A Fast and Sensitive Method for Detecting Specific Viral RNA in Mammalian Cells

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A quick and sensitive method to quantitate viral RNA synthesis has been developed. Utilizing glutaraldehyde to fix infected cells onto nitrocellulose paper, viral RNA can be probed directly in situ. Viral message can be detected from as few as 10⁴ infected cells. This technique can be used to study viral gene expression and can be adapted to screen the activity of antiviral agents such as interferon.

Advances in the molecular biology of viruses have been greatly facilitated by nucleic acid hybridization techniques. The standard approach often involves the isolation and purification of nucleic acids before transfer to nitrocellulose (NC) paper for subsequent hybridization reaction. Several methods have been developed to bypass the isolation and purification steps. Bresser et al. (1) described the use of NaI to denature and bind DNA and RNA to NC paper. Recently, other methods have been developed that rely on glutaraldehyde fixation and subsequent acetylation of the fixed samples (3, 5, 10). The requirement of the acetylation step limits the cell-immobilizing matrix to polylysine-coated glass slides or glass fiber filters, which are inconvenient to prepare.

We have found that glutaraldehyde can be used to fix cells directly onto NC paper. After treating cells with proteinase K, we can proceed to hybridization without the acetylation step using radioactive probes. Viral RNA can be detected from as few as 6 x 10⁵ infected cells. The procedure was designed to preferentially hybridize the probe to viral RNA, with minimal interference from a homologous DNA background. By using a replica filter treated with RNase, we can differentiate whether the hybridization target is DNA or RNA. We have used this technique to assay the effect of interferon on viral RNA replication.

MATERIALS AND METHODS

Cell lines and viruses. Cell lines and viruses were obtained from the American Type Culture Collection, Rockville, Md. Mouse L-929 fibroblasts and a human amniotic cell line (WISH) were grown in Dulbecco minimal essential medium (GIBCO) supplemented with 100 μg of streptomycin per ml-62 μg of penicillin per ml-2 mM glutamine-5% fetal bovine serum (Hyclone).

Murine encephalomyocarditis (EMC) virus was grown, and titers were determined on L-929 cells. Herpes simplex virus type 1 (HSV-1 strain MP) was grown on African green monkey kidney (Vero) cells, and titers were determined on both Vero and WISH cells.

Virus infection. At 24 h before infection, 2 x 10⁶ cells were plated onto 60-mm-diameter Corning tissue culture dishes. The medium was removed immediately before infection, and the monolayer was rinsed twice with phosphate-buffered saline (PBS). One of the cultures was trypsinized to calculate the total number of cells per dish. Then, 0.2 to 0.4 ml of diluted viral lysate was added to the monolayer at a multiplicity of infection of 1. Virus was allowed to adsorb at 37°C for 30 min, after which 3 ml of medium was added. For EMC virus, the infected cells were incubated for an additional 4 h, whereas for HSV-1, incubation was terminated after 6 h.

Interferon pretreatment of cells. To assay the effects of interferon on EMC virus replication, L-929 cells were treated at 12 h before infection with mouse alpha interferon (Lee Biomolecular) at 0.1, 1, and 10 U/ml. After the interferon treatment, virus was added as described above.

Fixation of cells to NC filter. After incubation, the monolayers were removed from the plastic surface with either a rubber policeman or by trypsinization. Cell concentration was adjusted to 10⁶ cells per ml in PBS. Serial twofold dilutions were made in PBS, and 100 μl of the diluted cells was spotted onto NC paper (Schleicher & Schuell) previously wetted with PBS, in a 96-well manifold filtering apparatus (Bethesda Research Laboratories).

The filter was then fixed in 3% NaCl-10 m NaH₂PO₄-40 mM Na₃HPO₄ (pH 7.4)-1% glutaraldehyde (EM Science) at 4°C for 1 h and rinsed three times with proteolytic buffer (50 mM EDTA, 0.1 M Tris hydrochloride [pH 8]). Fixed cells were digested in proteolytic buffer containing 20 μg of proteinase K (Boehringer-Mannheim) per ml at 37°C for 30 min. The filter was air dried briefly and placed directly in prehybridization buffer.

RNA isolation and Northern transfer. Total RNAs from both infected and uninfected cells were isolated as described by Chomczynski and Sacchi (2). Electrophoresis of RNA by using formaldehyde agarose gel and transfer to NC filter was done according to Maniatis et al. (7).

Hybridization probes. The recombinant plasmid pEM3 containing the major portion of the EMC viral genome was kindly provided by A. Palmenberg (8). The HSV-1 recombinant plasmid pRB201, encoding early genes corresponding to the HindIII H and M fragments of the HSV-1 genome map (9), was kindly provided by B. Roizman, Chicago, Ill.

The probes were radiolabeled by the oligonucleotide random-priming method. Briefly, the DNA (0.2 μg) was denatured by boiling and labeled in a reaction buffer containing 50 mM Tris hydrochloride (pH 7.2), 10 mM MgSO₄, 0.1 M dithiothreitol, 50 μg of bovine serum albumin, 1 mM each of dCTP, dGTP, dTTP, 100 mM α-32PdATP (Amersham). 1 μg of hexaoligonucleotide random primer, and 2 U of Escherichia coli DNA polymerase I Klenow fragment (Boehringer Mannheim) in a total volume of 30 μl. The reaction was incubated at 10°C for 12 h, after which the labeled DNA was purified by ethanol precipitation. This method routinely yields probes with specific activities of 10⁸

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RESULTS

Detection of the viral RNA in situ. To evaluate the in situ hybridization technique, we chose EMC virus, a picornavirus which has high rate of transcription and replicates via an RNA intermediate. Infected samples were harvested at 4 h, at which time maximum viral RNA has accumulated (11). Infected cells were then fixed and probed with \(^{32}\)P-labeled pEM3. The uninfected L-929 cells showed no hybridization, whereas \(6 \times 10^5\) infected cells were sufficient to give a positive signal (Fig. 1A).

To rule out the possibility that the hybridization signal was due to carry-over of the viral RNA from the lysate used to infect cells, a 30-min postinfection sample was included on the filter (Fig. 1A, vir.30 min). This sample showed no hybridization, indicating that the viral RNA detected by this technique was derived from the replication and transcription of EMC virus in infected cells.

We further demonstrated that if we omitted the glutaraldehyde fixation step, the hybridizing signal was drastically reduced (Fig. 1A, unfixed). Furthermore, the viral RNA fixed by glutaraldehyde was still susceptible to degradation by RNase. The addition of 1 mg of RNase (DNase free, Sigma) per ml to the prehybridization and hybridization buffers completely destroyed the target RNA (Fig. 1A, RNase).

As a parallel experiment, we performed a Northern (RNA) blot analysis under the identical conditions (Fig. 1B). The total RNAs isolated from \(5 \times 10^6\) cells were analyzed. The uninfected and 30-min postinfection RNAs show no hybridization signal, whereas the 4-h postinfection RNA shows a single hybridization band at the 7-kilobase position and represents the full-length EMC virus transcripts. The result of this analysis is consistent with the previous in situ hybridization results.

The sensitivity of in situ hybridization allows the detection of viral RNA from a minimum of \(6 \times 10^3\) virus-infected cells. We determined the feasibility of using the standard RNA extraction and Northern blot procedures to detect viral mRNAs from small samples. Total RNA was extracted from \(10^4\) cells and analyzed by Northern blot; however, there was no hybridization signal detected (Fig. 1B, lanes \(1 \times 10^4\)). The latter result could be attributed to low yield and loss of materials, which are common to multistep isolation procedures.

Detection of DNA virus transcripts. HSV-1 was used to test the possibility of using this technique to quantitate RNA transcripts of DNA viruses. The viral mRNAs are present along with several copies of the replicating viral DNA genome. A time-course analysis of HSV-1 DNA replication is presented in Fig. 2, using NaOH to fix the cells to NC filters (6). The results of the RNA experiment are summarized (Fig. 3). Specific viral mRNA was detected at 6 h after infection. To rule out the possibility that such a signal resulted from the hybridization of the probe to replicating viral DNA, a duplicate filter was treated with RNase. It showed no hybridization to the infected cells, indicating that under these conditions, the hybridization material was essentially viral RNA. Single-stranded (unlabeled) probe sequences were spotted on the filter as positive controls and gave comparable hybridization intensities on both filters.
This indicates that the RNase did not destroy the ability of the probe to hybridize to the available target.

**Application to interferon assay.** Since this in situ hybridization assay was very quick and sensitive, we adapted it to titrate the antiviral activity of interferon. L-929 cells were treated with different concentrations of recombinant mouse alpha interferon before infection, after which cells were fixed as described in Materials and Methods. Interferon at a concentration of 1 U/ml showed some inhibition of viral replication as measured by autoradiography (Fig. 4). At 10 U/ml there was a dramatic decrease in the hybridization signal, indicating that this level of interferon significantly inhibits viral RNA synthesis.

**DISCUSSION**

We have described here a fast and reproducible method for the detection of viral RNA in situ. This method is a modification of in situ hybridization techniques described elsewhere (3, 5, 10, 12). The major difference is the use of NC filter paper as the supporting matrix and the omission of the acetylation step.

Glutaraldehyde has been used as a fixative in the preparation of samples for electron microscopy. This compound reacts with proteins and glycogen by cross-linking (4). The interaction between glutaraldehyde and nucleic acids is not completely understood. It should be noted that glutaraldehyde fixation still leaves RNA susceptible to ribonuclease degradation. Thus, care must be taken not to introduce contaminating RNase into the hybridization reactions.

The number of cells immobilized in a dot is important. We frequently observed that if an excessive number of cells is loaded on NC filter paper (i.e., more than 10⁵ cells per 3-mm spot), the sample often detached from the filter during the hybridization. This may be caused by poor fixation resulting from the thickness of the sample. To ensure the linearity of this assay, the optimum number of cells immobilized per dot should be determined for the cell type in use.

The sensitivity of this method makes it applicable for assays measuring inhibition of viral replication, such as interferon or antiviral drugs. We are currently attempting to develop this method to detect levels of oncogene expression in a variety of tumor cells.

One major advantage of this method over others is the simplicity of sample preparation, since most steps are done directly on the NC paper. Under the given conditions, viral double-stranded DNA does not interfere with the hybridization of the probe to RNA, even though a sufficient amount of the viral DNA is present in the cell. Moreover, replica filters of the same samples can be treated with NaOH (6) and then hybridized to the same probe for DNA detection. Thus, parallel experiments on the same cells and virus can detect both viral DNA and RNA.

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**LITERATURE CITED**

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