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Amplification and detection of lentiviral DNA inside cells

(polymerase chain reaction/*in situ* hybridization/visna virus)

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ABSTRACT **Visna virus and human immunodeficiency virus are prototypes of animal and human lentiviruses, respectively, that persist and are disseminated despite the host immune response because cells in the tissues and the bloodstream harbor viral genomes in a covert state. To facilitate identification of these latently infected cells, the polymerase chain reaction has been adapted to amplify viral DNA in fixed cells for detection by *in situ* hybridization. By using a multiple primer set that generates DNA segments with overlapping cohesive termini, visna virus DNA can be amplified, retained, and detected in infected cells with sensitivities that exceed those of existing methods by more than 2 orders of magnitude. This advance in single-cell technology should prove useful in diagnosing and gaining insight into the pathogenesis of viral infections and provide new opportunities to look for viruses in chronic diseases of unknown etiology.**

The lentiviruses are a subfamily of retroviruses responsible for slowly evolving infections, such as AIDS in humans (1) and visna in sheep (2), in which the causative agents elude host defenses by residing inside cells in an immunologically "silent" state where too little viral antigen is produced for efficient detection and destruction of the infected cell by immune surveillance mechanisms (2, 3). We have used *in situ* hybridization to identify the relatively infrequent cell (1 in 10^2–10^6 or more) harboring viral genomes in this covert state (4) and now describe a technique combining polymerase chain reaction (PCR)-mediated amplification of viral genes inside cells with *in situ* hybridization. This method greatly increases the power of *in situ* hybridization to detect infected cells and should prove useful in following the course of slow, persistent, and latent infections.

MATERIALS AND METHODS

Experimental System. To develop a single-cell method capable of exploiting the power of the PCR (5, 6) to amplify low-abundance target sequences, we chose an experimental system particularly well-suited for method development, quantitative analysis, and safety, visna virus infection of tissue culture cells. In tissue culture, in contrast to the slow infection in animals, growth of virus is rapid; there is extensive synthesis of viral DNA, and the time course and extent of synthesis in individual cells and the population as a whole are known in considerable detail from previous studies employing *in situ*, solution, and solid-phase hybridization techniques (7).

Infection and Fixation. For these studies, permissive hosts, sheep choroid plexus (SCP) cells, were infected at a multiplicity of 3 plaque-forming units of visna virus (equivalent to about 30 copies of viral RNA) per cell and collected shortly after infection (3 hr), when 1–2 copies of viral DNA per cell have been reverse-transcribed from incoming viral RNA genomes; near the end of the latent period (20–24 hr), when the 20–40 RNA genomes introduced into the cell have been copied into DNA; and during exponential growth (48–66 hr), when superinfecting virions provide an additional increase in extrachromosomal DNA to 100 copies or more of viral DNA per cell (7). The cells were harvested by trypsinization, pelleted (600 × *g* for 5 min), washed once in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS-CMF), and fixed for 20 min at ambient temperature in freshly prepared 4% (wt/vol) paraformaldehyde in PBS-CMF (4). The cells were then centrifuged at 1600 × *g* for 5 min, resuspended in a small volume of PBS-CMF, diluted 25-fold in 70% (vol/vol) ethanol, and stored at 4°C.

PCR *in Situ*. Prior to amplification, aliquots containing 2 × 10^6 cells were pelleted, resuspended in PBS-CMF, and allowed to rehydrate for at least 20 min at ambient temperatures. After centrifugation, the pellets were suspended in 100–400 μl of PBS-CMF, transferred to 0.5-ml microcentrifuge tubes, and centrifuged at 1600 × *g* for 5 min. The PBS-CMF was aspirated, and the pellets were resuspended in 100 μl of a PCR reaction mixture consisting of 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleoside triphosphates, and 0.1 μM primers. The microcentrifuge tubes were placed in the heating block of a Perkin–Elmer/Cetus DNA thermal cycler, and DNA in the cells was denatured at 94°C for 10 min, followed by cooling to 4°C. *Thermus aquaticus* (*Taq*) DNA polymerase (0.5 μl containing 2.5 units of enzyme) was added, the cells were resuspended in the reaction mixture, and the reaction mixture was covered with 50–100 μl of mineral oil. The tubes were returned to the thermal cycler for 25 cycles of amplification (denaturation for 2 min at 94°C, annealing for 2 min at 42°C, extension for 15 min at 72°C). An additional 2.5 units of enzyme was added and the samples were subjected to 25 additional cycles of amplification.

Detection of Amplified DNA by *in Situ* Hybridization. The tubes containing the cells and PCR reaction mixture were centrifuged at 16,500 × *g* for 5 min, and the oil and reaction mixture were removed by aspiration. The pellets were suspended in 100 μl of PBS-CMF, transferred to 1.5-ml microcentrifuge tubes, and centrifuged at 16,500 × *g* for 5 min. After the PBS-CMF was aspirated, the pellets were suspended in 0.6 ml of PBS-CMF (for the nominally 2 × 10^6 cells), and the cells were then deposited by cytocentrifugation (450 rpm for 5 min, Shandon cytocentrifuge) onto two glass slides. Viral RNA was digested with ribonucleases A (100 μg/ml) and T_1 (10 units/ml) prior to postfixation of the cells for 2 hr in 4% paraformaldehyde to improve retention during subsequent denaturation, hybridization, and washing (4). After the DNA was denatured in 95% formamide/0.1× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0) for 15 min at 65°C, the slides were cooled to 4°C in 0.1× SSC, dipped in water, and dehydrated in graded ethanol solutions. Virus-specific probe (10^6 dpm) labeled with ^{125}I (see next

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Abbreviations: PCR, polymerase chain reaction; HIV, human immunodeficiency virus; SCP, sheep choroid plexus; MPS, multiple primer set; nt, nucleotide(s).

section) was added in 5 μl of a hybridization mixture containing 50% formamide, 0.6 M NaCl, 20 mM Hepes buffer (pH 7.2), 1 mM EDTA, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 5% polyethylene glycol, 100 μM aurintricarboxylic acid, and 500 μg of yeast RNA per ml. After incubation at 37°C for 12 hr, unreacted probe was removed from the slides by washing. The slides were then coated with nuclear track emulsion (Kodak NTB-2), developed after exposures at 4°C for designated times, and stained with hematoxylin and eosin according to established protocols (4).

^{125}I-Labeled Probes. Virus- and region-specific probes were prepared by nick-translation of cloned viral DNA with 5-[^{125}I]iodo-dCTP as precursor or by PCR using specific primer sets, at 1 μM, and 5-[^{125}I]iodo-dCTP as a precursor, as described by Schowalter and Sommer (8). The PCR probes were prepared as follows. To 19 μl of a reaction mixture as described above but lacking dCTP was added 1 μl containing 2 ng of bacteriophage λ DNA as carrier and 1 ng of cloned visna DNA (9) (10-kilobase genome) in plasmid pBR322 that had been linearized with restriction endonuclease *Cla* I. The reaction mixture was used to dissolve 0.5 mCi of lyophilized 5-[^{125}I]iodo-dCTP (2200 Ci/mmol; 1 Ci = 37 GBq) to give a final concentration of about 10 μM. The DNA was denatured, *Taq* polymerase was added, and the PCR was carried out for 50 cycles (denaturation for 1 min at 94°C, annealing for 2 min at 50°C, extension for 3 min at 72°C). The reaction mixture was removed from the thermal cycler, and the products were separated by gel filtration chromatography on Sephadex G-50 and concentrated by precipitation with ethanol and carrier RNA. The final probe products had specific activities of 3.7 × 10^9 dpm per μg and were reduced to about 150–300 base pairs (bp) in length as a consequence of radiolysis.

RESULTS AND DISCUSSION

PCR-Mediated Amplification in Cells. Despite attempts initiated shortly after the original description of PCR (5) to

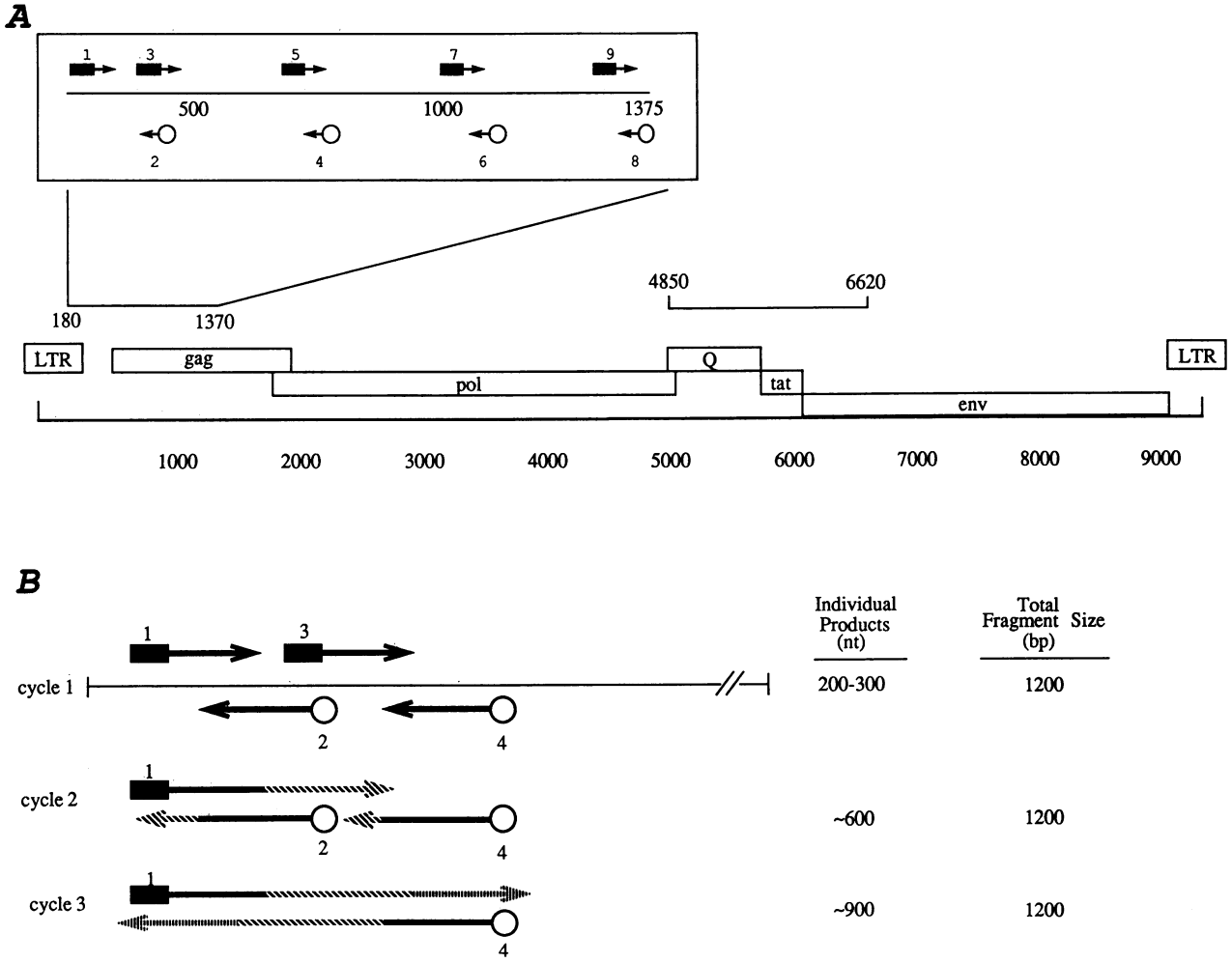

FIG. 1. (*A*) Schematic diagram of the visna genome and the location of amplified and nonamplified regions and primers. The long terminal repeats (LTRs) and the structural, replicative, and regulatory genes of visna virus DNA (10) are shown in the line diagram with an approximate scale in base pairs. The amplified region [nucleotides (nt) 180–1370] is shown above the line diagram. The odd numbered plus-strand primers are above this segment, with the 5′ → 3′ direction of polymerization indicated by an arrow (▬→). The minus-strand primers are shown below the segment (←○). The 5′ coordinates of the 20-nt primers are as follows: 1, 180; 2, 420; 3, 375; 4, 770; 5, 700; 6, 1070; 7, 1000; 8, 1370; 9, 1300. Another segment, nt 4850–6620, which was not amplified, was used as a control. Region-specific probes (nt 180–1370 and 4856–6620) were prepared with the PCR and flanking primer pairs (primers 1 and 8 for nt 180–1370). (*B*) Possible mechanism of amplification *in situ* with a multiple primer set (MPS) that will generate DNA products with cohesive termini. In cycle 1, extension of the primers (▬→; ←○) yields plus- and minus-strand DNA products of 200–300 nt, which, through base pairing in the region of the overlaps, produce a noncovalently linked structure equivalent in size to the entire 1200-bp region. In cycle 2, the DNA products are extended another 200–300 nt (—►). At each successive cycle, this process of elongation of existing strands, displacement synthesis, and base pairing in the overlaps maintains the fragment size and increases the yield of large covalently linked segments (estimates of the size of individual products are shown at right). To simplify the figure, only the left end of the region is shown.

exploit the technique at the single-cell level, we were only recently successful in doing so as a result of finding the special set of conditions that would satisfy the antithetical requirements of *in situ* amplification. The requirements are antithetical because amplification in cells would be expected to be maximized by increasing access of *Taq* polymerase and primers to target sequences in a short segment of DNA free of physical barriers. These are also conditions that are optimal for *in situ* hybridization, where the manipulations of the cells and probes are designed to overcome the constraints of diffusion-related processes peculiar to fixed cells (4), such as permeabilizing treatments and short probes. By contrast, the expected optimal conditions for retention of amplified DNA would be long segments crosslinked at intervals to proteins. Both sets of conditions can be satisfied in principle by using multiple primer pairs (Fig. 1) to amplify overlapping segments of DNA with cohesive termini. In the first few cycles, the polymerase need only transcribe and extend segments of about 300 nt since base pairing in the overlap of complementary strands will maintain a total fragment network of about 1200 bp. In subsequent cycles, the initial DNA products will be extended to form covalently linked segments of 1200 bp.

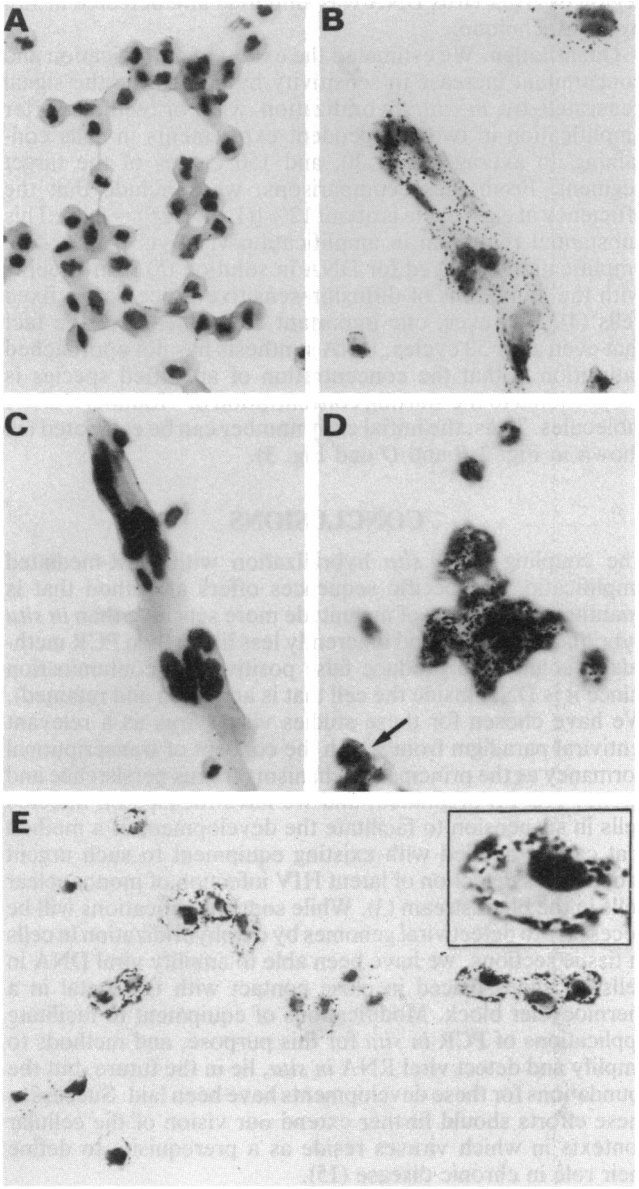

FIG. 2. Amplification and detection of visna virus DNA by *in situ* hybridization. SCP cells were infected, collected, and fixed as described in *Materials and Methods*. Viral DNA was amplified *in situ* and the cells were deposited on slides for ribonuclease digestion, postfixation, and *in situ* hybridization to region-specific probes labeled with ^{125}I. After washing and radioautography, the cells were stained and photographed. (*A*) Uninfected cells. (*B–D*) Multinucleated giant cells in cultures infected for 20 hr (*B*) or 48 hr (*C* and *D*). In *B* and *D*, amplification was with visna virus-specific multiple overlapping primers. The arrow in *D* indicates an uninfected cell in the field with two to three silver grains for comparison with the infected cell. In *C*, amplification was with random primers unrelated in sequence to visna virus DNA; omission of *Taq* polymerase, omission of visna virus-specific primers, or hybridization to a visna virus-specific probe in the region of nt 4850–6620 gave similar results to those shown in *C*. (*E*) Infected cells (20 hr), amplification with visna virus-specific primers that generate a 600-bp fragment (primers 1 and 4 in Fig. 1). The *Inset* emphasizes the ring-like pattern of grains in the radioautograph associated with amplification of a smaller segment of DNA. Exposure times were 3 hr (*A* and *C–E*) or 20 hr (*B*). (*A–D*, ×270; *E*, ×200; *Inset*, ×400.)

The length of the amplified product in cells indeed turned out to be as critical as this speculative model suggests. Whereas in *in situ* hybridization maximal efficiency is generally attained with probes of 100–200 bp and falls off rapidly with probes larger than 400–500 bp (4), the maximum signal in *in situ* amplification was generated with the 1200-bp segment transcribed with the visna MPS (Fig. 2 *B* and *D*). With primer pairs that generate products of about 300, 600, 900, and 1200 bp, respectively, we found that little amplified product was retained in the cell at 300 bp but the 600-bp segment was retained, albeit at an efficiency of about 5% of that achieved with the MPS. The silver grains were also concentrated in a ring-like pattern at the perimeter of the infected cells (Fig. 2*E*) or scattered about the cells (Fig. 2 *A* and *C*) as a consequence, we think, of binding of the probe to amplified DNA that had leaked from the cells. The 900-bp segment was amplified and retained at about one-half to one-third the efficiency achieved with the MPS (data not shown). In contrast to the results with the MPS, efficiency decreased by a factor of 10–20 at 1200 bp (when a single primer pair was used). This is in accord again with the model, which predicts just such an increase and then decrease in signal as the length of the DNA segment to be amplified reaches a point where the probability of unimpeded transcription of a segment of DNA inside a fixed cell is small. As

Table 1. Specificity of PCR-mediated amplification of viral DNA in cells

Cells	MPS*	*Taq* polymerase	Probe	Result†
Uninfected	Visna	+	Visna nt 180–1370	−
Infected	Visna	+	Visna nt 180–1370	+
	Non-visna	+	Visna nt 180–1370	−
	Visna	−	Visna nt 180–1370	−
	Visna	+	Visna nt 4840–6620	−
	Visna	+	HIV-specific	−

Uninfected SCP cells or SCP cells infected for 48–66 hr were fixed, and DNA was denatured and amplified *in situ*. After ribonuclease digestion and postfixation (see Fig. 2), the cells were hybridized *in situ* to ^{125}I-labeled probes prepared by nick-translation (11) of a segment of visna DNA corresponding to nt 180–1320 or by PCR amplification of cloned DNA with flanking primer pairs. PCR probes specific for a region other than the amplified segment in visna (nt 4840–6620) or for human immunodeficiency virus (HIV) were prepared as described (8).

*The visna MPS consisted of primers 1–9 (see Fig. 1); the non-visna MPS was a set of five plus-sense and four minus-sense primers of 20-nt sequences unrelated to visna virus.

†+, Grain counts 150–300 times those obtained with *in situ* hybridization without amplification; −, grain counts equivalent to *in situ* hybridization without amplification.

we also anticipated, the kinetics of DNA synthesis *in situ* were considerably slower than in solution, with an optimum extension time of 15 min.

Reproducibility and Specificity of Amplification *in Situ*. We reproducibly amplified visna DNA inside cells in 10 independent experiments and confirmed the specificity of amplification and detection as shown in Table 1 and Fig. 2 *A* and *C*. Uninfected sheep cells (Fig. 2*A*) did not yield detectable signal over background at radioautographic exposures (2–20 hr) that produced a large number of grains over background in infected cells (Fig. 2 *B* and *D*) in which viral DNA had been amplified and detected with nick-translated or PCR probes specific for the amplified region. Omission of *Taq* polymerase or visna virus-specific primers or substitution of a MPS of nine essentially random primers of equivalent size (20-mers) and base composition but complementary to sequences other than visna virus (e.g., sequences encoding actin, hemoglobin, etc.) produced only background levels of signal in infected cells (Fig. 2*C*). Similarly, a probe specific for a nonamplified region of the visna genome (nt 4850–6620) or a probe specific for HIV did not produce signal over background in cells that contained DNA amplified with multiple primers spanning nt 180–1370. These results, and the ribonuclease treatment prior to hybridization, all support the conclusion that a specific region of visna virus DNA was amplified and detected *in situ* by this technique.

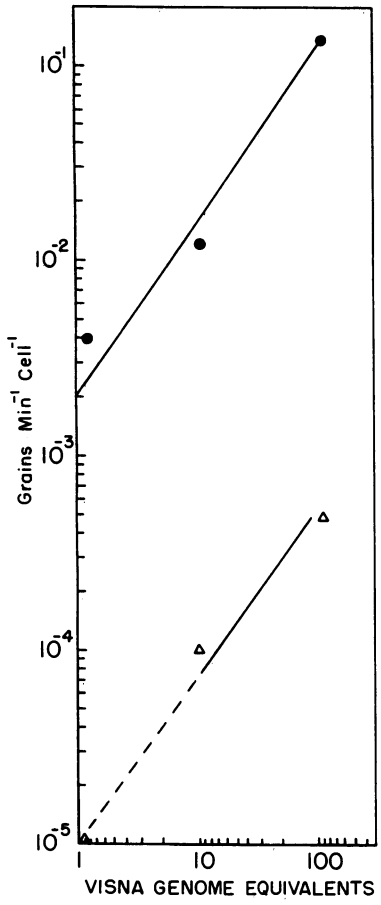

FIG. 3. Quantitative aspects of amplification *in situ*. In replicate experiments, uninfected SCP cells or cells infected for 3–48 hr were collected and hybridized *in situ* under optimal conditions with (●) or without (△) amplification with the ^{125}I-labeled probe to nt 180–1370 of the visna virus genome. Grains were enumerated (1000–1500 per slide) over 100 randomly selected cells in radioautographs exposed for 2–70 hr to establish the relationship between the number of 10-kilobase visna virus genome equivalents (from ref. 7) and averaged grain count (the variation in counts was <2-fold). Grain counts increase *pari passu* as the number of copies of viral DNA in cells increases during the viral life cycle; the broken line indicates extrapolation in the case of *in situ* hybridization for the earliest time point, where the one to two copies of the 1-kbp target do not generate a significant increase in signal over background. The increase in sensitivity with PCR was estimated to be 100- to 300-fold from comparisons of the signal (grains per min per cell) with or without amplification in cells with ≥10 genome equivalents.

Quantitation. We estimated the extent of amplification and concomitant increase in sensitivity by comparing the signal generated by *in situ* hybridization with or without prior amplification in two independent experiments in cells containing an average of 2, 20, and 150 copies of the target segment. From these comparisons, we conclude that the efficiency at each cycle is about 12% [$(1 + 0.12)^{50} \approx 300$]. This substantial reduction in amplification vis-a-vis the 10^6-fold amplification achieved for DNA in solution (5) is in keeping with the limitations of diffusion-sensitive processes in fixed cells (4). However, one important consequence of the fact that even after 50 cycles, DNA synthesis has not approached saturation is that the concentration of amplified species is proportional to the starting concentration of "founder" DNA molecules. Thus, the initial copy number can be estimated (as shown in Fig. 2 *B* and *D* and Fig. 3).

CONCLUSIONS

The coupling of *in situ* hybridization with PCR-mediated amplification of specific sequences offers a method that is quantitative, 2 orders of magnitude more sensitive than *in situ* hybridization alone, and inherently less likely than PCR methods in solution to produce false positives by contamination (since it is DNA inside the cell that is amplified and retained). We have chosen for these studies visna virus as a relevant lentiviral paradigm from which the concept of transcriptional dormancy as the principal mechanism of virus persistence and latency (12–14) originated, and we have focused on infected cells in suspension to facilitate the development of a method that can be applied with existing equipment to such urgent problems as detection of latent HIV infection of mononuclear cells in the bloodstream (3). While some modifications will be necessary to detect viral genomes by cytohybridization in cells in tissue sections, we have been able to amplify viral DNA in cells on slides placed in close contact with the metal in a thermocycler block. Modifications of equipment to facilitate applications of PCR *in situ* for this purpose, and methods to amplify and detect viral RNA *in situ*, lie in the future, but the foundations for these developments have been laid. Success in these efforts should further extend our vision of the cellular contexts in which viruses reside as a prerequisite to define their role in chronic disease (15).

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