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Detection of viral sequences of low reiteration frequency by *in situ* hybridization

(visna virus/gene expression)

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ABSTRACT **The sensitivity of *in situ* hybridization has been increased at least 10-fold by hybridizing in cDNA excess, by increasing the diffusion of the cDNA through the cells, by hybridizing at optimum temperature, and by stabilizing hybrids during autoradiography. Saturation of intracellular RNA with [^{3}H]cDNA has been achieved. The assay is quantitative. *In situ* hybridization has been used to detect and quantitate visna virus RNA in infected cells. By using [^{3}H]cDNA with specific activity of 2×10^8 dpm/μg and conditions that reduce background to negligible levels, 10–20 copies of viral RNA per cell can be detected and quantitated after 2 days of autoradiographic exposure.**

In situ hybridization is potentially a very powerful technique for localizing specific genes on chromosomes, following their expression in cells, and detecting and quantitating viral genes in infected cells. Unfortunately, until now the lack of sensitivity of the technique has restricted its use to the study of highly repeated genetic sequences such as the tandemly repeated genes coding for rRNA (1).

Visna disease is a slow persistent infection of sheep caused by a retrovirus (2, 3). During our investigation of the molecular pathogenesis of this disease, we showed that virus persistence is a consequence of restricted gene expression *in vivo* (4). Although proviral DNA is present in the tissues of infected animals, no viral proteins can be detected and the amount of infectious virus synthesized is minimal. In order to analyze this phenomenon further and, in particular, to determine the level at which the restriction is imposed, we have improved the sensitivity of *in situ* hybridization approximately 10-fold. This enables us to detect 1–10 copies of viral genome per cell with radioautographic exposures of a few days. It should now be possible, with this technique, to address a number of important issues in gene regulation by using radioactive cDNA transcribed *in vitro* from purified mRNA.

MATERIALS AND METHODS

Preparation of [^{3}H]cDNA. [^{3}H]DNA complementary to the visna virus genome was prepared in an endogenous reaction in which purified visna virus was used as a source of both RNA-dependent DNA polymerase and viral 70S RNA. The reaction mixture contained 50 mM Tris·HCl (pH 7.4), 8 mM $MgCl_2$, 1 mM dATP, dGTP, and dCTP, 0.1 mM [^{3}H]dTTP at a specific activity of 90 Ci/mmol or greater, 10 mM dithiothreitol, 100 μg of actinomycin D per ml, 0.02% (vol/vol) Triton X-100, and 250 μg of purified visna virus per ml. The total reaction volume was usually 4 ml. The reaction mixture was incubated at 45°C for 2 hr, and the reaction was terminated by addition of EDTA and Sarkosyl to final concentrations of 10 mM and 0.5% (wt/vol), respectively. The mixture was incubated for 10 min at 37°C; nonradioactive dTTP (in 20- to 100-fold excess) and 200 μg of yeast RNA were added, and the mixture was diluted with 1 vol of 150 mM NaCl/20 mM Tris·HCl, pH 7.4/2 mM EDTA. Nucleic acids were precipitated from the mixture by dropwise addition of 1.5 vol of 10 mM cetyltrimethylammonium bromide. After 20 min at 4°C the mixture was centrifuged at 13,000 rpm for 10 min in an RC2B Sorvall centrifuge. The precipitate was dissolved in 1 ml of 1 M NaCl, made 0.3 M in NaOH, and incubated overnight at room temperature. After neutralization and addition of 100 μg of yeast RNA, the nucleic acids were precipitated with 2 vol of ethanol for 1 hr at −80°C. After centrifugation the precipitate was dissolved in 100 μl of 3 mM EDTA. Between 1.5 and 2 μg of [^{3}H]cDNA was recovered at this stage. NaOH, NaCl, and EDTA were then added to final concentrations of, respectively, 0.6 M, 900 mM, and 10 mM, and the sample was layered on top of a 5–20% alkaline sucrose gradient made in 0.6 M NaOH/900 mM NaCl/10 EDTA. The gradient was centrifuged in siliconized nitrocellulose tubes for 15 hr at 40,000 rpm and 20°C in a SW41 rotor. Fractions in the lower two-thirds of the gradient were pooled, HCl was added to neutralize the NaOH, and the [^{3}H]cDNA was precipitated by 2 vol of ethanol after addition of 100 μg of yeast RNA. From 600 to 900 ng of cDNA was recovered. The characterization of the cDNA is described under *Results*. The size of the cDNA was reduced to about 50 nucleotides by incubation at 37°C for 8 min in 50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/1 mM dithiothreitol containing 0.6 μg of electrophoretically pure pancreatic DNase per ml. The reaction was terminated by adding Sarkosyl and EDTA to, respectively, 0.5% and 20 mM and incubating the mixture for 10 min at 37°C. The cDNA was precipitated with cetyltrimethylammonium bromide as described above. The precipitate was dissolved in 200 μl of 1 M NaCl and precipitated with ethanol. The final cDNA precipitate was dissolved in a small volume (usually 10 μl) of 3 mM EDTA and stored at −20°C. The size of this cDNA was determined by electrophoresis in 5% acrylamide gels containing 99% formamide (5).

***In Situ* Hybridization.** Microscope slides were incubated for 3 hr at 65°C in 450 mM NaCl/45 mM Na citrate, pH 7.0 containing 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin (all wt/vol) (6), dipped for 1 sec in water, and then fixed for 20 min at room temperature in ethanol/acetic acid, 3:1. This treatment prevents binding of cDNA to glass (see *Results*).

Sheep choroid plexus cells were trypsinized, washed once in phosphate-buffered saline, and deposited on treated microscope slides by a Shandon Elliott cytocentrifuge. Approximately 20,000–30,000 cells were deposited per slide. After a few minutes of drying, the specimens were fixed for 20 min at room

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Abbreviation: C_rt, initial concentration of RNA in mol of nucleotide per liter multiplied by time in sec; t_{opt}, optimum temperature.

temperature in ethanol/acetic acid, 3:1. The slides were then treated as follows: 20 min at room temperature in 0.2 M HCl, 30 min at 70°C in 300 mM NaCl/30 mM Na citrate, pH 7.0 (7), and 15 min at 37°C in 20 mM Tris·HCl, pH 7.4/2 mM $CaCl_2$ containing 1 μg of proteinase K per ml. Between the different treatments the slides were briefly washed in distilled water. The proteinase K digestion was followed by two washes in distilled water and dehydration in graded ethanol (70% twice, 95% once).

The slides were hybridized with 2 ng of [^{3}H]cDNA per slide for 48–60 hr. The hybridization medium contained 50% deionized formamide, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 600 mM NaCl, 0.02% (wt/vol) Ficoll, 0.02% polyvinylpyrrolidone, 1 mg of bovine serum albumin per ml, 100 μg of sonicated, denatured calf thymus DNA per ml, 1 mg of sheep rRNA per ml, 1 mg of sheep tRNA per ml, and 100 μg of poly(A) per ml. The hybridization mixture, including [^{3}H]cDNA, was heated to 100°C for 20 sec, quickly cooled to 0°C, and prehybridized in liquid for 1 hr at 50°C. Five microliters of the hybridization mixture was placed on the cells and covered with a 12-mm glass coverslip that had been siliconized and heated to 180°C for 2 hr. A zone around the cells and coverslip was delimited with a wax pencil, and this area was covered with a few drops of mineral oil to prevent evaporation. At the end of the hybridization period the slides were dipped twice in chloroform (5 min each time) and then twice in 50% formamide/10 mM Tris·HCl, pH 7.4/1 mM EDTA/600 mM NaCl. This was followed by extensive washing (three times, 2 liters each time, with stirring, for a total of 20 hr) in the same buffer. The slides were then washed twice in 300 mM NaCl/30 mM Na citrate, pH 7.0 (5 min each time), followed by dehydration in graded ethanol (70% and 90% ethanol containing 300 mM ammonium acetate). All washes were done at room temperature.

Kodak NTB-3 nuclear track emulsion was melted at 45°C and diluted 1:1 with 600 mM ammonium acetate. The slides were dipped in melted emulsion for 1–2 sec and allowed to dry in an upright position for 1 hr at room temperature. They were transferred to light-proof boxes containing silica gel desiccant and exposed at 4°C. The slides were developed in Kodak D-19 developer for 3 min at room temperature followed by a brief wash in 1% acetic acid and fixation for 3 min in Kodak fixer. After the slides were washed in distilled water, they were stained for 30–45 min with Giemsa stain diluted 1:50 in 10 mM phosphate buffer.

Liquid Hybridization. [^{3}H]cDNA was hybridized in liquid to a vast excess of purified cellular RNA as described (8).

RESULTS AND DISCUSSION

Characterization of [^{3}H]cDNA. In order to maximize the sensitivity of *in situ* hybridization the [^{3}H]cDNA must have high specific activity and a complexity equivalent to that of the viral genome from which it is copied. By using [^{3}H]dTTP (specific activity ≈90 Ci/mmol) as a radioactive precursor, we labeled the cDNA to a specific activity of 2.4×10^8 dpm/μg. A transcript with a complexity equivalent to that of its template was obtained by using a high concentration of nucleotide precursors (9), by incubating at 46°C (10), and by selecting from an alkaline sucrose gradient transcripts longer than 500 nucleotides. This cDNA had a modal length of ≈1000 nucleotides, as estimated by electrophoresis in polyacrylamide gels containing 99% formamide, and hybridized stoichiometrically to 70S [^{32}P]RNA. At a DNA:RNA ratio of 1, 100% of the 70S RNA was rendered resistant to RNase, demonstrating that the cDNA is a faithful copy of its template (data not shown). By contrast, shorter cDNA was less complex and was heavily contaminated with anti-complementary strand DNA, as shown by its ability to self-anneal (data not shown). With an excess of viral 70S RNA the long cDNA transcripts hybridized to 100% and the C_rt value for 50% hybridization was 10^{-2} mol·sec·liter^{-1}. (C_rt is the initial concentration of RNA in mol of nucleotides per liter multiplied by the time in sec.) We also found that about 20% of the cDNA hybridized to sheep rRNA or sheep tRNA. Although the extent of contamination by cellular sequences varied, this surprising result was obtained with all cDNA preparations and necessitated the prehybridization step in the experimental protocol.

Reducing the Background of *In Situ* Hybridization. Increasing the specific activity of the cDNA to levels in excess of 10^8 dpm/μg resulted in a concomitant increase of background due to nonspecific binding of the cDNA to the cells and the glass slide. Binding of the cDNA to glass was eliminated by treating the slides with serum albumin, Ficoll, and polyvinylpyrrolidone and by including these components in the hybridization medium. Binding to the cell was due to hybridization of cDNA to rRNA and tRNA (see above) and also to nonspecific binding to unidentified cellular components. It was eliminated in part by prehybridizing the cDNA in liquid with sheep rRNA and tRNA and by adding single-stranded heterologous DNA to the hybridization mixture. Finally, extremely low backgrounds were achieved by extensive washing of the slides after hybridization in a large volume of hybridization buffer. These maneuvers reduced background to four to eight grains per uninfected cells after autoradiographic exposures of several weeks (Fig. 1).

Increasing the Efficiency of *In Situ* Hybridization. During this study we found that homologous sequences can be annealed to saturation levels by hybridization *in situ* by (*i*) increasing the diffusion of the cDNA through the specimen, (*ii*) optimizing the temperature of hybridization, (*iii*) stabilizing hybrids during treatments after hybridization, and (*iv*) hybridizing with an excess of cDNA in order to drive the reaction with the nucleic acid in solution.

The rate of diffusion of the cDNA was increased by treatments designed to remove some proteins from the cells without altering their morphology. The slides were treated with 0.2 M HCl followed by heat, as already described by others (7), then subjected to a limited digestion with proteinase K (see *Materials and Methods*). These treatments increased efficiency 2-fold (Table 1), with no appreciable loss of RNA from the cells (see below) or alteration in morphology (Fig. 1). The diffusion of the cDNA during hybridization is also a function of its size. Fig. 2 shows that by reducing the size of the cDNA to about 50 nucleotides by a limited DNase digestion, the efficiency of *in situ* hybridization was increased 3-fold.

Table 1. Effect of treatments before and after hybridization on the efficiency of *in situ* hybridization

Sample	Grain/min per cell*
Complete procedure	0.42
Without pretreatments†	0.19
Without post-treatments‡	0.12
Control§	0.00091

Sheep choroid plexus cells were infected with visna virus for 72 hr, then hybridized *in situ* and exposed for 1 hr.

* A total of approximately 1500 grains per slide were counted. Two slides were used for each sample described.

† Pretreatment of the slide with 0.2 M HCl, heat, and proteinase K was omitted.

‡ Ammonium acetate was omitted from the ethanol solutions and from the Kodak NTB-3 emulsion.

§ No pre- or post-treatment. Hybridization was done in the same medium as for the complete procedure, but the temperature was 45°C and the cDNA was ≈1000 nucleotides.

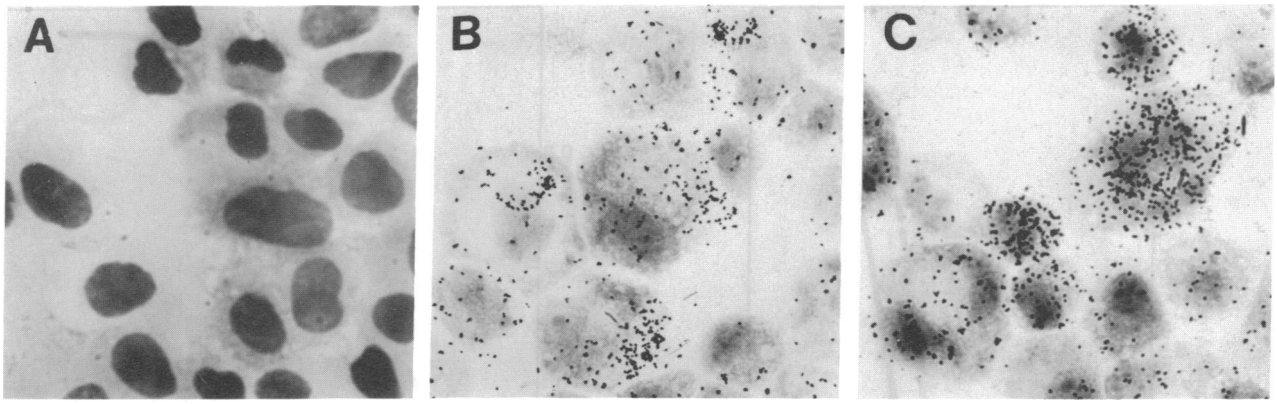

FIG. 1. *In situ* hybridization: uninfected sheep choroid plexus cells (*A*) or cells infected for 1 hr (*B*) or 72 hr (*C*). Cells were hybridized *in situ* with 2 ng/5 μl per slide of [^{3}H]cDNA (specific activity 2.3×10^8 cpm/μg). Cells in *B* contained an average of 20 copies of viral genome per cell; those in *C* contained an average of 3500 copies of viral genome per cell. Autoradiographic exposure was 1 hr for cells in *C* and 48 hr for those in *A* and *B*. (Final magnification ×640.)

It has generally been assumed that the optimum temperature (t_{opt}) for *in situ* hybridization is identical to that determined from filter hybridization (11); for visna virus RNA·DNA hybrids (42% G·C) this would be 45°C in 50% formamide/600 mM NaCl (12). Indeed, most published *in situ* hybridizations done in 50% formamide/high salt have been performed at temperatures ranging between 40° and 50°C (13). The data presented in Fig. 2 demonstrate that the t_{opt} for *in situ* hybridization is much lower. At 20–30°C the efficiency was 20-fold higher than at 45°C.

Hybrids formed *in situ* are detected by autoradiography. For this purpose the slides are dipped at 45°C in photographic emulsion. Although, in principle, RNA·DNA hybrids should be stable under these conditions, in practice hybrids may melt in water at room temperature (D. Spector, personal communication). We therefore included 300 mM ammonium acetate in the ethanol solutions used for dehydration prior to dipping and in the photographic emulsion itself. This salt was chosen because it is volatile, thereby avoiding the deposition of salt crystals on the slides. Table 1 demonstrates that this procedure increased the efficiency of the method 3- to 4-fold.

Specificity of *In Situ* Hybridization. Because of the surprisingly low temperature that was optimal for *in situ* hybridization, we were concerned about the specificity of the reaction. We therefore examined the thermal stability of hybrids formed *in situ* under the conditions that maximize annealing. The hybrids melted at a temperature of 50°C (Fig. 3) and with a sharp melting profile expected of DNA·RNA hybrids. The slope of the thermal transition curve for DNA·RNA hybrids formed under the most stringent conditions (visna virus 70S RNA·cDNA hybrids formed at 68°C in 0.6 M Na^+) was identical (Fig. 3). The melting temperature (t_m) was about 5°C higher, consistent with 3–5% base mismatching *in situ*. (14). Additional more compelling evidence for specificity is the fact that uninfected cells do not contain virus-specific sequences (8) and do not hybridize *in situ* (Fig. 1). Thus, an absolute criterion for specificity inherent in this system is satisfied.

Kinetics. The theoretical upper limit in efficiency of *in situ* hybridization is achieved when the sequences to be detected have been saturated with [^{3}H]cDNA. This can be attained if the cDNA in solution is in excess to drive the hybridization reaction. Another advantage of hybridizing in cDNA excess is that, as for filter hybridization (12), the kinetics of hybrid formation *in situ* is pseudo-first order with respect to the concentration of cDNA and, therefore, is independent of the amount of viral RNA on the slide (13). This has two important consequences: (*i*) the sole limitation to the detection of sequences of low reiteration frequency (single genes) is the specific activity of the cDNA and (*ii*) one can determine the time required to reach maximum hybridization regardless of the amount of viral RNA present in the specimen studied. For the visna virus genome, the kinetics of *in situ* hybridization in cDNA excess is shown in Fig. 4 as a plot of the extent of hybridization (number of grains per cell per min of radioautographic exposure) and C_0t, the product of the concentration of [^{3}H]cDNA and time corrected to standard conditions (15). Maximum hybridization is obtained at a C_0t value of $\approx 10^0$ mol·sec·liter^{-1}.

The rate of reaction *in situ* ($C_0t\ 1/2 \approx 1.5 \times 10^{-1}$ mol·sec·liter^{-1}) is about 1/10th that in liquid with cDNA in excess (data not shown) or 70S RNA in excess (8). Similar reductions of an order of magnitude or more have been reported for comparisons of liquid annealing with filter hybridization (16) and filter

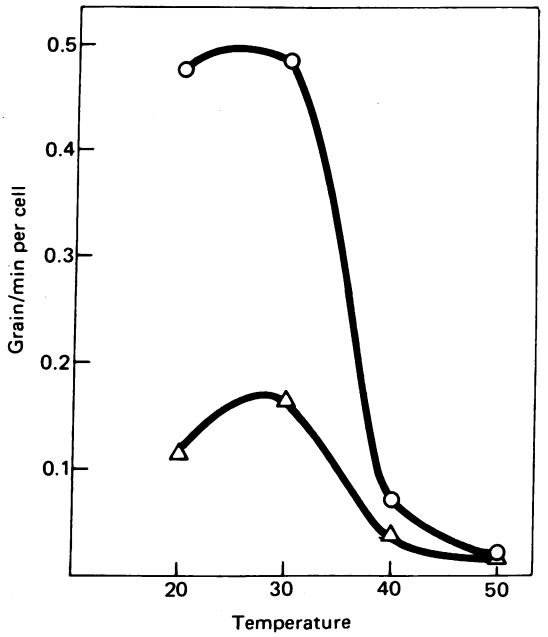

FIG. 2. Effect of temperature and size of [^{3}H]cDNA on the efficiency of *in situ* hybridization. [^{3}H]cDNA of modal length 500 (Δ) or 50 (O) nucleotides was hybridized *in situ* to cells infected for 72 hr as described in *Materials and Methods* except that the temperature of hybridization was varied. Extent of hybridization was determined by counting the number of grains present over the cells. It is expressed as the number of grains per min of autoradiographic exposure per cell. An average of 1500 grains were counted for each point.

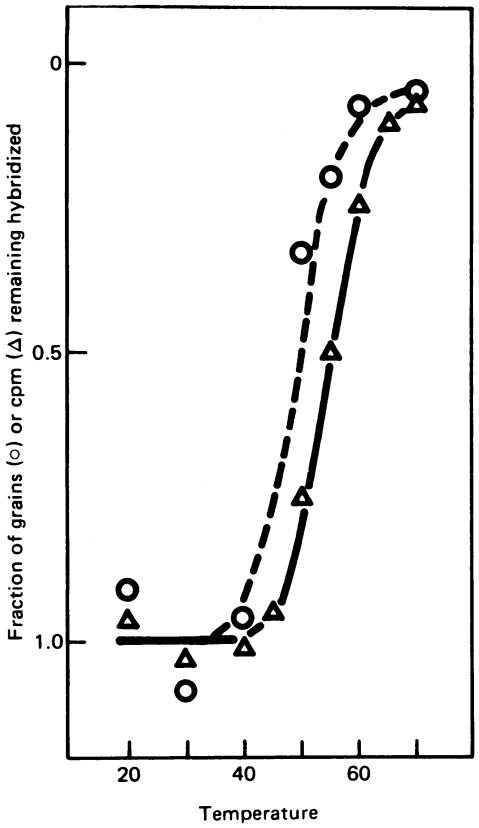

FIG. 3. Melting profiles of hybrids formed *in situ* (○) or in liquid under stringent conditions (△). Cells infected for 72 hr were hybridized *in situ* as described in *Materials and Methods* except that at the end of the hybridization the slides were incubated for 5 min in 50% formamide/300 mM NaCl/30 mM Na citrate at the temperatures indicated, followed by rapid cooling to 4°C in 300 mM NaCl/30 mM Na citrate. The usual procedure for washing the excess of cDNA and dipping in emulsion was then resumed. An excess of visna 70S RNA was hybridized to [^{3}H]cDNA to a C_rt of 2×10^{-1} mol·sec·liter^{-1}. The hybridization conditions were 68°C in 0.6 M NaCl/10 mM Tris·HCl, pH 7.4/2 mM EDTA/0.1% sodium dodecyl sulfate. The hybrid was precipitated with ethanol and dissolved in 50% formamide/300 mM NaCl/30 mM Na citrate, and aliquots were incubated for 10 min at the temperatures indicated, followed by quenching in ice water. The fraction of cDNA remaining hybridized was followed by its resistance to digestion with S1 nuclease (8).

hybridization with *in situ* hybridization (13). From the evidence presented (16), the slowed rate of reaction in solid-phase hybridization probably reflects constraints on diffusion of the reacting nucleic acids.

Quantitation. The number of grains developed after *in situ* hybridization is proportional to the amount of hybrid formed (13), which, at saturation, is equivalent to the amount of RNA sequences to be detected. This provides the basis for using the technique described in this article in a quantitative manner. We verified that *in situ* hybridization is quantitative by performing the following experiment. Sheep choroid plexus cells were infected with visna virus at a multiplicity of 3 plaque-forming units per cell and harvested after 1, 24, and 72 hr of infection. An aliquot of the cells was deposited on microscope slides and hybridized *in situ* to a C_0t of 1.5×10^0 mol·sec·liter^{-1}. Total RNA was extracted from the remainder of the cells, and the number of viral genomes per cell was determined by liquid hybridization (8). The relationship between the number of viral genomes and the number of grains developed is presented in Fig. 5. As expected, the straight line connecting the experimental points in Fig. 5*A* passes through the origin. The relationship between grain count and copy number shown in Fig.

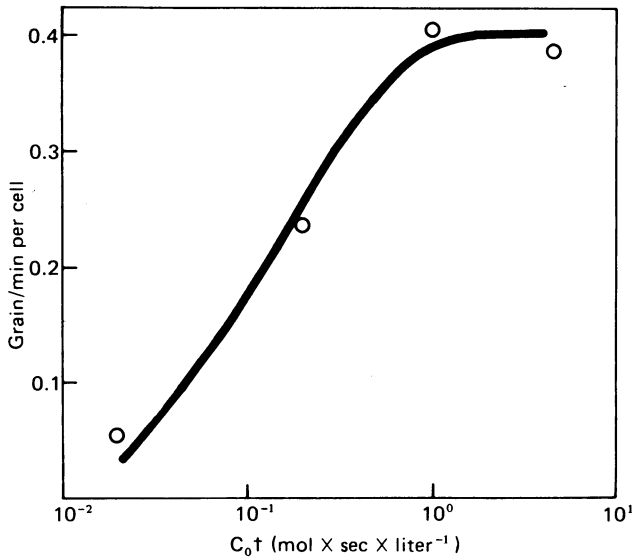

FIG. 4. Kinetics of *in situ* hybridization. [^{3}H]cDNA was hybridized *in situ* to cells infected for 72 hr. Concentration of [^{3}H]cDNA was 3 ng/μl; 5 μl was used per slide. Time of hybridization was varied and ranged from 7 min to 22 hr. An average of 1500 grains were counted for each point.

5*B* can be used to estimate the amount of viral RNA per cell over 3 orders of magnitude. At high copy number, the relationship deviates from linearity because the cDNA is no longer in vast excess. The last point of the curve corresponds to 3900 copies of viral RNA per cell, which is equivalent to 1.3 ng of viral RNA per slide (one viral genome = 1.7×10^{-8} ng of RNA; there are $\approx 2 \times 10^4$ cells per slide), a value close to the amount of cDNA per slide (2 ng). A linear relationship does obtain even at high copy numbers with 15 ng of cDNA per slide (data not shown); because this would be expensive in practice, we prefer to determine copy numbers from a calibration curve.

Efficiency of the Method. The slope of the straight line in Fig. 5*A* (5.8×10^{-4} grain per min per viral genome) gives the efficiency of *in situ* hybridization. This value can be compared to the maximum theoretical efficiency obtained by saturation hybridization. Knowing that one viral genome is equivalent to 1.7×10^{-11} μg of RNA and that the specific activity of the [^{3}H]cDNA was 2.4×10^8 dpm/μg, and assuming that the efficiency of autoradiography is 10% (1), saturation hybridization should yield 4.1×10^{-4} grain per min per viral genome. Con-

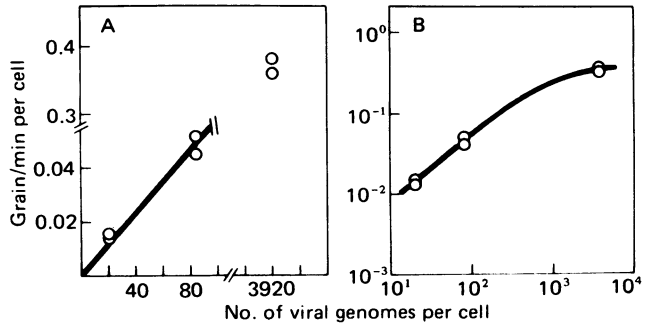

FIG. 5. Relationship between the amount of viral RNA present per cell and the number of grains developed after *in situ* hybridization. Infected cells were harvested at different times after infection and subjected in parallel to *in situ* hybridization and to determination of viral RNA content by liquid hybridization (8). The two points shown for each value of viral RNA content correspond to two independent experiments. An average of 1500 grains were counted for each point. (*A*) Linear plot; (*B*) logarithm-logarithm plot of same data.

sidering the error existing on grain counts and the uncertainty on the value of autoradiography efficiency, this value is close to that determined experimentally. The efficiency of the method described in this paper is therefore close to 100%. This is about 10-fold higher than previously reported in systems in which direct comparisons can be made. For example, Harrison *et al.* (17) detected globin mRNA *in situ* at an efficiency of 5%; similarly, in the recent quantitative study of *in situ* hybridization, sequences coding for ribosomal and 5S RNA were detected at an efficiency of 6–12% (13).

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