to gp120 is neutralizing and whether vaccination with live recombinant vaccinia virus or with inactivated materials from infected cells might have immunoprophylactic value in preventing the development of AIDS. It is important to note that use of live vaccinia virus in individuals that already have AIDS would be contraindicated because of the suppression of their immune systems.

We thank P. Earl for help in DNA sequencing, M. Sarngadharan and Z. Salahuddin for materials and helpful discussions, and M. Rust for editorial assistance.

Received 17 December 1985; accepted 11 February 1986.

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Expression of AIDS virus envelope gene in recombinant vaccinia viruses

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Acquired immune deficiency syndrome (AIDS) is an infectious disease characterized by severe impairment of the patient's cell-mediated immune system[1,2]. Several lines of evidence have indicated that the aetiological agent of AIDS is a group of T-lymphotropic retroviruses, variously known as lymphadenopathy-associated virus (LAV)[3], human T-lymphotropic virus type III (HTLV-III)[4,5] and AIDS-associated retrovirus (ARV)[6]. Serological surveys have indicated that as many as one million people in the United States may have been infected by LAV/HTLV-III[7], and the spread of AIDS has become a global concern. The need for a better understanding of the viral immunology and for a vaccine against AIDS is self-evident. To this end, we have constructed recombinant vaccinia viruses containing the envelope (*env*) gene of LAV, and demonstrate here that cells infected with these viruses express immunoreactive proteins similar to those present on LAV virions. Experimental animals infected with these recombinant viruses elicited antibodies that specifically recognized LAV envelope proteins.

The proviral genome of LAV has been cloned[8] and its complete nucleotide sequence determined[9]. As deduced from open reading frames in the nucleotide sequence, the genome of LAV contains not only the typical retroviral genes coding for the core proteins (*gag*), the reverse transcriptase (*pol*) and envelope proteins (*env*), but also two novel genes of unknown function, Q and F (ref. 9). We chose to express the *env* gene of LAV as for many viruses it is the major envelope glycoprotein that elicits neutralizing or protective responses.

To insert the LAV *env* gene into vaccinia virus vectors, we used a general procedure described by Mackett *et al.*[10]. Briefly, a foreign gene is first inserted into a plasmid vector (pGS20) downstream from a vaccinia transcriptional control element (7.5K promoter). This chimaeric gene in the recombinant plasmid is flanked by vaccinia sequences encoding the viral thymidine kinase gene (*tk*). The plasmid is then introduced into cells previously infected by wild-type vaccinia virus (strain WR); recombination occurs in the *tk* region, which is homologous for both the viral DNA and the plasmid, and allows the insertion of the chimaeric gene into the genome of the vaccinia virus. The resultant recombinant virus will be TK^- and will grow in selective medium containing 5-bromodeoxyuridine. By using this approach, we constructed two recombinant vaccinia viruses, v-env5 and v-env2. Recombinant v-env5 contains the entire envelope coding sequence (nucleotides 5,767 to 8,349)[9] as well as 96 base pairs (bp) of 5′-proximal and 223 bp of 3′-proximal untranslated sequences. Recombinant v-env2 contains the *Kpn*I fragment (5,889–8,572) and does not carry the sequence encoding the N-terminal 42 amino acids of *env*. The *Kpn*I fragment was inserted into v-env2 using oligonucleotide linkers so that a translational initiation codon was introduced in-phase with the reading frame of the envelope coding sequences (Fig. 1).

To determine whether the LAV *env* gene was expressed by the recombinant vaccinia viruses, we infected two cell lines (BSC-40 and HeLa) with v-env5 and v-env2 and used Western blotting to assay for LAV-specific proteins in the infected cell lysate. Three major proteins immunoreactive with pooled serum from seropositive individuals were detected in the v-env5-infected cells (Fig. 2). The relative molecular masses (M_rs) of these proteins were estimated to be 150,000 (150K), 120K and 41K, similar to those of LAV envelope glycoproteins gp150, gp110 and gp41 (refs 11, 12 and F. Clavel, personal communication). Radioisotope labelling with glucosamine indicated that these recombinant-made proteins, like the envelope proteins of LAV, were also glycosylated (Fig. 3*a*, lane 7). Differences in the glycosylation patterns could account for the slight variations observed in the electrophoretic mobilities of recombinant-made proteins compared with LAV virion glycoproteins (data not shown). It has been suggested that gp150 of LAV is the precursor from which an exterior protein gp110 and a transmembrane protein gp41 are derived[9,11,12]. Our results (Fig. 3*b*, lanes 7–12) indicated the same precursor–product relationship for the recombinant-made 150K, 120K and 41K proteins. The processing of the 150K protein appeared to be slow and inefficient in HeLa cells, as by 6 h after pulse-labelling less than 50% of the radioactivity in the 150K protein was chased into 120K and 41K proteins. Preliminary results indicated that this processing was more efficient in certain types of human peripheral blood cells infected with the same recombinant virus (data not shown). As would have been expected for gp110 of LAV, the recombinant-made 120K protein was also detected in infected cell medium (Fig. 3*c*, lane 3). These results demonstrated that the recombinant virus v-env5 is able to express the LAV *env* gene and to produce immunoreactive proteins that are processed in a pattern similar to that of authentic LAV envelope glycoproteins.

Recombinant virus v-env2 lacked the putative signal sequence for LAV *env*, but was able to produce at least three immunoreactive polypeptides: 99K, 68K and 40K (Fig. 2, lane 4). Results from pulse-chase experiments indicated that the *env* sequence in v-env2 was expressed as an unmodified 87K precursor (780

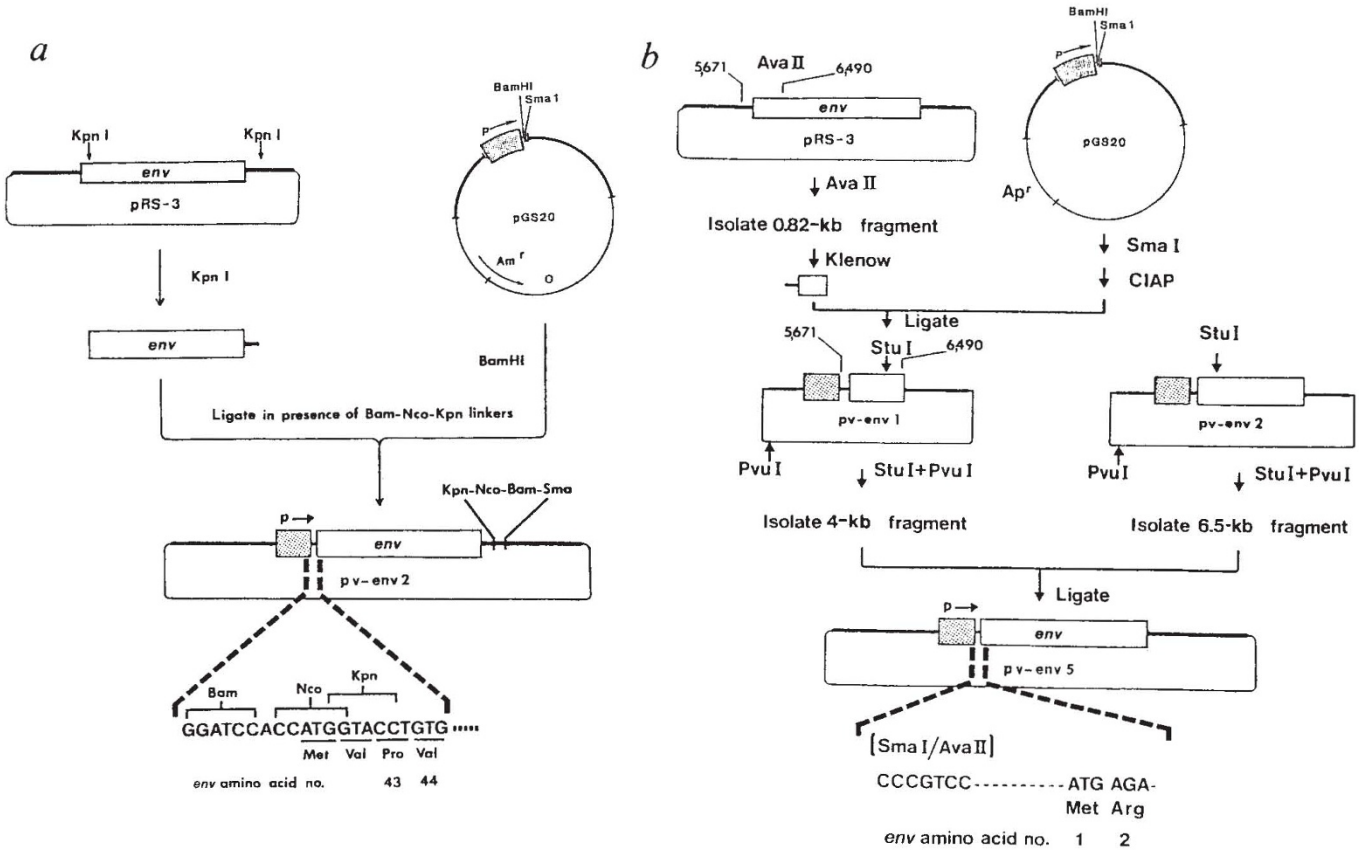
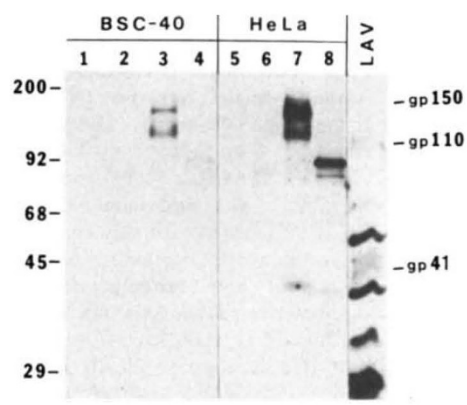


Fig. 1 Construction of plasmid vectors containing vaccinia promoter and LAV envelope coding sequences. *a*, Construction of vaccinia–LAV recombinant plasmid lacking the sequence coding for the first 42 amino acids of *env*. Plasmid pRS-3, which contains LAV-specific sequences from the *Eco*RI site at nucleotide 5,289 (ref. 9) to the *Sst*I site at 9,129 inserted at the corresponding sites of plasmid vector pUC18, was digested with *Kpn*I and a 2.68-kilobase pair (kb) fragment was isolated. This fragment contained LAV-specific DNA (nucleotides 5,889–8,572) and included sequences encoding envelope proteins from amino acid 42 to the carboxy-terminus. This fragment was ligated with *Bam*HI-digested pGS20 DNA in the presence of the oligodeoxynucleotide linkers 5′-GATCCACCATGGTAC-3′ OH and 5′-CATGGTG-3′ OH. These linkers were designed (1) to allow ligation of the *Bam*HI and *Kpn*I cohesive ends of the two different DNA molecules and (2) to provide a translational initiation codon in-phase with the envelope coding sequences. The ligation mixture was used to transform *Escherichia coli* strain MC1000. Plasmid DNA from ampicillin-resistant tranformants was tested for the orientation of the insert and the regeneration of *Bam*HI, *Nco*I and *Kpn*I sites at the ligation junction. The nucleotide sequence at the ligation junction of the desired plasmid, pv-env2, is as indicated. The vaccinia 7.5K promoter is represented by the shaded area and the direction of transcription by the arrow. *b*, Construction of vaccinia–LAV recombinant plasmid containing the entire envelope coding sequence. This plasmid was constructed in two steps. First, a 0.82-kb *Ava*II fragment from pRS-3 was purified. This fragment contains LAV-specific sequences (nucleotides 5,671 to 6,490) including those encoding the N-terminal portion of the envelope protein and 95 bp of 5′-untranslated sequences. The fragment was blunt-ended with Klenow enzyme and ligated with plasmid pGS20 that had been linearized with *Sma*I and dephosphorylated with calf-intestine alkaline phosphatase (CIAP). Recombinant plasmid with the structure indicated was isolated and designated pv-env1. The second stage of construction involved rejoining the entire *env* sequence at the *Stu*I site present in both pv-env1 and pv-env2; this was done by ligating the 4-kb *Stu*I and *Pvu*I fragment of pv-env1, which contained the vaccinia promoter and the 5′ portion of the *env* gene, with the 6.5-kb fragment of pv-env2 generated by the same restriction enzymes and containing the remaining portion of the *env* gene. The resultant plasmid, pv-env5, contained the entire envelope gene as well as 95 bp of 5′-proximal and 223 bp of 3′-proximal adjacent sequences. To insert the chimaeric vaccinia–LAV *env* gene into the vaccinia virus genome, the generalized method described by Mackett *et al.*[10] was used. All recombinant DNA techniques used are described in ref. 21.

Fig. 2 Expression of LAV envelope-related proteins derived from vaccinia–LAV recombinants. Confluent monolayers of BSC-40 or HeLa cells were infected with recombinant virus v-env5 or v-env2 at a multiplicity of infection (MOI) of 50 PFU per cell; 12 h after infection the cells were washed twice in phosphate-buffered saline (PBS), resuspended in Laemmli sample buffer and boiled for 4 min. Proteins from infected cells were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred to nitrocellulose membrane, and reacted with pooled serum from LAV/HTLV-III-seropositive individuals. Immunoreactive proteins were detected using protein A, which was labelled with ^{125}I by the chloramine-T method. The sources of protein in lanes 1–8 are: 1 and 5, mock-infected cells; 2 and 6, cells infected with wild-type vaccinia virus; 3 and 7, v-env5-infected cells; 4 and 8, v-env2-infected cells. LAV virion proteins purified on a sucrose gradient were used as a control (LAV) and the positions of envelope proteins gp150, gp110 and gp41 were as indicated. M_r standards are shown on the left ($\times 10^{-3}$).



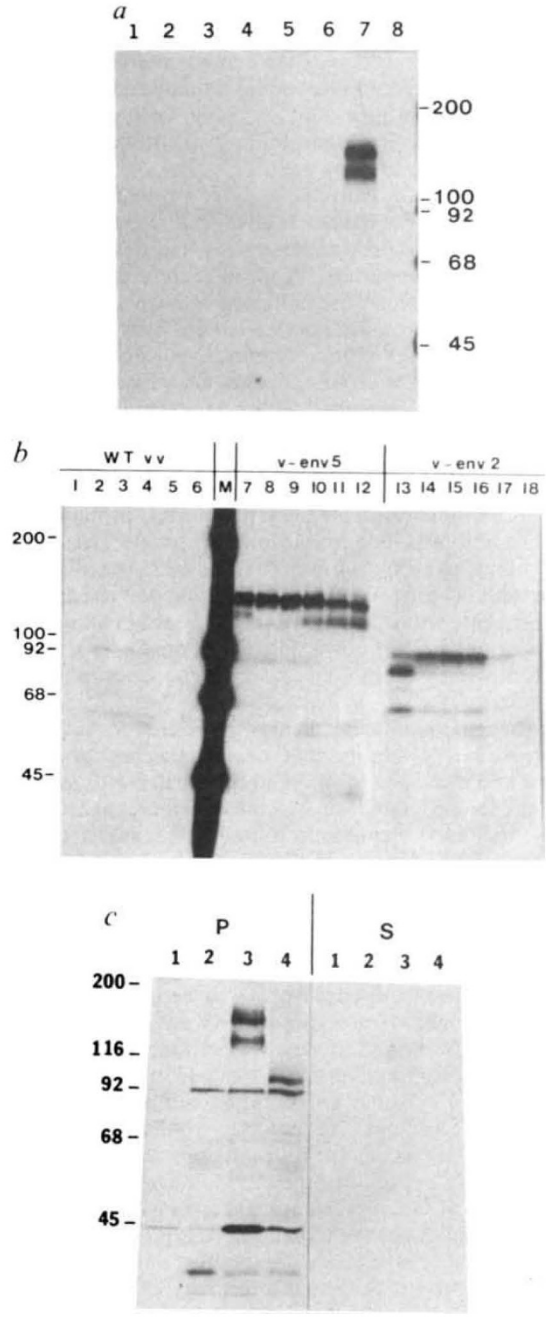


Fig. 3 Radioimmunoprecipitation analysis of LAV envelope-related proteins produced by vaccinia–LAV recombinant viruses. ***a***, ^{3}H-glucosamine labelling of recombinant-produced proteins. HeLa cells were either mock-infected (lanes 1, 5) or infected separately with wild-type vaccinia virus (lanes 2, 6), recombinant v-env5 (lanes 3, 7) or v-env2 (lanes 4, 8), all at a MOI of 50 PFU per cell. ^{3}H-glucosamine (0.25 μCi, 23 mCi mg^{-1}; Amersham) was added to culture medium from 4 to 16 h after infection. Cells were washed twice with PBS and lysed in buffer containing 0.1 M NaCl, 0.01 M Tris *p*H 7.4, 1 mM EDTA, 1% Nonidet P-40 (NP40) and 0.5% sodium deoxycholate. Aliquots of cell lysates were mixed with either normal human serum (lanes 1–4) or pooled serum from LAV/HTLV-III-seropositive individuals (lanes 5–8). Immunoreactive proteins were precipitated by fixed *Staphylococcus aureus* cells bearing protein A, resolved by SDS-PAGE and detected by fluorography. ***b***, 'Pulse-chase' analysis of LAV envelope-related proteins produced by vaccinia–LAV recombinants. Confluent monolayers of HeLa cells were infected with wild-type vaccinia virus (lanes 1–6), recombinant v-env5 (lanes 7–12) or v-env2 (lanes 13–18), all at a MOI of 50 PFU per cell. At 10.5 h post-infection, cells were labelled with ^{35}S-methionine (>800 Ci mmol^{-1}; Amersham) at 100 μCi ml^{-1} for 15 min. At the end of the labelling period, cells were washed once with 2 ml of pre-warmed chase medium (Dulbecco's modified Eagle's medium, 3 mg ml^{-1} L-methionine, 5% calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) and re-fed with 1 ml of the same medium before being returned to the incubator. At various times afterwards, cells were washed and lysed as described for *a* and proteins from cell lysates were immunoprecipitated with pooled serum from LAV/HTLV-III-seropositive individuals. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by fluorography. The duration of each chase was as follows (h): 0 (lanes 1, 7, 13); 0.5 (lanes 2, 8, 14); 1 (lanes 3, 9, 15); 2 (lanes 4, 10, 16); 6 (lanes 5, 11, 17) and 12 (lanes 6, 12, 18). ***c***, Presence of LAV envelope-related protein in the growth medium of cells infected with vaccinia–LAV recombinant viruses. Confluent monolayers of HeLa cells were either mock-infected (lane 1), or infected separately by wild-type vaccinia virus (lane 2), recombinant v-env5 (lane 3) or v-env2 (lane 4), all at a MOI of 20 PFU per cell. Cells were labelled with ^{35}S-methionine (>800 Ci mmol^{-1}; Amersham) at 100 μCi ml^{-1} from 10 to 12 h after infection. At the end of the labelling period, medium was removed and clarified by centrifugation for 2 min at 12,000*g* before immunoprecipitation using pooled serum from LAV/HTLV-III-seropositive individuals. Proteins from infected cells immunoprecipitated by the same serum are shown in the panel labelled P (for pellet) and those from the medium in the panel labelled S (for supernatant).

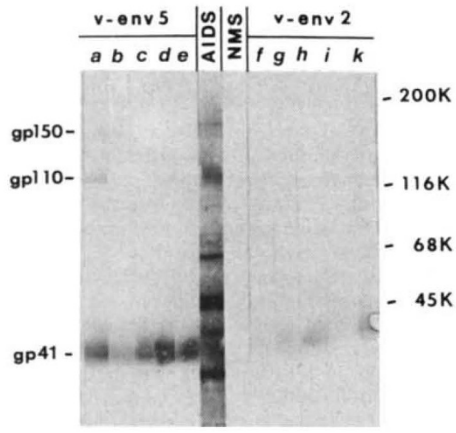


Fig. 4 Western blot analysis of serum samples from mice immunized with vaccinia–LAV recombinant viruses. *a–e*, Serum samples from five mice inoculated with v-env5; *f–k*, samples from individuals inoculated with v-env2. Pooled sera from LAV/HTLV-III-seropositive individuals (AIDS) and non-immunized C57BL/6J mice (NMS) were used as positive and negative controls, respectively. The positions of the LAV envelope glycoproteins gp150, gp110 and gp41 are indicated.

Methods. Male inbred mice (C57BL/6J, Jackson Laboratory) at 5–7 weeks old were immunized by tail scarification, in which a 10-μl inoculum containing 2×10^{7} PFU of recombinant vaccinia virus was applied to abrasions generated with a bifurcated needle at the base of the tail. Animals were bled from the retro-orbital sinus at 8 weeks post-inoculation and the serum kept frozen until use. Aliquots of serum samples diluted 50-fold in PBS plus 0.2% NP40 plus 3% non-fat dry milk were reacted with LAV virion proteins resolved by SDS-PAGE and immobilized on nitrocellulose filters by electro-transfer. LAV proteins recognized by these sera were detected using goat anti-mouse immunoglobulin conjugated with alkaline phosphatase.

amino acids), which was processed to a 99K intermediate and cleaved into the 68K and 40K polypeptides (Fig. 3*b*, lanes 13–18). As would have been expected for proteins lacking signal peptides, no LAV envelope-related polypeptide was detected in the infected cell medium (Fig. 3*c*, lane 4) and no *N*-linked glycosylation with ^{3}H-glucosamine was observed (Fig. 3*a*, lane 8).

We examined the immunogenicity of proteins expressed by recombinant vaccinia viruses v-env5 and v-env2. Male mice (strain C57BL/6J) were inoculated by tail scarification with 2×10^7 plaque-forming units (PFU) of each of the recombinant viruses. Serum samples were collected at 2-week intervals for 8 weeks post-inoculation and were analysed by Western blotting for reactivity with LAV virion proteins. By 8 weeks post-inoculation, all animals immunized with the recombinant viruses produced antibodies that reacted with LAV envelope glycoprotein gp41 (Fig. 4). Serum from some of the animals immunized with v-env5 (Fig. 4*a* and our unpublished results) also recognized gp150 and gp110, indicating the ability of this recombinant virus to elicit an immune response to all major glycoproteins of LAV. Using enzyme-linked immunosorbent assay (ELISA) and Western blot analyses, we found that the LAV-specific antibody titres elicited by both recombinants continued to rise throughout the experiment, and that recombinant v-env5 was also able to elicit LAV-specific responses in an out-bred strain of mouse (ICR) following several routes of immunization (data not shown).

The description of neutralizing antibodies to LAV/HTLV-III[13] raises the possibility that neutralizing epitopes can be identified and a vaccine formulated for the prevention of AIDS. One promising approach for vaccine development is the use of vaccinia virus as an expression vector[14,15]. A vast database has been established detailing the safety and efficacy of vaccinia virus as a successful smallpox vaccine, and there is an increasing body of knowledge concerning the use of recombinant vaccinia viruses to stimulate humoral[16–19] and cell-mediated[20] immunity against various pathogens. The vaccinia–LAV recombinants that we have described here could prove to be useful tools for the study of the antigenic properties of LAV envelope proteins and eventually for the design of an effective vaccine against AIDS.

We thank the staff at Genetic Systems Corporation for assistance, in particular Bruce Travis for providing plasmid pRS-3, Jan McClure for LAV virion proteins and human serum samples, and Elaine Thomas for helpful discussions. The genomic clone of LAV (λJ19)[8] was provided by Luc Montagnier. We also thank Tony Purchio for encouragement; Peter Linsley, Jeff Ledbetter and Paul Beaumier for technical advice; and Bonnie Kirk and Anne Little for preparation of the manuscript.

Note added in proof: The immunogenicity of v-env5 has been examined in one species of sub-human primate (*Macaca fascicularis*). Seven out of 8 vaccinated animals developed specific antibodies to the envelope glycoprotein of LAV.

Received 2 January; accepted 11 February 1986.

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Requirement for c-*ras* proteins during viral oncogene transformation

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Many retroviral oncogenes have been classified into one of several categories based on structure, enzymology and cellular localization[1]. These genes originated from host cells and are probably derived from genes normally involved in the control of cell proliferation[2]. The cellular counterparts of three oncogenes have been identified as a growth factor or growth factor receptor[3–6]; related oncogenes include receptor-like membrane proteins which often express tyrosine kinase activity. These growth factor-related oncogenes are structurally and biochemically distinct from the membrane-associated *ras* gene family, which bind and hydrolyse GTP[7–9]. Oncogenes localized primarily in the cytoplasm which probably have serine kinase activity, have also been identified[10–12]. Although the structure and biochemistry of many oncogenes have been extensively studied, relatively little is known about the functional relationships of oncogene proteins within the cell. An opportunity to study such interaction is provided by the identification of a monoclonal antibody that neutralizes cellular *ras* proteins when microinjected into cells[13]. It has been shown previously that the injected antibody inhibits the initiation of S-phase in NIH 3T3 cells[14]. In the present study we injected this monoclonal antibody into NIH 3T3 cells transformed by a variety of oncogenes. The results show that transformation by three growth factor receptor-like oncogenes depends on c-*ras* proteins, while transformation by two cytoplasmic oncogenes appears to be independent of c-*ras* protein.

Anti-*ras* monoclonal antibodies originally prepared by Furth *et al.*[15] have been analysed extensively. Monoclonal antibody Y13-259 (which binds the c-*ras* proteins of a variety of species[15]) neutralized the activity of co-injected, purified *ras* protein, and

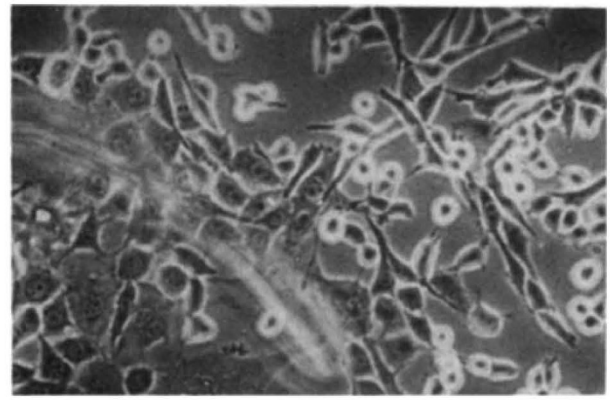

Fig. 1 Morphological reversion of *src*-transformed cells following injection of anti-*ras* antibody. These NIH 3T3 cells were transformed by the Rous sarcoma viral genome (see Table 1). Neutralizing anti-*ras* antibody 259 was injected into the cells adjacent to or within a circular mark on the underside of the coverslip to which the cells were attached. A small section of this circular mark is visible, allowing identification of the injected area in the lower left half of this phase-contrast photomicrograph (×82). After 16 h, cells in the injected area had reverted to the flattened, non-refractile apperance of untransformed cells. The nuclei of most injected cells, for example, are clearly visible. Uninjected cells (or cells injected with control antibody 238; data not shown) remained refractile, with the spherical or spindle shape of transformed cells.