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Subcellular localization of low-abundance human immunodeficiency virus nucleic acid sequences visualized by fluorescence *in situ* hybridization

(nuclear transport/viral infection/transcription site/Epstein–Barr virus)

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ABSTRACT **Detection and subcellular localization of human immunodeficiency virus (HIV) were investigated using sensitive high-resolution *in situ* hybridization methodology. Lymphocytes infected with HIV *in vitro* or *in vivo* were detected by fluorescence after hybridization with either biotin or digoxigenin-labeled probes. At 12 hr after infection *in vitro*, a single intense signal appeared in the nuclei of individual cells. Later in infection, when cytoplasmic fluorescence became intense, multiple nuclear foci frequently appeared. The nuclear focus consisted of newly synthesized HIV RNA as shown by hybridization in the absence of denaturation and by susceptibility to RNase and actinomycin D. Virus was detected in patient lymphocytes and it was shown that a singular nuclear focus also characterizes cells infected *in vivo*. The cell line 8E5/LAV containing one defective integrated provirus revealed a similar focus of nuclear RNA, and the single integrated HIV genome was unequivocally visualized on a D-group chromosome. This demonstrates an extremely sensitive single-cell assay for the presence of a single site of HIV transcription *in vitro* and *in vivo* and suggests that it derives from one (or very few) viral genomes per cell. In contrast, productive Epstein–Barr virus infection exhibited many foci of nuclear RNA per cell.**

A sensitive high-resolution detection of human immunodeficiency virus (HIV) nucleic acids in single cells would provide information and insights into the cellular life cycle of this virus. In addition, improved methods for sensitive, accurate, and rapid detection of HIV sequences in cells would be valuable for screening and diagnosis. Examples of such applications are the testing of infants who are seropositive due to maternal antibodies (1) or for quantitation of virally infected lymphocytes in the peripheral blood during therapeutic regimens. In contrast to other molecular methods, *in situ* hybridization allows identification and precise quantitation of the number of cells infected in a given cell sample, particularly valuable in situations when infected cells are rare (2). *In situ* hybridization (2–4) and immunofluorescence (5) have found that an extremely small percentage (1 in 10^4–10^5) of peripheral blood cells from HIV-infected individuals actively express virus. In addition, *in situ* hybridization is also capable of evaluating the amount of viral nucleic acid per cell. We have demonstrated a fluorescence *in situ* hybridization methodology capable of detecting as little as one Epstein–Barr virus (EBV) genome within a single cell (6) or EBV nuclear transcripts within latently infected cells (7). The dual aims of the present work were to apply this sensitive high-resolution methodology to the detection of HIV in infected cells as well as the investigation of the subcellular localization of HIV sequences.

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MATERIALS AND METHODS

Cells and Cell Preparation. Normal T lymphocytes were cultured for 3–5 days with interleukin 2 and phytohemagglutinin and infected with HIV-1 (strain IIIB) at a low multiplicity of infection. At daily intervals, samples were taken for *in situ* hybridization. Cells were washed in isotonic phosphate-buffered saline (PBS) and applied to multi-well serologic slides (5-mm wells, Cel-line) at 250×10^6 cells per ml (2).

The following reagents were obtained through the AIDS Research and Reference Reagents Program: C8166-45 (8) and 8E5/LAV cells (9). The 8E5/LAV cell line contains (9) a single viral genome with a defective *pol* gene. Cytogenetic preparations of 8E5/LAV cells were prepared and hybridized as described (6).

***In Situ* Hybridization.** Hybridization was performed essentially as described and was the same for biotin- or digoxigenin-labeled probes (6, 7). The probes were labeled by nick-translation as described (2). After fixation in 4% (wt/vol) paraformaldehyde/PBS for 5 min, slides were dehydrated through a graded ethanol series and air-dried. The nick-translated probe mixture (40 μl containing 40 ng of HIV DNA plus carrier) was lyophilized, resuspended in 10 μl of deionized formamide, and heated at 75°C for 10 min. Hybridization buffer was prepared by mixing 30 μl of 20× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 30 μl of bovine serum albumin [2% (wt/vol), molecular biology grade; Boehringer Mannheim], 60 μl of 50% (wt/vol) dextran sulfate, and 30 μl of H_2O. The heated probe (10 μl) was mixed rapidly with an equal amount of hybridization buffer, placed in a serological well, covered with Parafilm, and placed for 3 hr in a humidified 37°C incubator. Duplicate samples were tested in each experiment. Slides were rinsed for 30 min in 50% (vol/vol) formamide/2× SSC at 37°C and for 30 min in 1× SSC at room temperature.

Detection. Detection was by a direct one-step staining procedure using fluorescein-conjugated avidin (6) and bound probe was visualized by epifluorescence microscopy. Samples were incubated in avidin-fluorescein (2 μg/ml; Enzo Biochemicals) in 4× SSC/1% bovine serum albumin for 30 min at room temperature. Samples were rinsed three times at room temperature in 4× SSC, with 0.1% Triton in the first rinse. Samples were mounted in 2.5% (wt/vol) DABCO [(triethylenediamine) in 90% (vol/vol) glycerol/1× PBS] antibleach compound containing propidium iodide (4 μg/ml). Photographs were taken on a Zeiss ICM microscope using Ektachrome 400 film.

Probes labeled by nick-translation with digoxigenin-dUTP (Boehringer Mannheim) were detected by a rhodamine-conjugated anti-digoxigenin antibody (a gift from Boehringer

Abbreviations: HIV, human immunodeficiency virus; EBV, Epstein–Barr virus.

Mannheim) at 2 μg/ml in 4× SSC/1% bovine serum albumin. Although this label worked well for most purposes, including single genome detection, preliminary results suggested nonspecific labeling of a subset of patient lymphocytes.

RESULTS AND DISCUSSION

Detection of Single Cells Infected *in Vitro*. In cultured T lymphocytes fixed at daily intervals after infection, fluorescence *in situ* hybridization unambiguously detected infected cells at the earliest time point and detected increasing numbers of infected cells on later days (Fig. 1). At early times after infection, most positive cells exhibited weak to moderate staining, whereas, in cultures several days after infection (Fig. 2*A*), positive cells (bright yellow) were easily distinguished from negative cells (red) and varied markedly from intense to very weak, possibly reflecting cycles of infection within the culture. Since fluorescence intensity is proportional to the quantity of sequence hybridized, as described for EBV (6), this approach allows quantitation not only of the number of positive cells but also of the approximate amount of signal within each cell. In experiments in which this fluorescence detection technique was directly compared to other detection methods utilizing either avidin-alkaline phosphatase or autoradiography (2), the fluorescence approach detected 2-fold more positive cells per equivalent sample of cells, for reasons detailed below.

Subcellular Localization and Early Detection of Primary Viral Transcripts. High-resolution fluorescence microscopy revealed the subcellular localization of HIV sequences. One or more intensely fluorescent foci (Fig. 2*B*) were apparent in all weakly and in most strongly positive cells, representing detection of highly localized concentrations of HIV sequences. This distinct pattern was observed after infection with laboratory viral strains as well as patient viral isolates. The bright nuclear foci were ≈0.4 μm in diameter and often slightly elongated. The foci appeared to be in the nucleus, as indicated by double staining for HIV sequences with fluorescein and total DNA with propidium iodide (see Fig. 2 *A* and *E*). However, to verify this, isolated nuclei were prepared (6) and found to contain bright foci after hybridization,

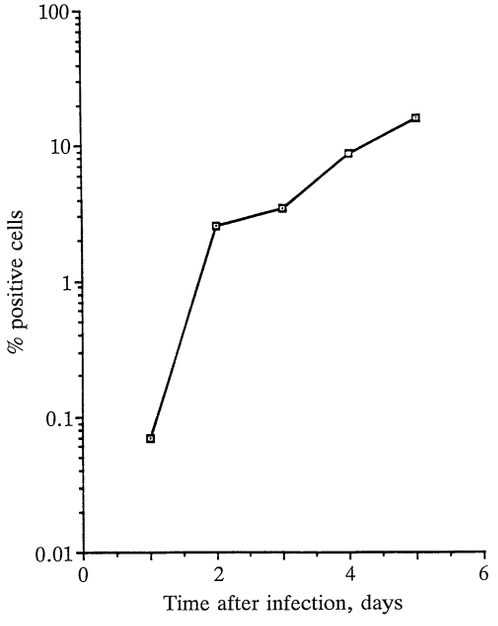

FIG. 1. Kinetics of HIV detection using high-resolution fluorescence *in situ* hybridization. Cells were infected and samples were taken at daily intervals.

further indicating that this focal concentration of HIV sequences resides within the nucleus (data not shown). Cells with weak to moderate cytoplasmic fluorescence generally had just one focus. Greater than 99% of cells with this diffuse fluorescence characteristic of the cytoplasm exhibited a distinct nuclear focus. In cells with very bright cytoplasmic fluorescence, multiple nuclear foci were present. Table 1 summarizes the frequency of these observations. These fluorescent foci were absent from control samples evaluated in multiple experiments; hence, the presence of the characteristic nuclear focus was sufficient to identify an individual cell as HIV positive. From the data in Table 1, it can be seen that at least half of the cells scored as infected by HIV were identified because of this distinct and very characteristic staining pattern made possible by the high resolution of epifluorescence microscopy.

A prominent focus in cells with marginally detectable cytoplasmic staining may signify an early event in HIV expression prior to substantial movement of HIV nucleic acids from sites of transcription into the cytoplasm. Therefore, this focus should be detectable soon after infection. To verify this, C8166 cells, known to have rapid kinetics of viral replication (10), were infected and fixed 12, 24, or 48 hr after infection. Unambiguously infected cells were first observed 12 hr after infection, when cells with barely visible cytoplasmic fluorescence and a bright nuclear focus were observed (Fig. 2*C*). At later time points (e.g., 48 hr), the cell cytoplasm also showed unambiguous fluorescence. Hence, the kinetics of appearance of the nuclear focus precedes significant accumulation of viral nucleic acids throughout the rest of the cell.

To determine if the HIV nuclear focus consists primarily of single- or double-stranded nucleic acids, denaturation of cellular sequences prior to hybridization was omitted (7). In repeated experiments, this did not appreciably diminish the size or intensity of the nuclear focus; hence, this structure is predominantly or entirely single-stranded. To address further whether this signal was RNA or DNA, slides were treated with RNase A at 100 μg/ml for 1 hr prior to hybridization. This treatment essentially eliminated both the nuclear focus and cytoplasmic fluorescence. In a small percentage of cells, a much less intense fluorescent focus remained visible. Results of replicate experiments showed that these subdued fluorescent spots after RNase A treatment were still present in the absence of denaturation, supporting the conclusion that these represent small traces of single-stranded DNA or RNA not totally removed by enzymatic digestion.

If the focus represents newly synthesized viral transcripts, it should be removed by inhibition of viral transcription with actinomycin D. Nuclear foci were almost entirely eliminated by a 2-hr treatment and were totally eliminated by 4 hr in actinomycin D (cf. Fig. 2 *D* and *E*), reflecting the loss of transcripts synthesized prior to inhibition. Only cytoplasmic fluorescence remained throughout actinomycin treatment. Thus, the presence of the nuclear focus results from active synthesis of HIV RNA. This fluorescence *in situ* hybridization technique makes possible the assessment of HIV transcriptional activity (recent or ongoing) within individual cells. In addition, it may serve as a means to evaluate the transcriptional activity of specific viral genes.

Visualization of a Single HIV Genome and Its Expression. Within the first few days after infection of primary lymphocytes when cytoplasmic fluorescence is low, there is generally one predominant site of HIV RNA synthesis within the nucleus. This may represent the transcription from a single virus, integrated (provirus) or unintegrated. To compare these results with those for cells known to contain one viral DNA copy (in G_1 phase cells), as well as to test further the sensitivity of our technique, we investigated the 8E5/LAV cell line reported to carry a single integrated provirus defec-

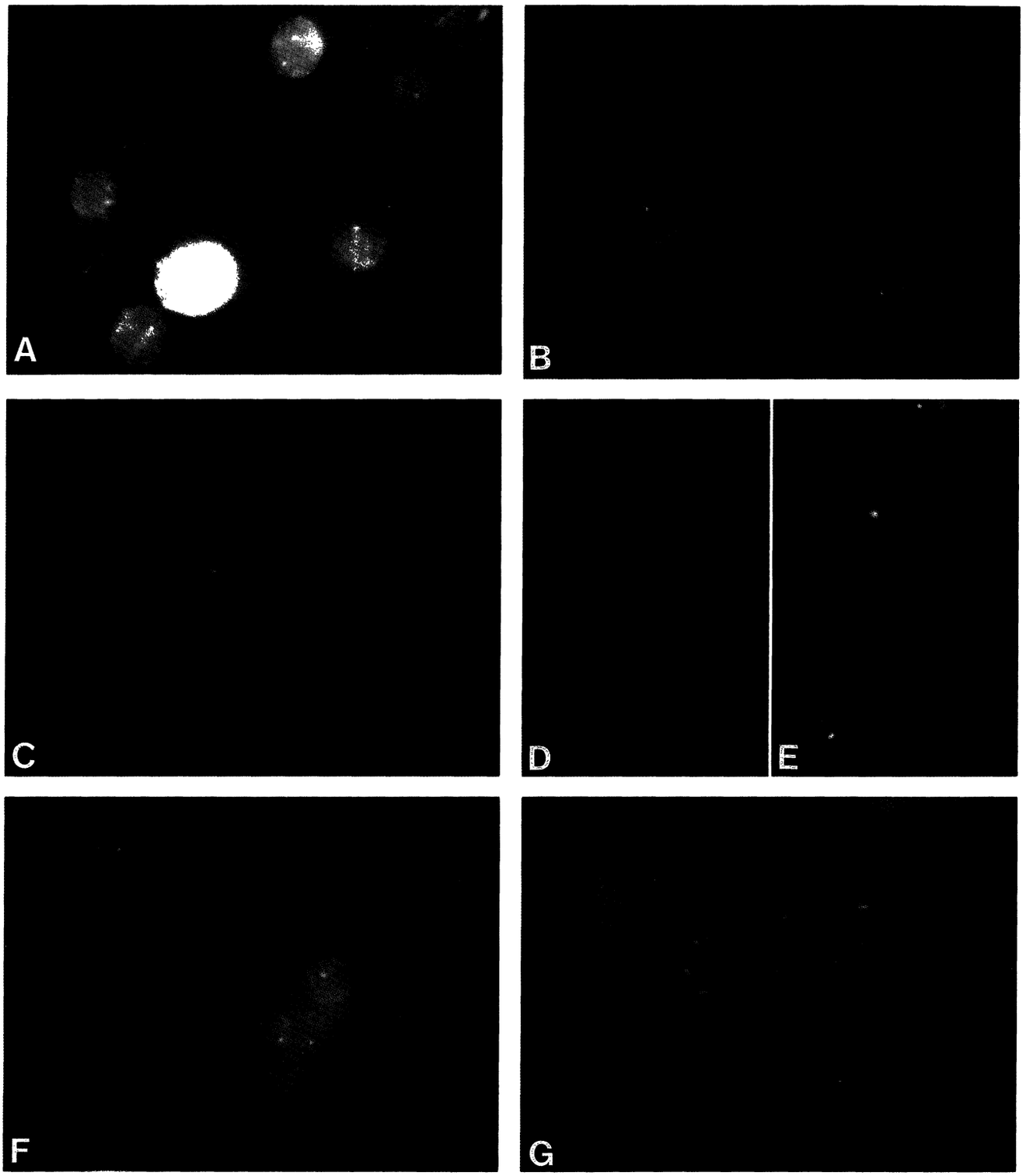

FIG. 2. Visualization of viral RNA in infected cells. Cells were hybridized with a genomic HIV probe and HIV sequences were detected. Hybridization conditions utilized were optimized for RNA detection and do not allow hybridization to double-stranded DNA in the absence of denaturation (7). Yellow cells are positive for HIV and are the result of green (fluorescein signal) superimposed on the red (propidium iodide) DNA stain. No fluorescein signal was observed in uninfected cells probed with HIV or in infected cells probed with pBR322. (*A*) Normal lymphocytes infected in culture for 5 days as in Fig. 1. Cells were stained with propidium for DNA (red). The hybridized probe is detected as yellow. (*B*) Foci of infection are in the same cells as in *A* but after only 3 days of infection. The propidium (DNA) fluorescence has been omitted so that nuclear foci are clearer, hence the uninfected cells that fill the field are not visible. (*C*) C8166 cells 12 hr after infection (no propidium). Again, uninfected cells that fill the field are not visible due to the low nonspecific background of the technique. (*D* and *E*) Cells were as in *A*, except that 4 hr before harvesting, a sample of the cells was treated with actinomycin D at 4 μg/ml. Note the disappearance of foci but the persistent cytoplasmic signal in the treated cells. (*D*) Cells were treated with actinomycin. (*E*) Cells from same hybridization that were not treated with actinomycin. (*F*) 8E5 cells known to have a single integrated defective provirus. The focus of hybridization results from transcription

Table 1. Nuclear foci within HIV-infected cells

Total cells, no.	Total positive cells, %	Single-focus cells, %			Multifocal cells, %
		Cytoplasmic fluorescence			
		Weak	Moderate	Strong	
644	57	28	13	3	13

Normal T lymphocytes were infected in culture and after 5 days fixed for *in situ* hybridization. Positive cells were scored and categorized further as to whether the individual cell contained a single focus or multiple foci. The cells containing a single focus could be further categorized by intensity of cytoplasmic fluorescence into weak, moderate, or strong signal, whereas the multifocal cells showed more uniformly strong cytoplasmic fluorescence. The number of foci per cell was then correlated with the level of cytoplasmic fluorescence. To emphasize this correlation, cells with weak cytoplasmic fluorescence (28%) were scored for single versus multiple foci and 98% of these were found to contain only a single nuclear focus. In contrast, of the cells with strong cytoplasmic fluorescence (16%), only 23% contained a single focus and 76% contained multiple nuclear foci. Similar results were obtained using C8166 or H9 cells or with patient viral isolates. In multiple experiments, in more than 100,000 uninfected control cells examined, fluorescent nuclear foci were not observed.

tive in *pol* (9). By utilizing methods developed to detect EBV integration (6), the integrated HIV genome was visualized at a single site on a D-group chromosome within cytogenetic preparations of this cell line (Fig. 3*A*). The signal generated by a single genome was unequivocally identified due to the identical labeling of sister chromatids, the consistent labeling of a D-group chromosome, and the negligible nonspecific label indicated by control samples or the homologous chromosome not containing integrated HIV. The integrated viral genome was detected in at least 60% of metaphase figures and the viral genome was also observed in most interphase nuclei as a single fluorescent spot. When paraformaldehyde-fixed preparations of intact 8E5/LAV cells were hybridized (without prior denaturation of nuclear DNA), a singular nuclear focus of viral RNA was present in expressing cells (Fig. 2*F*), as was seen in the cells infected with exogenous virus (Fig. 2 *A–C* and *E*). Cells were not observed with multiple nuclear foci, unlike productively infected cells late in infection. This demonstrates that a single integrated HIV genome is capable of producing this nuclear focus of viral RNA and indicates that the focus is a concentrated site of transcription for this single viral genome.

Substantial quantities of HIV DNA in tissues (11) and high amounts of viral RNA in cultured cells (10) have led to the suggestion that numerous copies of HIV DNA may be present and active per cell. Based on the fluorescence intensity relative to that obtained using a single genome as target, we estimate that the nuclear focus in peripheral blood lymphocytes or C8166 cells contains roughly 30–100 genome equivalents of HIV RNA. This makes it difficult to distinguish small amounts of DNA that may be present within this focus from residual RNA after RNase digestion. However, the fact that the signal after denaturation and RNase digestion was very weak indicates that few proviral genomes were present at this site (not more than four or five). These observations suggest that the single nuclear focus represents transcription of, most likely, a single viral genome localized at a single nuclear site, rather than an accumulation of transcripts from viral genomes distributed throughout the nucleus. Multiple HIV DNA genomes may be present and active later in the productive infection of a single cell, as suggested by multiple secondary foci.

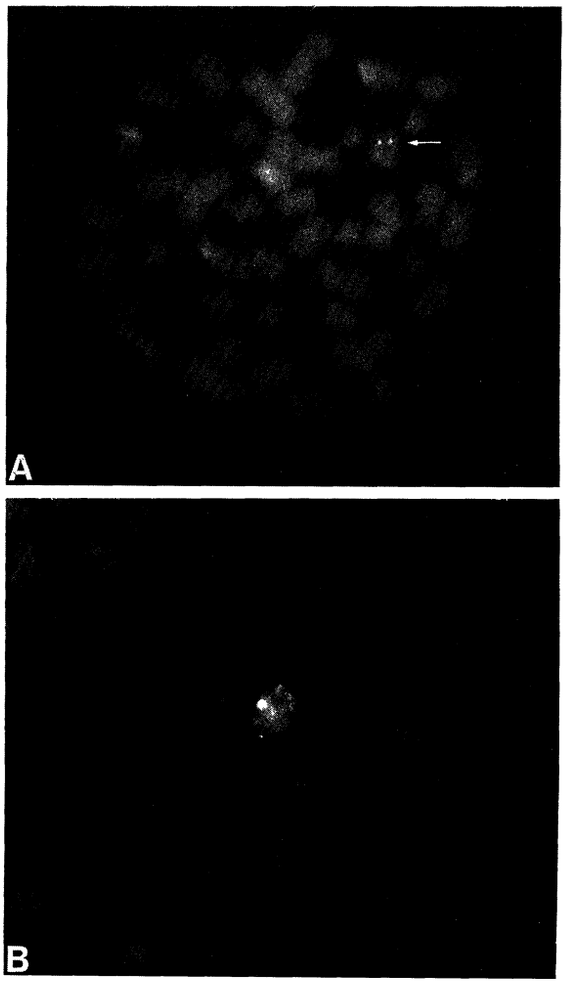

FIG. 3. Detection of a single HIV genome in 8E5 cells and HIV RNA in patient samples. (*A*) 8E5 cells (9) were prepared for DNA single-copy *in situ* hybridization by techniques as described (6). Note the identical labeling of sister chromatids of a D-group chromosome (arrow), which is visible above the generalized fluorescence of the propidium iodide-stained chromosomes. All spreads examined showed labeling of this D-group chromosome, as expected for this clonal line. (*B*) HIV RNA detected in peripheral blood lymphocyte taken directly from a seropositive patient. Note the prominent fluorescent focus suggestive of ongoing transcriptional activity. (*A*, ×2300; *B*, ×700.)

Comparison to EBV Productive and Latent Infection. The nuclear focus of HIV RNA may be contrasted to observations on nuclear transcripts synthesized from EBV, a herpes virus. A cell line (Namalwa) with two integrated copies of EBV generally has only one or two "tracks" of nuclear RNA (7). These tracks in the EBV latent infection were highly elongated relative to the tracks in the 8E5/LAV cells. However, the most-elongated tracks were observed in cytogenetic preparations, with less-elongated formations being observed in paraformaldehyde-fixed cells; because less of the EBV RNA is transported to the cytoplasm, the RNA transcript tracks may be more elongated due to their accumulation within the nucleus (7).

To compare these two viruses in a productive infection, we determined the pattern of hybridization for B95-8 cells carrying numerous copies of the EBV genome, which is pro-

of a single DNA copy. (*G*) B95-8 cells persistently infected with EBV, of which 5% of the population becomes productive. Many copies of the EBV genome persist per cell and these give rise to numerous tracks of nuclear RNA in actively expressing cells after hybridization with a probe for EBV RNA. (*A–F*, ×1000; *G* ×2000.)

ductively expressed in about 5% of the cell population (12). As shown in Fig. 2*G*, 10–30 foci or slightly elongated "tracks" of RNA were observed in productively infected B95-8 cell nuclei, in contrast to the singular focus for productive HIV infection. A singular focus of nuclear hybridization (observed in most HIV-positive cells) was never observed in the EBV-producing cell population. Hence, the singular focus of concentrated newly synthesized RNA is characteristic of HIV-infected cells and strongly suggests a single site of viral RNA synthesis.

Detection of HIV-Expressing Cells Within Patient Samples. The efficacy of this technique was evaluated for detecting patient peripheral blood lymphocytes expressing HIV and for determining whether the single nuclear RNA focus was also a characteristic of cells infected *in vivo*. Ten lymphocyte preparations from seropositive hemophiliacs were hybridized under nondenaturing conditions, so that only RNA was detected. After brief scanning of 50–100,000 cells per patient sample, at least three patients were found to contain positive cells, with a frequency of 1 in 30,000 to 1 in 100,000 [consistent with other reports (refs. 2–4)]. As shown in Fig. 3*B*, the HIV-expressing cells were clearly apparent partly because of their weak cytoplasmic fluorescence and because they consistently had one central focus of intense fluorescence, indicating the presence of newly synthesized HIV RNA.

The nuclear focus of viral sequences is a unique identifying feature of positive cells that enhances both the accuracy and sensitivity of single-cell detection. This may be diagnostically useful in identifying the rare HIV-positive cells in the peripheral mononuclear cell population of infected individuals. Our current observations suggest that, at this stage, fluorescence detection is more sensitive for detecting fewer nucleic acid copies per cell than other methods of detecting *in situ* hybrids. The use of enzymatic detection, however, has advantages in that it is permanent and slides may be rapidly scanned using standard bright-field microscopy (2). We have not directly compared the sensitivity of this fluorescence *in situ* hybridization methodology with that of the polymerase chain reaction, which is a highly sensitive technique for detection of viral nucleic acids in solution but which does not provide cellular information (i.e., amount of virus in or number of infected cells). As a clinical research tool, our approach can be used to evaluate the replicative activity of the virus under various conditions, such as in evaluating drugs or transfected viral genes (e.g., *rev*) for their effects on viral transcription or nuclear RNA transport. Recent evidence suggests that latent virus can exist in as much as 1% of patient cells expressing the CD4 receptor (13). The demonstrated capability of this technology to detect the unexpressed viral genome should prove valuable when rigorously applied to the investigation of viral latency in patient cells.

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1. Scott, G. B., Buck, B. E., Letterman, J. G., Bloom, F. L. & Parks, W. P. (1984) *N. Engl. J. Med.* **310,** 76–81.
2. Singer, R. H., Byron, K. S., Lawrence, J. B. & Sullivan, J. L. (1989) *Blood* **74,** 2295–2301.
3. Harper, M. E., Marselle, L. M., Gallo, R. C. & Wong-Staal, F. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 772–776.
4. Busch, M. P., Rajagopalan, M. S., Gantz, D. M., Fu, S., Steimer, K. S. & Vyas, G. N. (1987) *Am. J. Clin. Pathol.* **88,** 673–680.
5. Fauci, A. S. (1988) *Science* **239,** 617–622.
6. Lawrence, J. B., Villnave, C. & Singer, R. H. (1988) *Cell* **52,** 51–61.
7. Lawrence, J. B., Singer, R. H. & Marselle, L. M. (1989) *Cell* **57,** 493–502.
8. Salahuddin, S. Z., Markham, P. D., Wong-Staal, F., Franchini, G., Kalyanaraman, V. S. & Gallo, R. C. (1983) *Virology* **129,** 51–64.
9. Folks, T. M., Powell, D., Lightfoote, M., Koenig, S., Fauci, A. S., Benn, S., Rabson, A., Daugherty, D., Gendelman, H. E., Hoggan, M. D., Venkatesan, S. & Martin, M. A. (1986) *J. Exp. Med.* **164,** 280–290.
10. Somasundaran, M. & Robinson, H. L. (1988) *Science* **242,** 1554–1557.
11. Shaw, G. M., Harper, M. E., Hahn, B. H., Epstein, L. G., Gajdusek, D. C., Price, R. W., Navia, B. A., Petito, C. K., O'Hara, C. J. & Groopman, J. E. (1985) *Science* **227,** 177–182.
12. Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C., Tuffnell, P. S. & Barrell, B. G. (1984) *Nature (London)* **310,** 207–211.
13. Schnittman, S. M., Psallidopoulos, M. C., Lane, H. C., Thompson, L., Baseler, M., Massari, F., Fox, C. H., Salzman, N. P. & Fauci, A. S. (1989) *Science* **245,** 305–308.