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Simultaneous *in situ* detection of viral RNA and antigens

(*in situ* hybridization/immunocytochemistry)

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ABSTRACT **We have combined *in situ* hybridization and immunocytochemistry to detect RNA and proteins in the same cell. We envision a wide range of applications for this method, and, as one example, we show that viral genomes and capsid antigens can be detected simultaneously in paraffin sections of the central nervous system of mice infected with Theiler's virus, the causative agent of a slow demyelinating disease.**

Methods to detect genes and their products in tissue sections (*in situ*) have a wide range of applications in biology. Nucleotide sequences, either DNA or RNA, can be detected and quantitated with highly sensitive *in situ* hybridization methods (1, 2), and polypeptides can be detected with specific antibodies by immunofluorescence or immunocytochemical techniques (3). Reagents for *in situ* hybridization and immunocytochemistry are now readily available with the revolutionary advances in cloning nucleotide sequences and producing monoclonal antibodies to specific antigens.

In many instances, the simultaneous detection, in the same tissue section, of a specific nucleotide sequence and a specific antigen would be of great advantage. For example, in the field of virology, it would be possible to study, at the single-cell level, the distribution of viral genes and their expression in the form of viral antigens, or, with cell-type-specific antigenic markers, one could identify the cells harboring viral genes in a complex organ such as the central nervous system (CNS). In this article, we describe a general method for the simultaneous detection, in tissue sections, of antigens and single-stranded viral RNA, and we illustrate the use of the method in the persistent and demyelinating infection of the CNS of mouse due to Theiler's murine encephalomyelitis virus (TMEV), a murine picornavirus (4–6).

MATERIALS AND METHODS

Virus, Cells, Animals. The DA strain of TMEV was plaque-purified 3 times on baby hamster kidney (BHK) cells.

SJL/J mice, 3–4 weeks old, were purchased from The Jackson Laboratory. Mice were inoculated intracranially with 40 μl of phosphate-buffered saline (P_i/NaCl) containing 10^4 plaque-forming units (pfu) of TMEV. All inoculated animals had intense inflammatory and demyelinating CNS lesions 2 months after inoculation.

Preparation of Cells and Tissue Sections. Microscope slides were cleaned and treated with Denhardt's medium (7) followed by acetylation, as described (1, 2).

BHK-21 cells were infected at a multiplicity of infection (moi) of 10 pfu per cell and were harvested by trypsinization 5 hr after infection. Cells were washed once in P_i/NaCl and deposited on treated slides using a cytocentrifuge. After air drying for a few minutes the cells were fixed for 20 min in ice-cold fixative containing 0.5% formaldehyde/0.5% glutaraldehyde/0.1 M phosphate buffer, pH 6.0/1.6% glucose/0.002% $CaCl_2$/1.0% dimethyl sulfoxide. The fixative was prepared as described (8). After fixation, the cells were quenched by immersion for 20 min in ice-cold 0.15 M ethanolamine (pH 7.5) followed by washing for 5 min in cold P_i/NaCl. If not processed immediately, slides were stored at 4°C in P_i/NaCl containing 0.35% bovine serum albumin/0.02% NaN_3. Storage was for up to 1 week.

Antigens and RNA were stable for several months at room temperature, when the slides were treated as follows: washing in P_i/NaCl was followed by dehydration (70% ethanol, twice; 95% ethanol, twice; 10% ethanol, twice; 100% xylene, twice; 5 min for each step) and paraffin embedding (2 times, 10 min in Paraplast at 62°C). Prior to immunochemistry *in situ* hybridization the paraffin was removed from the slides by dipping 3 times in xylene and twice in 100% ethanol (5 min each time).

Mice were perfused under ether anesthesia with 20 ml of P_i/NaCl followed by 20 ml of cold fixative (described above) (8). During perfusion with fixative, animals were encased in ice. Duration of perfusion was ≈15 min. After dissection, the brain and spinal cord were fixed further by immersion in ice-cold fixative for 30 min. Fixation was terminated by quenching in cold 0.15 M ethanolamine (pH 7.5) for 1 hr. After fixation, the tissues were transferred to 70% ethanol and processed for paraffin embedding using routine histological techniques. Ten-micrometer-thick sections were cut and picked on treated microscope slides after floating on warm water (45°C) containing 1% Elmer's white glue. After drying overnight at 37°C, the slides were stored at 4°C for up to 2 or 3 weeks. Prior to immunocytochemistry and *in situ* hybridization, the slides were dipped twice in xylene and twice in ethanol, 5 min each time to remove the paraffin.

In some experiments (see *Results*), the fixative was a mixture of 2% paraformaldehyde/0.075 M lysine/0.01 M $NaIO_4$/0.037 M phosphate buffer, pH 7.5 (PLP) prepared as described (9). Cells were fixed for 15 min at 4°C followed by two 5-min washes in P_i/NaCl at room temperature. Animals were perfused with cold PLP. Dissected brain and spinal cord were fixed further for 2 hr at 4°C by immersion in PLP followed by washing for 30 min at room temperature in P_i/NaCl, dehydration, and embedding in paraffin.

Immunocytochemistry and *in Situ* Hybridization. A hyperimmune antiserum against TMEV capsid proteins was obtained as follows. TMEV was purified from BHK cell culture fluid as described (6). One New Zealand White rabbit was immunized in the four foot pads with 150 μg of purified virus emulsified in complete Freund's adjuvant. One month later, the animal was challenged with 100 μg of purified virus

Abbreviations: CNS, central nervous system; TMEV, Theiler's murine encephalomyelitis virus; pfu, plaque-forming units; P_i/NaCl, phosphate-buffered saline; PLP, 2% paraformaldehyde/0.075 M lysine/0.01 M $NaIO_4$/0.037 M phosphate buffer, pH 7.5; DAB, diaminobenzidine tetrahydrochloride; NaCl/Cit, 0.15 M NaCl/0.015 M Na citrate; PFG, 0.5% paraformaldehyde/0.5% glutaraldehyde/0.1 M phosphate buffer, pH 6.0/1.6% glucose/0.002% $CaCl_2$/1.0% dimethyl sulfoxide.

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injected intravenously in 300 μl of P_i/NaCl. Antiserum was obtained 10 days later.

BHK cells or tissue sections with paraffin removed were permeated by immersion for 4 min at room temperature in P_i/NaCl containing 0.1% Triton X-100, followed by two 5-min washes in P_i/NaCl. The slides reacted successively with normal goat serum, anti-TMEV rabbit antiserum diluted 1:300 or 1:3000, biotinylated anti-rabbit IgG prepared in goat, avidin biotinylated horseradish peroxidase complex, 0.01% H_2O_2/diaminobenzidine tetrahydrochloride (DAB) (0.5 mg/ml) prepared in 0.05 M Tris·HCl (pH 7.5). The biotinylated anti-rabbit antiserum and the avidin biotinylated horseradish peroxidase complex were from Vector Laboratories (Burlingame, CA) (Vectastain ABC kit). The conditions of the reactions were those recommended by the manufacturer.

After staining with DAB, slides were washed twice in distilled water and treated for *in situ* hybridization. Treatments with 0.2 M HCl/2× NaCl/Cit at 70°C/proteinase K (1 μg/ml) were as described (1) (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate).

After two washes in distilled water, the slides were transferred to 0.1 M triethanolamine (pH 7.5) and acetic anhydride was added to a final concentration of 0.25% (10). After mixing, acetylation was carried out for 10 min at room temperature followed by two washes in distilled water and dehydration in graded ethanol (70% twice; 95% once). Acetylation is critical to prevent nonspecific binding of cDNA probes to the DAB stain precipitate (see *Results*).

Acetylated slides were hybridized *in situ* with [^{3}H]cDNA (specific activity, 2×10^8 dpm/μg) synthesized by reversed transcription of TMEV 35S RNA as described (6). The conditions for hybridization have been described (1, 2). [^{3}H]cDNA concentration was 0.4 ng/μl for BHK cells and 0.13 ng/μl for tissue sections. Hybridization was for 42 hr at room temperature. Washing the excess [^{3}H]cDNA was as described (2) including a 1-hr wash in 2× NaCl/Cit at 55°C.

Washed slides were dipped in NTB-2 Kodak emulsion diluted (1:1) with 0.2 M ammonium acetate (1). Exposure times are indicated in the text for each experiment. After development in Kodak D-19 and fixation, the slides were counterstained with 1% methyl green in water (BHK cells) or hematoxylin (CNS sections), as described (2, 6).

RESULTS

To detect both nucleic acids and proteins in the same cells, three conditions must be satisfied: (*i*) reactivity of nucleic acids and antigens must be preserved; (*ii*) two different signals must be generated and retained; and (*iii*) recognizable details of cell structure must be maintained. We have developed methods of fixation and procedures compatible with these requirements.

Optimal Conditions for Fixation. The fixative must not only preserve morphological details but also retain antigenicity and allow a maximal level of hybridization *in situ*. Best results were obtained with a mixture of 0.5% paraformaldehyde/0.5% glutaraldehyde (PFG) used in the cold for short periods of time (see *Materials and Methods*). This fixative was developed recently by others (8) for optimal retention of antigenicity of CNS surface markers.

We compared *in situ* hybridization levels after fixation with PFG or ethanol acetic acid (3:1, vol/vol; 20 min at room temperature), a fixative of reference for this procedure (1, 2). Table 1 shows that hybridization levels on infected BHK cells were identical.

It is likely that PFG is suitable for picornaviruses in general and for many other systems. However, in some cases it might be necessary to use other fixatives. In our experience, PLP (9) used as described above is an alternative. Preservation of morphology and of TMEV capsid antigen was good. *In situ* hybridization levels, however, were decreased by a factor of 2 to 3 when compared to ethanol acetic acid or PFG (not shown). With labile antigens, it is possible to use frozen sections picked on treated slides coated with Elmer's white glue. After immunoperoxidase staining, the slides are fixed with ethanol acetic acid or PFG and hybridized *in situ*.

Table 1. Efficiency of *in situ* hybridization alone or after immunocytochemistry

Treatment		Efficiency of *in situ* hybridization, grains per min per cell
Fixative	Dilution of antibody	
Ethanol/acetic acid	—	0.20
PFG	—	0.25
PFG	1:300	0.11
PFG	1:3000	0.22

The sample consisted of TMEV-infected BHK cells harvested 4 hr after infection. Cells were fixed either with ethanol acetic acid or with PFG. Fixed cells were processed for immunocytochemistry using a 1:300 or a 1:3000 dilution of primary anti-TMEV antiserum. After staining with peroxidase substrate (DAB), samples were acetylated. —, Immunocytochemistry and acetylation were omitted. Autoradiography exposure time was 19 hr. For each sample, grains were counted over 50 randomly selected cells.

Immunocytochemistry Followed by *in Situ* Hybridization. The method is predicated on the observation that the insoluble DAB polymer deposited in immunocytochemical reactions withstands the treatments required for *in situ* hybridization (Fig. 1). As expected, detection of antigen had to be done first, as antigens were destroyed by hybridization procedures.

In preliminary experiments, we found that DAB polymer interfered with *in situ* hybridization by binding radioactive cDNA nonspecifically. Fig. 1*A* shows the binding of heterologous [^{3}H]cDNA (cDNA against visna virus genome) to TMEV-infected BHK cells stained for TMEV antigens. We found that this nonspecific binding could be blocked by prior acetylation of the cells or tissue section with acetic anhydride (Fig. 1*B*). Acetylation did not impair the binding of specific TMEV [^{3}H]cDNA (Fig. 1*C*).

The binding of TMEV cDNA after acetylation was prevented by prior digestion of the cells, or sections, with RNase A at 100 μg/ml in 2× NaCl/Cit followed by extensive washing with 2× NaCl/Cit, thus confirming that binding was due to hybridization to RNA (not shown).

The cells used in this experiment were harvested 4 hr after infection at a moi of 10. One hundred percent of the cells contained viral RNA and proteins, although considerable variations in levels were observed from cell to cell, reflecting asynchronous virus replication in a cell population (Fig. 1 *B* and *C*). As expected, cells that contained high levels of viral RNA stained more intensely for viral capsid antigens.

Sensitivity. First, we determined the sensitivity of *in situ* hybridization after fixation with PFG and paraffin embedding. BHK cells were infected at moi of 0.1, 1, 3, and 6.4 pfu per cell, using dilutions of a single virus suspension. The cells were harvested with trypsin at the end of virus absorption (1 hr after infection). Cells infected at moi of 3 and 6.4 also were harvested 2, 4, and 6 hr after infection. An aliquot of each harvest was used to extract cytoplasmic RNA and to measure the number of viral genomes per cell. Copy numbers were obtained by comparing the extent of hybridization of ^{32}P-labeled TMEV cDNA to known amounts of cytoplasmic RNA and purified viral RNA immobilized on nitrocellulose after denaturation with formaldehyde (11, 12). Another aliquot was deposited on treated microscope slides, fixed with PFG, dehydrated, and embedded in paraffin. After re-

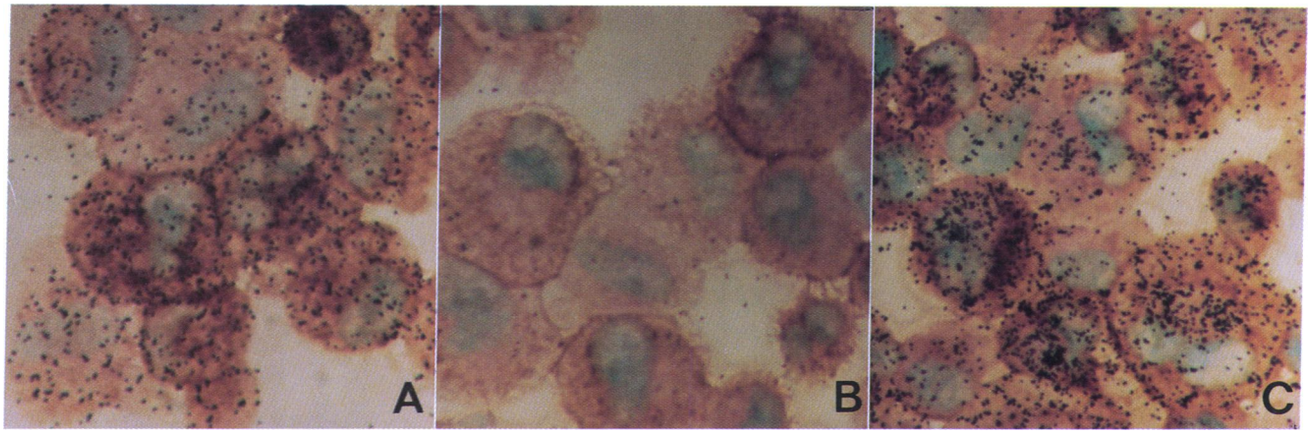

FIG. 1. Simultaneous detection of TMEV capsid antigens and TMEV RNA in infected BHK cells. Cells were harvested 4 hr after infection and fixed with PFG. Immunocytochemical techniques were carried out using anti-TMEV primary antibody diluted 1:300. *In situ* hybridization was carried out using 0.4 ng of [^{3}H]cDNA per μl, with a specific activity of 2×10^8 dpm/μg. Autoradiography exposure time was 19 hr in all cases. Slides were counterstained with methyl green. (*A*) Sample was not acetylated prior to *in situ* hybridization with a cDNA specific for visna virus genome (heterologous cDNA). (*B*) Sample was acetylated before hybridization with visna virus-specific cDNA. (*C*) Sample was acetylated before hybridization with TMEV-specific cDNA.

moval of paraffin, the cells were hybridized *in situ* and the number of grains per cell was counted after suitable times of autoradiographic exposure. Fig. 2 shows the result of the experiment. Cells infected at a moi of 1 contained an average of 120 copies of viral RNA 1 hr after infection, indicating that the particle to pfu ratio was ≈100 in the virus suspension used. As expected, copy numbers 1 hr after infection were proportional to moi. A linear relationship was obtained between the number of grains per cell per minute of autoradiographic exposure and the number of viral genomes per cell. Ten copies per cell were detected in 3 weeks of exposure (≈12 grains per cell over background).

Slides from the same experiment were also used to detect viral antigens by immunocytochemistry. One hour after infection, antigens were detected in 100% of cells infected at a moi of 1 or higher but not in cells infected at a moi of 0.1 (not shown). One pfu corresponds to 120 viral genomes (see above). Since this amount of RNA corresponds to inoculum, the cells contained 7200 copies of each viral structural protein (picornavirus are made of 60 capsomers containing one copy of each four structural proteins). The serum used detected two viral proteins out of four, as shown by immunoblotting (not shown); therefore, immunocytochemistry detected 14,400 molecules of proteins per cell.

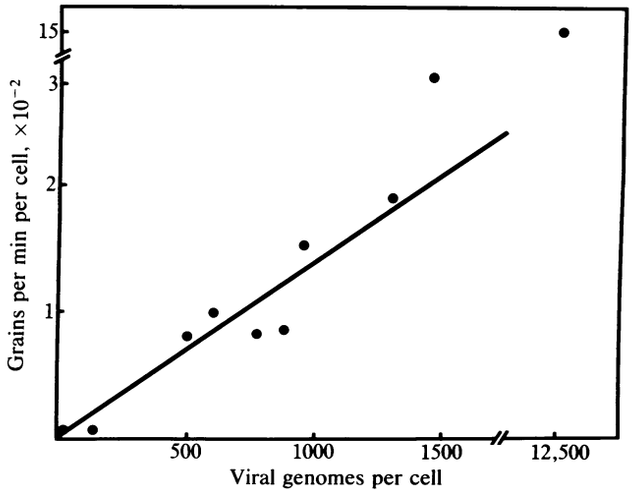

FIG. 2. Sensitivity of detection of viral RNA by *in situ* hybridization after fixation with PFG and paraffin embedding. BHK cells were infected at moi of 0.1, 1, 3, and 6.4 pfu per cell and were harvested 1 hr later. Cells infected at moi of 3 and 6.4 were harvested also 2, 4, and 6 hr after infection. The number of viral RNA genomes per cell was measured by filter hybridization and the level of *in situ* hybridization (grains per min per cell) was obtained by counting grains over 100 randomly selected cells. Background, determined by hybridizing uninfected cells, was subtracted in each case. Autoradiographic exposure times ranged from 3 hr to 28 days. The straight line was computed by linear regression (correlation coefficient = 0.99).

In a separate experiment, we compared the levels of hybridization (number of grains per cell) on infected BHK cells hybridized *in situ* with or without prior detection of viral antigens by immunocytochemistry. Table 1 shows that hybridization level was decreased by a factor of ≈2.5 when we used a 1:300 dilution of primary antibody for immunocytochemistry. We also observed that this decrease in hybridization level was a function of the amount of DAB stain precipitated in the cells. Hybridization levels were raised to control level by using a 1:3000 dilution of primary antibody, with some loss in intensity of staining (Table 1) or by using shorter incubation times with DAB stain solution (not shown). The highest level of sensitivity was obtained when *in situ* hybridization was carried out immediately after immunocytochemistry. We observed that storage of the slides at room temperature for 60 hr between DAB staining and hybridization reduced hybridization levels noticeably.

Simultaneous Immunocytochemistry *in Situ* Hybridization in Paraffin Sections. The simultaneous detection of antigens and RNA sequences would be particularly useful for examination of tissues. To test this technique in this context, we analyzed the spinal cords of SJL/J mice 2 months after intracerebral inoculation with TMEV. At this time, animals enter the late phase of the disease, characterized by primary demyelination and limited virus expression. Infectivity titers in the CNS are very low or nil, and viral antigens are detected only with highly sensitive techniques such as immunocytochemistry.

Mice were perfused with PFG or PLP fixatives and CNS tissues were postfixed and embedded in paraffin. Tissue sections were cut, paraffin was removed, and sections were processed sequentially through immunocytochemistry and *in situ* hybridization, including acetylation. The reagents were anti-TMEV hyperimmune antiserum and TMEV-specific [^{3}H]cDNA. Fig. 3 shows examples of the results obtained with PFG fixative. Morphological details of both gray

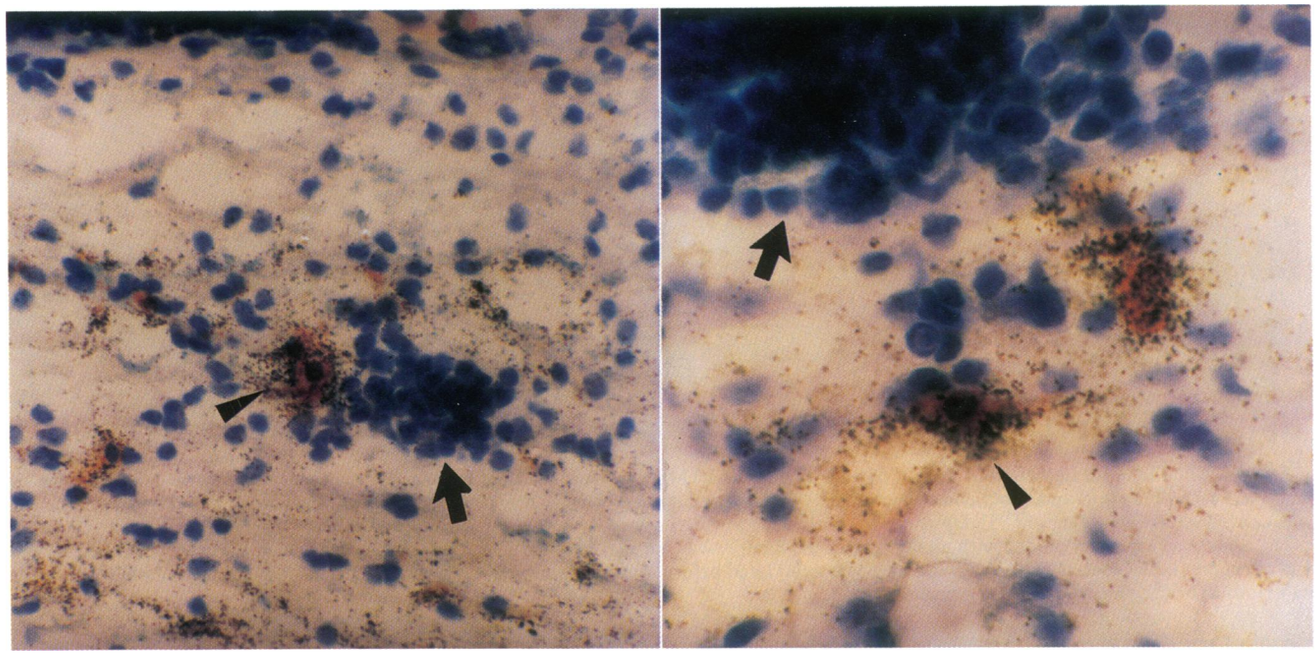

FIG. 3. Simultaneous detection of TMEV capsid antigens and TMEV RNA in paraffin sections of the spinal cord of infected mice. The animals were perfused with PFG 2 months after intracerebral inoculation with TMEV. CNS tissues were embedded, cut, and processed for immunocytochemistry and *in situ* hybridization. Primary anti-TMEV antiserum was diluted 1:300. Slides were hybridized with 0.13 ng of [^{3}H]cDNA per μl specific for TMEV RNA. Autoradiography exposure time was 2 weeks. Counterstain was hematoxylin (2). Arrows point to inflammatory infiltrates; arrowheads indicate cells containing both TMEV RNA and antigens.

and white matter were well-preserved and inflammatory foci were found scattered throughout the white matter of spinal cord. In areas with inflammation, a considerable number of cells contained viral RNA and a much smaller number stained for viral antigens. As expected, cells positive for viral antigens also contained viral RNA.

DISCUSSION

Detection of specific proteins and nucleic acids in the same cell should prove a useful technology for addressing a number of issues in gene expression. We have illustrated the potential of this technique in analyzing one of the central problems of viral pathogenesis—how a virus persists in the face of a vigorous immune response. Restriction in virus gene expression clearly provides one explanation for virus persistence (6), and even in the preliminary analysis presented here, it is evident that the restriction is effected at more than one level. In general, infected cells have only a few copies of viral RNA, and in many cells with RNA, capsid antigens could not be detected.

What is the sensitivity of the simultaneous assay? With a cDNA of specific activity 2×10^8 dpm/μg, we can detect by *in situ* hybridization 10 picornavirus genomes per cell (12 grains per cell above background in 3 weeks of exposure). Since the simultaneous assay decreases hybridization by a factor of 2 (Table 1), 20 genomes per cell can be detected. The sensitivity can be increased by using ^{35}S-labeled cDNA probes. Reverse transcribed cDNA containing ^{35}S-labeled dATP (specific activity, $\approx$1000 Ci/mmol; 1 Ci = 37 GBq) has a specific activity of $\approx 10^9$ dpm/μg, and ^{35}S is 5 times more efficient than ^{3}H in producing grains. The assay described in this paper combines, at the single-cell level, a very high degree of sensitivity for the detection of RNA sequences with the highest sensitivity presently available for the detection of antigenic determinants. Using published protocols for DNA *in situ* hybridization should make it possible to assay DNA and proteins in the same cell.

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1. Brahic, M. & Haase, A. T. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 6125–6129.
2. Haase, A. T., Brahic, M. & Stowring, L. (1984) in *Methods in Virology*, eds. Maramorosch, K. & Koprowski, H. (Academic, New York), Vol. 7, in press.
3. Sternberger, L. A. (1979) *Immunocytochemistry* (Wiley, New York).
4. Theiler, M. (1937) *J. Exp. Med.* **65,** 705–719.
5. Lipton, H. L. (1975) *Infect. Immun.* **11,** 1147–1155.
6. Brahic, M., Stroop, W. G. & Baringer, J. R. (1981) *Cell* **26,** 123–128.
7. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23,** 641–646.
8. Morris, R. G. & Barber, P. C. (1983) *J. Histochem. Cytochem.* **31,** 263–274.
9. McLean, I. W. & Nakane, P. K. (1974) *J. Histochem. Cytochem.* **22,** 1077–1083.
10. Hayashi, S., Gillam, I. C., Delaney, A. D. & Tener, G. M. (1978) *J. Histochem. Cytochem.* **26,** 677–679.
11. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5201–5205.
12. White, B. A. & Bancroft, F. C. (1982) *J. Biol. Chem.* **257,** 8569–8572.