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Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus

(Leu-3$^+$ T-cell line/viral cytopathic effect/virus propagation *in vitro*/reverse transcriptase)

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ABSTRACT **We have developed a continuous human T-cell line (A3.01) for the study of acquired immunodeficiency syndrome (AIDS)-associated retrovirus that mimics normal peripheral blood lymphocytes in susceptibility to viral cytopathic effect without the need for cell activation or conditioned medium. Following infection, substantial quantities of virus are produced during a 3- to 5-day period; the associated killing of cells can be monitored in a microtiter assay as a function of virus input. Southern blot hybridization of infected cellular DNAs indicated that no gross alteration occurred in the restriction maps of the proviral DNA during the transfer of virus to and its passage in A3.01 cells. This cell system offers an alternative to other AIDS retrovirus cell systems because it permits the monitoring of viral cytopathic effects.**

The acquired immunodeficiency syndrome (AIDS) retrovirus (RV), also known as lymphadenopathy-associated virus (LAV) and human T-lymphotropic virus type III (HTLV-III), replicates preferentially and produces cytopathic effects (CPE) in activated human Leu-3$^+$ cells (1, 2), the same T-lymphocyte subset that is dramatically reduced in patients with the disease (3, 4). Although the virus can be propagated in normal human peripheral blood lymphocytes (PBLs), susceptibility to infection varies in cells obtained from different donors. In addition, the requirement for phytohemagglutinin stimulation and interleukin 2 make detailed biological and molecular studies difficult to carry out in this system. To overcome some of these deficiencies, T-lymphoid leukemia (5) and Epstein–Barr virus-transformed B-cell (6) lines that continuously produce the AIDS RV have been developed. Unfortunately, neither line is killed by the virus, thereby precluding investigations of viral CPE. In this report, we describe a hypoxanthine/aminopterin/thymidine (HAT)-sensitive derivative of CEM (7) cells that supports AIDS RV replication and exhibits virus-induced CPE. This line (A3.01) is readily propagated in the absence of exogenously added interleukin 2 and, since it is >95% Leu-3$^+$, nearly all of the cells are susceptible to infection.

MATERIALS AND METHODS

Preparation and Culture of Virus-Infected Cells. Normal PBLs were cryopreserved and stored in liquid nitrogen until needed. Prior to use, the lymphocytes were quickly thawed, washed, and prepared for infection as described (8). A3.01 cells, a HAT-sensitive derivative of the CEM (7) line, were cultured in RPMI 1640 medium containing 10% fetal calf serum until needed and were routinely checked for surface Leu-3 positivity prior to infection. PBLs were infected with various dilutions of LAV virus (generously provided by L. Montagnier, Institut Pasteur) as described by Barré-Sinoussi *et al.* (8). A3.01 cell cultures were infected in the presence of Polybrene (Sigma) at 1 μg/ml, but no interleukin 2 or rabbit anti-human interferon was added. Medium was changed daily and supernatants were harvested, spun at 800 × *g* to remove cell debris, and stored at −70°C. Reverse transcriptase assays were carried out as described (9) on pellets from cell-free supernatants, centrifuged in a Beckman J3 Airfuge at 100,000 × *g* for 36 min.

For cytopathogenicity assays, a subclone of A3.01 cells (A8.7.10) was added in quadruplicate (10^3 cells per well) to Costar 3596 microculture plates. The LAV-A virus stock (a high-titer isolate prepared by a single passage in A3.01 cells) was added at various dilutions (25 μl per well) in a total volume of 250 μl. Polybrene (1 μg/ml) was included in all cultures. Each microculture was refed every 3 days by replenishing 100 μl of medium per well. On day 9 the supernatants from quadruplicate cultures were pooled, spun at 800 × *g*, and monitored for reverse transcriptase activity. One microcurie of [^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) then was added to each well for 4 hr at 37°C, after which the cells were harvested with a PHD cell harvester (Cambridge Technologies, Cambridge, MA).

Preparation of Cells for Fluorescence Analysis. Infected (day 9) and uninfected A3.01 cells (10^6) were incubated with a 1:25 dilution of pooled sera from AIDS patients (previously adsorbed with uninfected A3.01 cells) for 45 min at 4°C, washed, and incubated with a 1:100 dilution of fluorescein-conjugated goat anti-human IgG (Atlantic Antibodies, Westbrook, ME) for 30 min at 4°C. Cells were also stained with 2 μl of fluorescein-conjugated monoclonal antibodies (Becton Dickinson) for 30 min at 4°C. All cells were finally washed twice and fixed in 1% paraformaldehyde followed by flow-cytometric analysis on a FACS-A (Becton Dickinson).

Characterization of Viral Nucleic Acids Present in AIDS RV-Infected Cells. AIDS RV RNA was purified from virus obtained from large-scale (14-liter) cultures of infected A3.01 cells and used for the preparation of ^{32}P-labeled cDNA (ref. 10 and unpublished work). A 17.5-kilobase (kb) *Bam*HI segment was then cloned from a λJ1 (11) library of LAV-infected A3.01 cellular DNA screened with the cDNA probe. The cloned insert contained viral DNA sequences extending from the 5′ long terminal repeat to the *Bam*HI site at 8.4 kb (see Fig. 4) as well as 8.7 kb of 5′ flanking cellular DNA. Two

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Abbreviations: AIDS, acquired immunodeficiency syndrome; RV, retrovirus; LAV, lymphadenopathy-associated virus; CPE, cytopathic effect; PBL, peripheral blood lymphocyte; HAT, hypoxanthine/aminopterin/thymidine; kb, kilobase(s).

subclones (pBENN-4 and pBENN-6, see Fig. 4) were used in DNA or RNA blot-hybridization experiments.

DNA was purified from $\approx 2 \times 10^7$ infected PBLs or infected A3.01 cells as described (12), digested with restriction enzymes as recommended by their suppliers, electrophoresed in 0.6% horizontal agarose slab gels (13), and transferred to nitrocellulose membranes as outlined by Southern (14). The cloned internal 6.5-kb *Hin*dIII fragment (pBENN-6, Fig. 4) of AIDS RV DNA was labeled by the nick-translation procedure (15) to a specific activity of 2×10^8 cpm/μg and used as a hybridization probe of Southern blots of restriction-enzyme digested cellular DNA as described (13).

Total cellular RNA was prepared from 4×10^7 uninfected or infected A3.01 cells, by modification of the guanidinium isothiocyanate/cesium chloride method of Chirgwin (16, 17). Samples (2 μg) of RNA were electrophoresed through 1% agarose gels containing 2.2 M formaldehyde (18) for 14–16 hr at 40 V and analyzed by blot hybridization as previously described (19). The probe was the cloned AIDS RV insert present in pBENN-4, labeled with ^{32}P to specific activities of $5–10 \times 10^8$ cpm/μg by random hexamer priming of Klenow fragment DNA synthesis (20).

RESULTS

A cell line (CEM), originally isolated by Foley *et al.* (7) from a child with acute lymphocytic leukemia, was cultured in the presence of 8-azaguanine to derive a cloned HAT-sensitive cell (A3.01) suitable for human T-lymphocyte fusions. The A3.01 cells were >95% Leu-3$^+$, Leu-8$^+$, Leu-1$^+$,Tac$^-$, and transferrin-receptor positive and were sensitive to infection with the LAV virus. Further surface characterization by HLA typing indicated that the parental CEM line (obtained as CCRF-CEM from ATCC) was of type A1,19 B40 Cw3,7 DR$^-$; the HLA phenotype of the HAT-sensitive A3.01 cells could not be determined because of complement sensitivity.

In an initial group of experiments, the sensitivity of A3.01 cells to AIDS RV infection was compared to that of phytohemagglutinin-stimulated normal human lymphocytes. The infectivity of LAV was greatest on primary human lymphocytes but was clearly demonstrable at a 10^{-1} dilution on A3.01 cells. In a second experiment, a high-titer virus pool (LAV-A), prepared by a single passage in A3.01 cells, contained at least 10^5 infectious virus per ml as titered in primary lymphocytes and 10^3, as titered in A3.01 cells (Table 1). The kinetics of LAV-A infection in A3.01 cells is illustrated in Fig. 1. Electron micrographs of infected cells taken on day 9 revealed typical budding AIDS RV particles (data not shown). Prior to the peak of reverse transcriptase activity on day 10, the number of infected cells began to level off and it fell precipitously between days 10 to 12. As shown in Fig. 2, flow-cytometric analysis indicated that by day 9 a

Table 1. Production of AIDS RV by human cells (as judged by reverse transcriptase activity at 4–10 days after infection)

Virus	Cells	Virus dilution	Reverse transcriptase activity on day 4	5	6	7	8	9	10
LAV	Primary lymphocytes	10^{-1}	+	+	+	+	−	−	−
		10^{-3}	−	+	+	+	+	−	−
	A3.01	10^{-1}	−	−	−	+	+	+	−
		10^{-3}	−	−	−	−	−	−	−
LAV-A	Primary lymphocytes	10^{-1}	+	+	+	+	−	−	−
		10^{-3}	+	+	+	+	−	−	−
		10^{-5}	−	+	+	+	+	−	−
	A3.01	10^{-1}	−	+	+	+	+	+	−
		10^{-3}	−	−	−	−	+	+	+
		10^{-5}	−	−	−	−	−	−	−

The indicated dilutions of virus were added to 2×10^6 phytohemagglutinin-stimulated lymphocytes or 10^6 A3.01 cells. Supernatants were collected each day from the infected cultures and tested for reverse transcriptase activity. +, Reverse transcriptase activity > 500,000 cpm/ml of infected-cell supernatant.

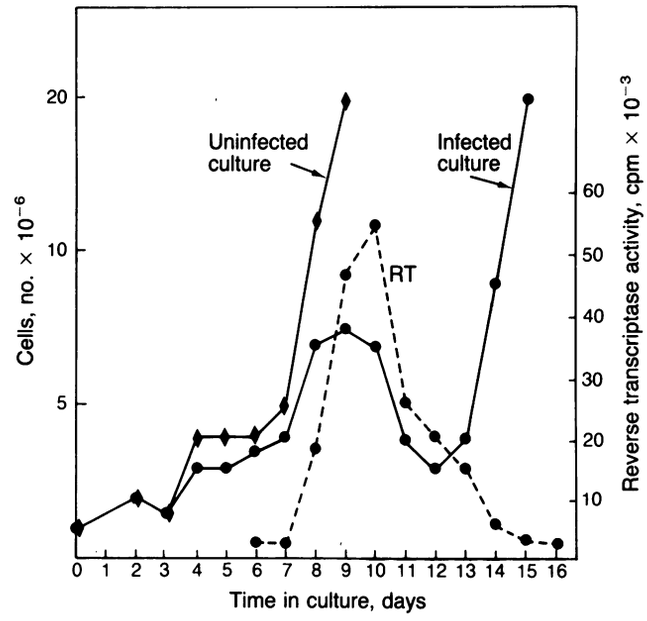

FIG. 1. Kinetics of LAV-infected A3.01 cells. A3.01 cells (10^6) were infected with a 10^{-3} dilution of the LAV-A virus stock. Cell number (solid lines), in terms of total cells per culture, and reverse transcriptase (RT) activity (broken line) were monitored daily. ●, Infected cells; ◆, uninfected cells.

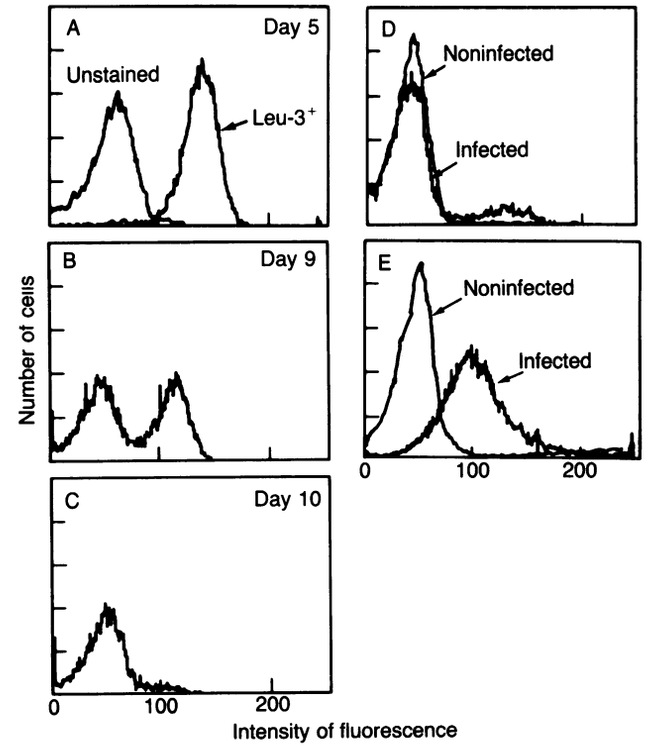

FIG. 2. Immunofluorescent staining of A3.01 cells infected with the LAV-A virus stock. A3.01 cells (10^7) were infected with LAV-A virus and stained with fluorescein-conjugated Leu-3 on days 5, 9, or 10 (*A–C*, respectively). On day 9, cells were also stained with (*E*) or without (*D*) preadsorbed pooled AIDS sera.

sizeable fraction of infected A3.01 cells had lost the Leu-3$^+$ phenotype and that by day 10, >90% of the cells were Leu-3$^-$. In a separate experiment, pooled sera from AIDS patients, which had been preadsorbed with uninfected cells, were used to stain A3.01 cells 9 days after infection. A shift in the mean channel fluorescence was observed in >95% of the infected cells (Fig. 2*E*) but not when the same sera were applied to uninfected A3.01 cells.

The rapid decline in the number of Leu-3$^+$ A3.01 cells, coupled with a decrease in the number of viable cells following infection with the AIDS RV, suggested that a CPE assay might be developed to quantitate the infectivity of a virus inoculum. Accordingly, a subclone of the A3.01 cells (A8.7.10) was seeded in quadruplicate in a 96-well microtiter plate (10^3 cells per well) and infected with dilutions of the LAV-A virus stock. At various times after infection, aliquots were removed and assayed for reverse transcriptase activity. On day 9, the infected cells in each well were exposed to [^{3}H]thymidine (1 μCi) for 4 hr, after which the cells were harvested and the radioactivity incorporated was measured. As shown in Fig. 3, low levels of [^{3}H]thymidine incorporation were detected in cells infected with 10^{-1}–10^{-3} dilutions of the LAV-A virus stock. This correlated directly with the results of reverse transcriptase assays done on day 9: at a 10^{-1} dilution, the peak of reverse transcriptase activity occurs on day 7, whereas at 10^{-2} or 10^{-3} dilutions of the LAV-A stock, the peak occurs on day 9 (Fig. 1). In three of four cultures infected at 10^{-4} dilution of virus, only small amounts of labeled thymidine were incorporated. At a 10^{-5} dilution of the LAV-A virus, no incorporation of [^{3}H]thymidine was detected in one of the four cultures examined, suggesting that a virus-induced cytopathic effect had occurred even though reverse transcriptase activity was low. This occurred at a time (9 days) prior to the peak of reverse transcriptase activity for this dilution of the LAV-A stock and suggested that cell viability, as measured by [^{3}H]thymidine uptake, can be used as an earlier and more quantitative measure of virus infection than the transcriptase assay.

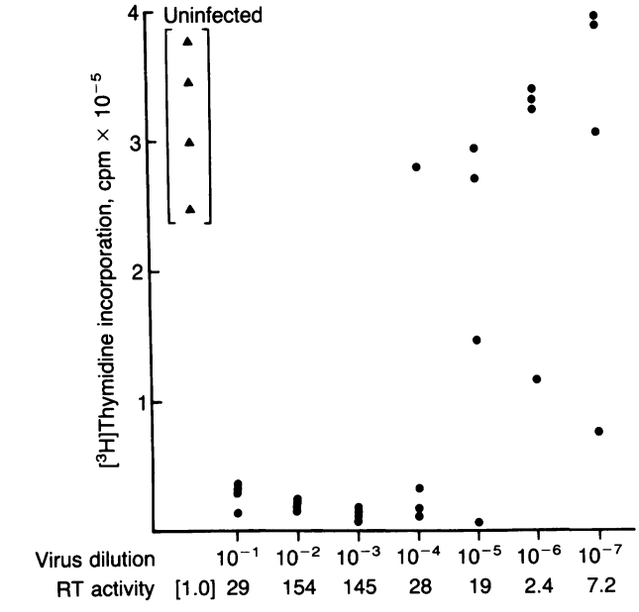

FIG. 3. Microtiter CPE assay of infected A3.01 cells. Microtiter plates containing 10^3 A8.7.10 cells per well were infected in quadruplicate with dilutions of the LAV-A virus stock. Infected (●) or uninfected (▲) cells were incubated for 4 hr on day 9 with 1 μCi of [^{3}H]thymidine and then harvested for determination of incorporated radioactivity. Supernatants from the quadruplicate cultures were also pooled and assayed for reverse transcriptase (RT) activity (expressed as cpm × 10^{-3}) on day 9.

The A3.01 cell line was also used to molecularly clone AIDS RV proviral DNA. RNA was prepared from virus (purified from 14 liters of A3.01 cells 9–12 days postinfection by centrifugation on sucrose gradients) and used as template for the synthesis of ^{32}P-labeled cDNA. A phage λJ1 (11) library containing *Bam*HI-digested cellular DNA from LAV-infected A3.01 cells was screened with the viral cDNA probe. A cloned, integrated proviral DNA segment consisting of AIDS RV sequences extending from the 5′ long terminal repeat to the *Bam*HI site at 8.4 kb (21) as well as 8.7 kb of 5′ flanking cellular DNA (see Fig. 4) was obtained (unpublished work).

One of the questions that could be addressed once molecular clones of viral DNA became available was whether differences in restriction enzyme cleavage patterns attended the propagation of LAV virus in A3.01 cells compared to normal transformed lymphocytes. DNA was prepared from both types of infected cells at the peak of reverse transcriptase activity; cleaved with *Kpn* I, *Hin*dIII, or *Bgl* II; and analyzed by Southern blot hybridization with a recombinant plasmid (pBENN-6) DNA probe containing the 6.5-kb inter-

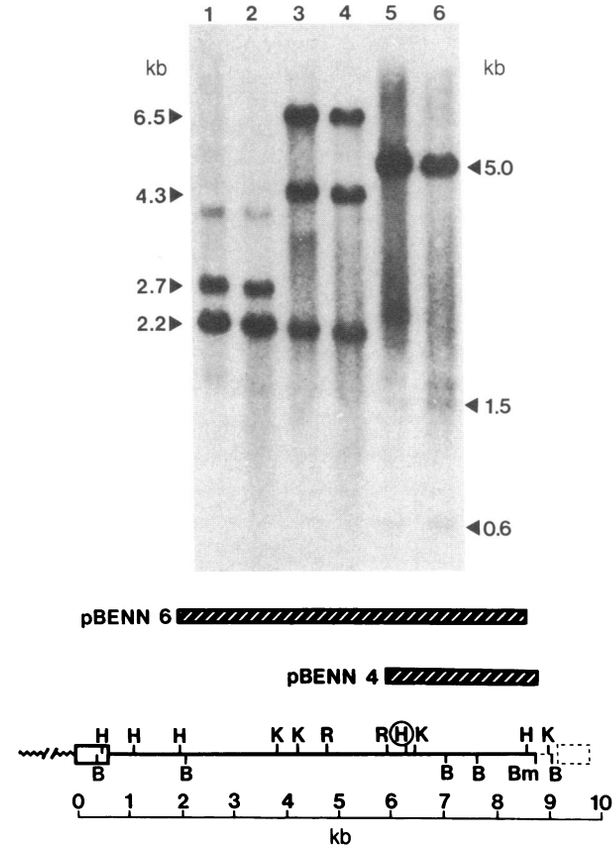

FIG. 4. Stability of the LAV provirus in primary human lymphocytes and A3.01 cells. DNA (5.0 μg) from LAV-A-infected transformed normal human lymphocytes (lanes 1, 3, and 5) or A3.01 cells (lanes 2, 4, and 6) was digested with *Kpn* I (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), or *Bgl* II (lanes 5 and 6), electrophoresed through 0.6% agarose, transferred to a nitrocellulose membrane, and hybridized to ^{32}P-labeled pBENN-6 DNA. The restriction map of a cloned, integrated, 17.5-kb *Bam*HI fragment containing 8.4 kb of AIDS RV DNA (pBENN-3) is shown at the bottom. Restriction enzyme cleavage sites: B, *Bgl* II; H, *Hin*dIII; K, *Kpn* I; Bm, *Bam*HI. The circled *Hin*dIII site is not present in the cloned AIDS RV DNA but is found in a variant present in the LAV virus stock (18) and is detected in lanes 3 and 4. Map positions of the pBENN-4 and pBENN-6 subclones are also indicated. Scale at the bottom starts at the junction between the 5′ long terminal repeat (boxed on map) and the flanking cellular sequence.

nal *Hin*dIII fragment of the provirus (Fig. 4). The results of this experiment indicate that the LAV provirus has undergone no detectable change following its introduction into A3.01 cells (Fig. 4). The sizes of the reactive cleavage products agree with those previously published for LAV (21) or present in the cloned 17.5-kb *Bam*HI fragment. A LAV variant containing an extra *Hin*dIII site at 6.3 kb (Fig. 4) and previously reported to be present in the LAV virus stock (21), replicated efficiently in both normal transformed lymphocytes and A3.01 cells, as evidenced by the appearance of 4.3- and 2.2-kb fragments in addition to the predicted 6.5-kb *Hin*dIII cleavage product.

An analysis of LAV-specific RNA over the duration of tissue culture infection of A3.01 cells is presented in Fig. 5. Cells (3×10^7) were harvested daily beginning 5 days after infection and used for the preparation of total cellular RNA; aliquots of the tissue culture supernatant were assayed for reverse transcriptase activity, which began to rise above background levels on day 10 and continued to increase on days 11 and 12. By day 13 the infected cultures exhibited extensive cell death.

Blot hybridization of total cellular RNA to the ^{32}P-labeled segment of cloned LAV proviral DNA present in pBENN-4 is shown for days 7–13 in Fig. 5*A*. This 2.7-kb probe encompasses the putative *env* region of LAV and should react with full-length as well as subgenomic (and presumably spliced) viral RNAs. Viral RNAs of 9.0 and 4.0–5.0 kb were detected as early as day 9; the quantities of these species peaked 12 days postinfection. A longer exposure of the autoradiogram (not shown) reveals the presence of AIDS RV mRNA as early as day 7, 3 days before the rise in supernatant reverse transcriptase levels. The sizes of the genomic RNA and putative *env* mRNA are both larger than the corresponding 8.2- or 3.0-kb RNAs present in murine leukemia virus-infected cells and electrophoresed in the same gel (Fig. 5*B*).

DISCUSSION

In this study, we have described a cloned T-cell line in which the cytopathic nature of AIDS RV can be studied. Clones of this cell line have been shown to be >95% susceptible to the cytopathic effects induced by this virus. This cell line needs no activation, conditioned medium, or anti-human α-interferon in order to show infectivity. In contrast to the H9 T-cell line or the FR8 Epstein–Barr virus-transformed B-cell line, both of which are continuous producers of the AIDS RV and exhibit minimal viral CPE, infected A3.01 cells die over a 3- to 5-day period coinciding with the peak of reverse transcriptase activity. The A3.01 cell line has been successfully used for virus titration by CPE or reverse transcriptase assays, large-scale production of virus, isolation of molecular clones of the AIDS RV, and the identification of unique species of viral mRNA.

The data presented in Table 1 clearly indicate that normal transformed lymphocytes are more sensitive than A3.01 cells to infection by the AIDS RV. In this regard, it has been our experience that transformed lymphocytes are the cell of choice for new virus isolation. This result is somewhat paradoxical, since only 2–10% of phytohemagglutinin-stimulated normal lymphocytes are infected with the AIDS RV as monitored by indirect immunofluorescence (22), leading one to predict that viral infection of these cells should be characterized by slower replication kinetics and lower virus yields. However, exactly the opposite is the case, suggesting that the AIDS RV replicates significantly more efficiently in mitogen-transformed lymphocytes than in A3.01 cells. Nonetheless, we have successfully transferred seven of seven new virus isolates into A3.01 cells, following the preparation of high-titered stocks in normal transformed lymphocytes, and are now propagating these isolates in the A3.01 cells (unpublished observations). Changes in cytopathic properties or detectable alterations in proviral restriction maps do not appear to accompany the adaptation of the virus to A3.01 cells.

As shown in Fig. 2, the course of viral replication in A3.01 cells could be correlated with the loss of Leu-3 surface molecules. Cells surviving infection were Leu-3$^-$ but still expressed the Leu-1 and transferrin-receptor surface molecules. Surviving cells neither produced (as judged by reverse transcriptase activity) nor were infectable with the AIDS RV. On the other hand, preliminary results indicate that virus can be induced from these cells after treatment with 5-iododeoxyuridine. Since 1–4% of the A3.01 cells are Leu-3$^-$ prior to infection, it is possible that this subpopulation becomes infected and harbors the virus in a latent form. Alternatively, the survivor cells could have arisen from a small fraction of the infected Leu-3$^+$ cells that have undergone alteration in expression of surface antigens. Further work is needed to evaluate the state of viral DNA in these cells as well as to monitor AIDS RV mRNA expression prior to and subsequent to induction.

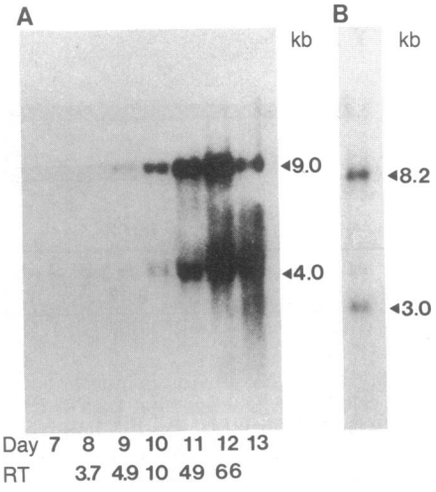

FIG. 5. Blot hybridization analysis of electrophoretically fractionated RNA from LAV-A-infected A3.01 cells. (*A*) RNA prepared at the indicated times postinfection was electrophoresed in a formaldehyde-containing agarose gel, transferred to nitrocellulose membranes, and hybridized to the ^{32}P-labeled pBENN-4 DNA insert (see Fig. 4). Reverse transcriptase activity (RT, expressed as cpm $\times$ 10^{-3}) was assayed in supernatants from the infected cells. (*B*) Cellular RNA from murine leukemia virus-infected cells was electrophoresed in the same gel and hybridized with a murine leukemia virus *env*-specific probe.

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