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Production of Acquired Immunodeficiency Syndrome-Associated Retrovirus in Human and Nonhuman Cells Transfected with an Infectious Molecular Clone

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We constructed an infectious molecular clone of acquired immunodeficiency syndrome-associated retrovirus. Upon transfection, this clone directed the production of infectious virus particles in a wide variety of cells in addition to human T4 cells. The progeny, infectious virions, were synthesized in mouse, mink, monkey, and several human non-T cell lines, indicating the absence of any intracellular obstacle to viral RNA or protein production or assembly. During the course of these studies, a human colon carcinoma cell line, exquisitely sensitive to DNA transfection, was identified.

A group of T-lymphotropic retroviruses (RVs), with a genomic structure characteristic of lentiviruses (34), has been isolated from patients with acquired immunodeficiency syndrome (AIDS), AIDS-related complex, and generalized lymphadenopathy (2, 9, 23). During its propagation in vitro, the virus selectively infects and ultimately kills the subset of human T lymphocytes exhibiting the OKT4-Leu-3 phenotype (7, 20), the same cells that are profoundly affected in individuals suffering from the disease (14, 15).

Mechanisms responsible for cell- and species-specific tropism(s) have been studied in a number of retroviral systems. For both avian and murine RVs, restriction affecting the interaction of the viral envelope glycoprotein and a specific cell receptor has been demonstrated repeatedly and is responsible for the unique host range of xenotropic and ecotropic mouse leukemia viruses (for a review, see reference 36). In the murine system, at least, retroviral restriction or tropism may also be determined at a point subsequent to adsorption and penetration. For example, the thymotropism exhibited by many mink cell focus-forming murine leukemia viruses very likely reflects the properties of enhancer elements present within the long terminal repeats of these recombinant murine leukemia viruses (5). Furthermore, *Fv-1* (29) restriction involves the interaction of viral capsid proteins (18, 19, 31) and certain cell component(s) during the early phase of infection.

In this report, the potential restriction of AIDS RV replication in non-T4 lymphocytes was evaluated. Because the transfection of an infectious molecular clone of the AIDS RV provirus would bypass any barrier imposed by the interaction of virus particles with their receptors, intracellular restriction affecting the expression of viral genes can be readily examined. Cloned AIDS RV DNA was introduced into 3 human lymphoid, 11 human nonlymphoid, and 3 nonhuman cell lines. The progeny, infectious virions, were detected in 14 of the 17 transfected cell lines, indicating the absence of any intracellular obstacle to viral RNA or protein synthesis and assembly.

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MATERIALS AND METHODS

Cells. The cell lines used in these studies are listed in Table 1. The three human lymphoid cell lines were propagated and maintained in RPMI 1640 medium supplemented with heat-inactivated (56°C for 30 min) 10% fetal calf serum. The adherent cell lines listed in Table 1 were cultured in Dulbecco modified Eagle minimal essential medium containing heat-inactivated 10% fetal calf serum. Normal human peripheral blood lymphocytes (PBLs) were cryopreserved and stored in liquid nitrogen until needed. Before use, the PBLs were quickly thawed, washed, and prepared for infection as described previously (2).

Construction of an infectious molecular clone. The origin of the NY5 and LAV AIDS RV isolates has been outlined previously (2, 3). Molecular clones of integrated NY5 and LAV proviruses were obtained from bacteriophage lambda DNA libraries constructed from *Eco*RI-restricted preparations of virus-infected PBLs. NY5 proviral DNA, like other North American isolates, contained a single *Eco*RI site at 5.7 kilobases (kb) and was cloned as two separate restriction fragments containing either 5′ or 3′ flanking cellular DNA. Integrated NY5 proviruses, cloned in *Eco*RI-digested λ Charon 4A (4), were identified by hybridization to ^{32}P-labeled pBenn6, which contains 6.5-kb AIDS RV sequences mapping between 1.7 and 8.2 kb (8). Molecular clones containing sequences located 3′ to the *Eco*RI site at 5.7 kb of LAV proviral DNA were isolated from a λJ1 (28) library after hybridization with ^{32}P-labeled pBenn4 (8). The cloned NY5 and LAV proviruses were then transferred to *Eco*RI-digested pUC18 and subsequently used to construct full-length clones.

Transfection assays. Nonlymphoid and lymphoid cells were transfected by the calcium phosphate precipitation (16, 37) and electroporation (30) techniques, respectively, with 10 μg of uncleaved plasmid DNA in each assay. Virus production was monitored in non-T4 cells by cocultivation with CD4$^+$ A3.01 cells (10^6 cells of each type) 2 days after transfection. Reverse transcriptase (RT) assays were carried

TABLE 1. Cell lines used for transfection

Cell line	Description	Virus production[a]		Source or reference
		Co-culture	Filtrate	
A3.01	Human T-cell leukemia	+	+	8
BJAB	Human B-cell lymphoma (EBV−)[b]	+	ND	22
Raji	Human B-cell lymphoma (EBV+)[c]	−	ND	ATCC CCL86
A-204	Human rhabdomyosarcoma cells	+	+	ATCC HTB82
CHP126	Human neuroblastoma cells	−	ND	1
HMB2	Human melanoma cells	+	ND	39
CAPAN-1	Human pancreatic carcinoma cells	+	+	ATCC HTB79
SK-OV-3	Human ovarian carcinoma cells	+	+	ATCC HTB77
T47D	Human breast carcinoma cells	−	ND	39
CaCO-2	Human colon carcinoma cells	+	+	ATCC HTB37
SK-CO-1	Human colon carcinoma cells	+	ND	ATCC HTB39
HT-29	Human colon carcinoma cells	ND	+	ATCC HTB38
SW480	Human colon carcinoma cells	ND	+	ATCC CCL228
SW1463	Human rectal carcinoma cells	ND	+	ATCC CCL234
Mink	Mink lung epithelial cells	+	ND	ATCC CCL64
COS-1	Monkey kidney cells transformed by simian virus 40	+	+	12
NIH 3T3	Mouse fibroblast cells	+	ND	ATCC CCL92

[a] +, Production; −, no production. ND, Not determined.
[b] EBV−, Epstein-Barr virus negative.
[c] EBV+, Epstein-Barr virus positive.

out at various times after cocultivation as described previously (8).

In situ hybridization. Transfected cell preparations were sedimented onto polylysine-coated glass slides, fixed in periodate-lysine-paraformaldehyde-glutaraldehyde, and pretreated with HCl and proteinase K to allow the labeled probe to enter the cells (10, 11). Cells were prehybridized in 10 mM Tris (pH 7.4)–2× standard saline citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4])–50% formamide–1× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.], 0.02% bovine serum albumin)–200 μg of yeast tRNA per ml at 45°C for 2 h and were hybridized in this solution plus 10% dextran sulfate, 5 μM dithiothreitol, and 10^6 cpm of ^{35}S-labeled AIDS RV RNA in 10-μl reaction mixtures. Subgenomic viral DNA fragments present in pB1 (3), pBenn6 (8), pB11 (3), and a recombinant plasmid (pRG-B) which contains a 1.35-kb *Hin*dIII fragment mapping between 8.25 and 9.6 kb on the proviral DNA were subcloned into SP6/T7 vectors (Promega Biotec, Madison, Wis.), and the pooled DNAs were transcribed with ^{35}S-labeled UTP (Amersham Corp., Arlington Heights, Ill.). The labeled RNAs were incubated with 40 μM $NaHCO_3$–60 μM Na_2CO_3 [pH 10.2] before hybridization to facilitate their entry into cells.

Hybridization was performed at 45°C for 8 h. The samples were then washed in 2× SSC at 22°C for 10 min, with two changes; 2× SSC–0.1% Triton X-100 at 60°C for 30 min; 2× SSC plus RNase A (40 μg/ml) and RNase T_1 (10 U/ml) at 37°C for 30 min; and 2× SSC at 60°C for 10 min. All solutions except those with RNase A contained 5 μM dithiothreitol and 1 μM EDTA. Autoradiography was performed for 1 to 2 days as described previously (11).

CAT assays. Chloramphenicol acetyltransferase (CAT) assays with pSV2CAT DNA have been described previously (13). The uptake of pSV2CAT DNA into transfected cells was monitored by dot blot hybridization using ^{32}P-labeled pUC18 DNA.

RESULTS

Construction of full-length clones of the AIDS RV provirus. Our previous experience indicated that only a minority of cloned murine leukemia virus proviral DNAs were infectious after their introduction into susceptible cells. Therefore, we decided to take advantage of the single *Eco*RI site that virtually bisected (located at 5.7 kb) the proviruses of North American AIDS RV isolates (3), and we elected to mix and match 5′ and 3′ halves of integrated proviral DNAs that had been obtained from different infected cellular DNA libraries. Representative examples are shown in the top portion of Fig. 1. λN5′ consisted of a 20.2-kb *Eco*RI fragment in λ Charon 4A that was cloned from a library of NY5-infected PBLs. λL3 and λL4 were isolated from LAV-infected cellular DNA preparations with a 3′ AIDS RV probe and consisted of 7.5- and 13.6-kb *Eco*RI inserts, respectively, in a λJ1 phage vector. An 8.3-kb *Bam*HI-*Eco*RI subclone of λN5′ was introduced into *Bam*HI-*Eco*RI-digested pUC18 to generate a plasmid designated pN5′. Because pN5′ contained a single *Eco*RI site, *Eco*RI fragments containing the 3′ half of the AIDS RV provirus could be readily introduced, thereby generating full-length and potentially infectious constructs. The *Eco*RI segments from λL3 (both orientations) and λL4 were inserted into pN5′ (Fig. 1). Of note is the presence of a unique *Hin*cIII site at 8.7 kb in pNL4-3; a *Hin*cII site at this position (representing a restriction enzyme site polymorphism in the parental LAV virus stock) had been previously identified in an isolate from Alabama (3).

Infectivity of full-length AIDS RV proviral DNA constructs was monitored after electroporation into A3.01 cells. We have previously reported that the A3.01 cell line is >95% Leu-3$^+$ Leu-8$^+$ Leu-1$^+$ and is exquisitely sensitive to AIDS RV infection, exhibiting the same viral cytopathic effect observed in infected PBLs, including cell death (8). Only the pNL4-3 DNA construct was infectious after its introduction into A3.01 cells (Fig. 2). Syncytia were visualized in cultures transfected with pNL4-3 DNA as early as day 9. This was followed by a peak of RT activity on day 16, as well as by a profound reduction in the number of viable cells. pNL3-2 DNA failed to elicit any cytopathic effect or RT activity after its electroporation into A3.01 cells. Because pNL3-2 contained the same 5′ 5.7 kb of AIDS RV sequences present in the infectious plasmid pNL4-3, an alteration affecting viral sequences mapping 3′ to the *Eco*RI joint most likely explains the loss of biological activity.

Transfection of the infectious molecular clone of the AIDS RV into nonhuman T cells. Viral infectivity studies indicate that, with few exceptions (24, 27), the AIDS RV can only be propagated in the Leu-3-OKT4 helper-inducer subset of human T lymphocytes. This selective cell tropism most likely reflects the interaction of AIDS RV particles with a

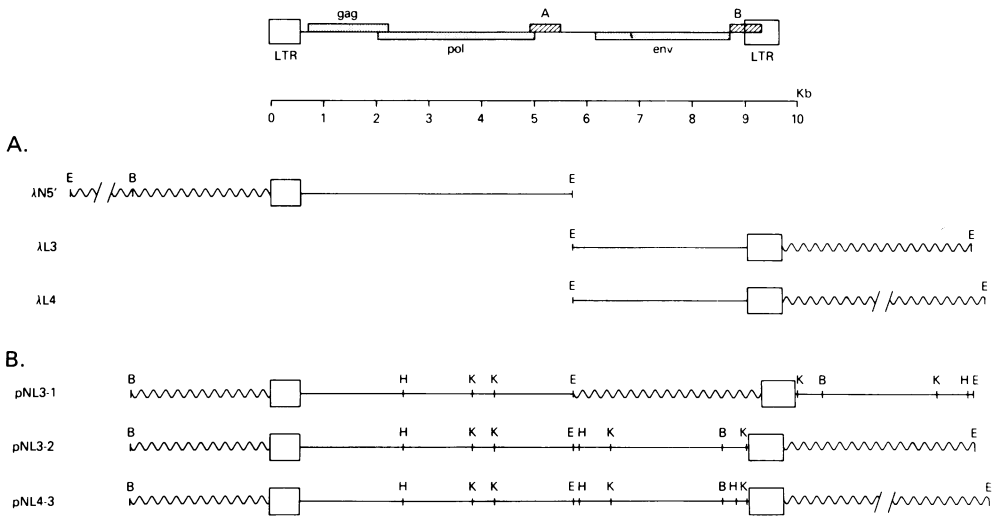

FIG. 1. Construction of full-length clones of AIDS RV. Integrated NY5 and LAV proviruses were cloned in λ Charon 4A (λN5′) or λJ1 (λL3 and λL4), respectively (A). The *Eco*RI inserts of λL3 (both orientations) and λL4 were introduced into the *Eco*RI site of plasmid pN5′ DNA, which contained the *Bam*HI-*Eco*RI segment from λN5′ (see Materials and Methods) (B). Only inserts are shown. A schematic diagram of AIDS proviral DNA is shown at the top of the figure. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*cII; K, *Kpn*I.

specific receptor(s) (7, 21, 26) on the surface of $CD4^+$ lymphocytes. However, intracellular restriction involving, for example, the activity of viral promoter or enhancer or the assembly of viral proteins and genomic RNA into infectious virions has not been formally ruled out. The existence of such an intracellular block could readily be evaluated with an infectious clone of the AIDS provirus, since the barrier imposed by a specific cell receptor would no longer be a factor.

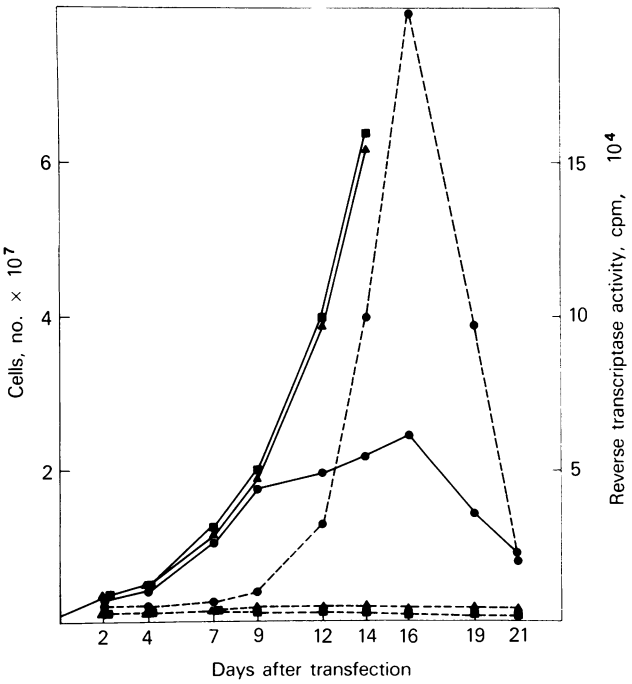

FIG. 2. Kinetics of virus infection after transfection of A3.01 cells. On day 0, pNL4-3 (●), pNL3-2 (▲), or pNL3-1 (■) was introduced into A3.01 cells by electroporation, and 10^6 viable cells were placed in culture. The number of viable cells in the culture (——) and RT activity (– – –) were monitored on the days indicated.

pNL4-3 DNA was introduced into the 16 non-T cell lines listed in Table 1, which included both human and nonhuman cells. Because none of these transfected cells would be expected to express the putative receptor for the AIDS RV, it seemed very unlikely that progeny, virus particles produced as a result of the initial transfection, would reinfect other cells in the culture. Because insufficient numbers of virions would be generated to be detected directly by the RT assay, $CD4^+$ A3.01 cells were added to the cultures 48 h after transfection to amplify any virions that appeared. The presence of virus was monitored by RT assays at various times after cocultivation. Of the 16 non-T cell lines, 13 yielded infectious AIDS RV particles as a consequence of transfection with pNL4-3 DNA (Fig. 3 and Table 1). In most cases, the peak of RT activity was detected 14 to 16 days after transfection (12 to 14 days after cocultivation with A3.01 cells); cocultures of COS-1 (monkey) and SK-OV-3 (human ovarian carcinoma) cells exhibited more rapid kinetics of virus production. A repeat of the experiment depicted in Fig. 3 gave similar results. In a parallel experiment that omitted cocultivation with A3.01 cells, no RT was detected. To rule out the possibility that the non-T cell lines might harbor a cryptic lymphotropic virus with RT activity, each was cocultivated with A3.01 cells for 4 weeks; no infectious virus was detected. In addition, when the noninfectious molecular clone of the AIDS RV, pNL3-1, was transfected into each of the cell lines listed in Table 1, no infectious virions were generated, even after 4 weeks of coculture.

We were interested in ascertaining whether the progeny, virus particles synthesized in non-T cells, entered A3.01 cells by cell-to-cell transfer or whether they budded into the tissue culture medium and subsequently adsorbed to viral receptors on the $CD4^+$ target cells. Cell-free supernatants obtained from eight (A204, CAPAN-1, CaCO-2, HT-29, SK-OV-3, SW480, SW1463, COS-1) of the cell lines 48 h after transfection and prepared by filtration of tissue culture medium through 0.45-μm-pore membranes were used to infect phytohemagglutinin-stimulated PBLs. Infectious AIDS RV was present in all of the supernatants tested, with

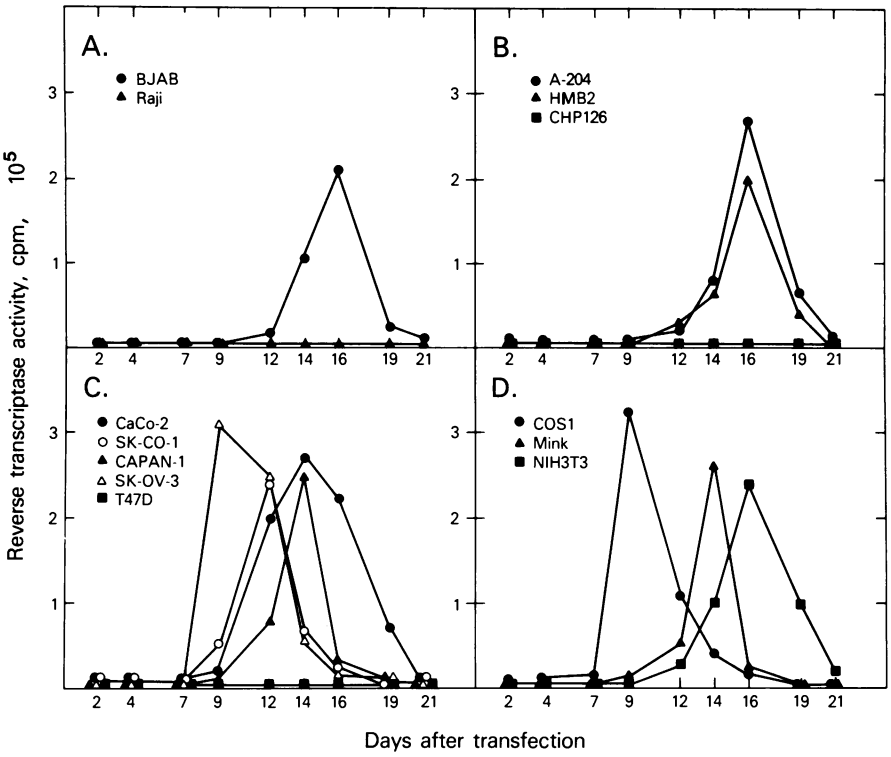

FIG. 3. Kinetics of RT activity in transfected cells cocultivated with A3.01 cells. On day 2 after transfection with the infectious molecular clone pNL4-3, 10^6 cells were cocultured with 10^6 A3.01 cells, and RT activity was monitored on the days indicated. See Table 1 for the description of each cell line.

peaks of RT detected 5 to 14 days after infection of the PBLs (Table 1). No virus was detected when the supernatants of mock-infected cultures were examined.

Replication of the AIDS RV in non-T cells was also monitored by in situ hybridization. Viral RNA was readily detected in the A204 rhabdomyosarcoma cell line 2 days after transfection (Fig. 4A). RNase treatment of the transfected cell preparation before hybridization reduced the signal to background levels (zero to two grains per cell). Assuming that five grains above background (seven or more grains per cell) indicated the presence of AIDS RV RNA, approximately 6.7% of the A204 cells were synthesizing viral RNA 48 h after transfection. The number of cells expressing viral RNA fell precipitously thereafter, to 0.2% on day 3 and to 0 (out of 10^5 A204 cells examined) on day 7. The recovery of virus by cocultivation with A3.01 cells on days 2 and 3 but not on day 7 (data not shown) is consistent with these in situ hybridization results. Similar transient expression of AIDS RV RNA was observed in BJAB, SW480, and NIH 3T3 cells transfected with pNL4-3 DNA (data not shown).

Failure to obtain a chronic AIDS RV-producing line. Because the non-T cell lines listed in Table 1, which synthesized infectious virus particles after transfection, apparently lacked the receptor(s) that would permit the initial burst of virions to spread throughout the culture, the possibility existed that cells continuously producing virus might be isolated from the mass culture of the transfected cells. Therefore, A204 cells were cotransfected with pSV2NEO and pNL4-3 DNAs (in a molar ratio of 1:10, respectively), and G418-resistant cells were selected as described previously (35). In a companion experiment, no G418-resistant clones were obtained from mock-transfected cells. None of the 92 G418-resistant cell clones that were isolated during a 3-week period synthesized infectious AIDS RV after cocultivation with A3.01 cells. This result suggested that the resistant A204 cells, although containing the pSV2NEO gene, harbored either a defective copy or no proviral DNA. This finding also raised the possibility that nonlymphoid cells undergoing productive infection with the AIDS virus may also be killed in the process.

Identification of a human cell line that is exquisitely sensitive to transfection by cloned AIDS RV proviral DNA. During the examination of cell-free filtrates obtained from nonlymphoid cells transfected with the infectious clone of the AIDS provirus, we observed that one of the supernatants contained high levels of RT activity. A more careful evaluation of the human colon carcinoma cell line SW480 revealed that substantial amounts of RT activity were detectable 24 h posttransfection (Fig. 5); these levels gradually fell to background levels over a 3-week period. When the experiment depicted in Fig. 5 was monitored by in situ hybridization, 25 and 19% of the transfected SW480 cells were found to be synthesizing viral RNA on days 1 and 2, respectively (Fig. 4B). In comparison with a cloned human $CD4^+$ lymphocyte cell line (S. Koenig, unpublished data) productively infected with the AIDS RV (Fig. 4D), SW480 cells, expressing viral gene products, contained two to five times more grains.

We considered the possibility that the unique susceptibility of SW480 cells to cloned AIDS RV proviral DNA might represent a more global sensitivity to DNA transfection. Therefore, pSV2CAT DNA was introduced into the seven cell lines indicated in Table 2; conversion of chloramphenicol to its acetylated form, as well as DNA uptake, was measured in each case. When normalized for DNA uptake, SW480 cells were at least five times more efficient in CAT gene expression than any of the other cells lines examined.

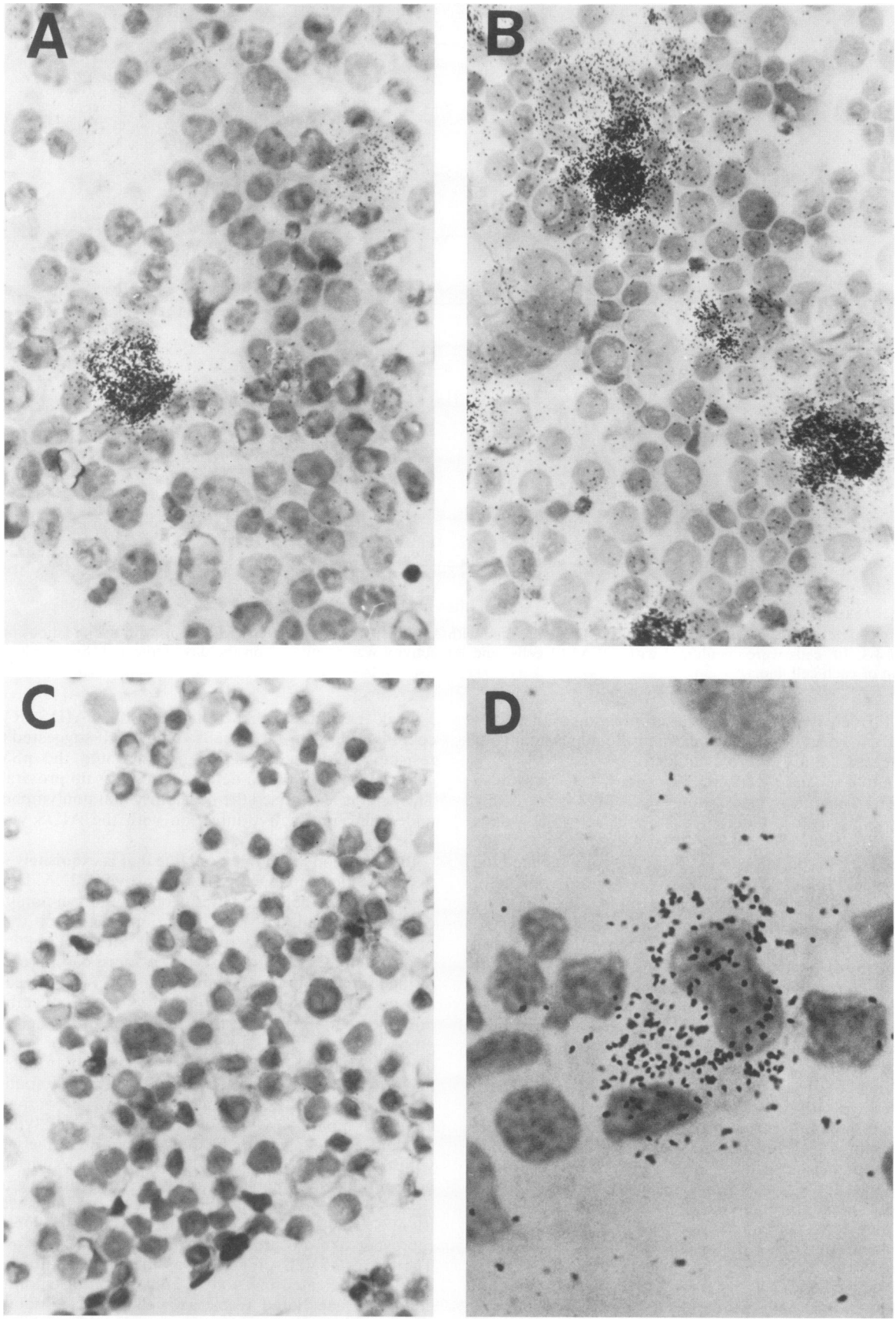

FIG. 4. In situ hybridization of cells transfected with the infectious molecular clone of the AIDS RV. A-204 (A) and SW480 (B) cells were subjected to in situ hybridization 48 h after transfection with pNL4-3 DNA. Mock-transfected A-204 (C) and LAV-infected $CD4^+$ lymphocyte (D) cells are also shown. Magnification in panels A, B, and C, ×100; magnification in panel D, ×400.

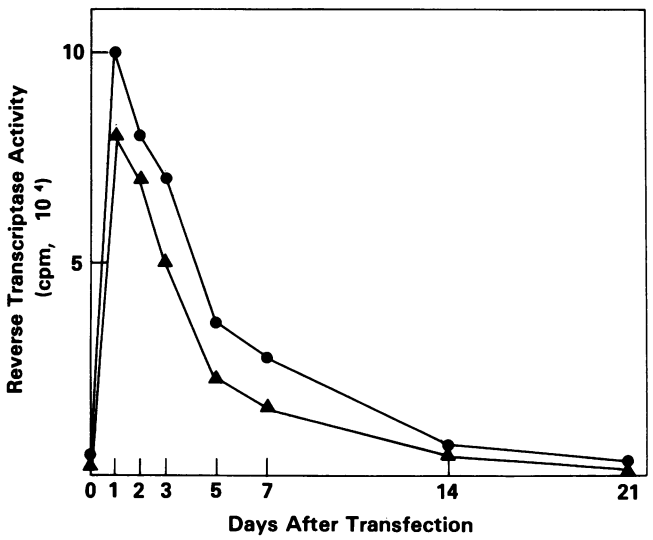

FIG. 5. Kinetics of RT activity in SW480 cells transfected with the infectious clone pNL4-3. On day 0, 3×10^6 SW480 cells were transfected with the infectious clone pNL4-3 by the calcium phosphate precipitation method, and RT activity in the tissue culture medium was monitored on the days indicated. ● and ▲ indicate the results of two independent experiments.

DISCUSSION

A major conclusion of our experiments involving the transfection of an infectious molecular clone of the AIDS virus into several different cell lines is that the interaction of AIDS RV particles with their receptor(s) is clearly the principal determinant of cell tropism. Once this cell surface restriction is negotiated, viral RNA and proteins are synthesized and assembled into infectious and cytopathic virions, irrespective of cell type. Although this result might have been inferred from earlier studies which showed that the AIDS RV-*trans*-activating determinant functioned in a variety of cell types (33), this is the first report to demonstrate that non-T cells of four different mammalian species are capable of generating infectious virus.

Unlike the situation with the cloned provirus of HTLV-II, which required cotransfection and selection with G418 (6), infectivity with pNL4-3 DNA was observed after its introduction into the $CD4^+$ A3.01 cells without the use of selective pressure. A characteristic feature of transfecting non-T cells with the cloned AIDS RV provirus is the relatively short period (usually up to 7 days) during which progeny are synthesized. This transient production of virions, shown graphically in Fig. 5, most likely reflects the failure of the input DNA to stably integrate into the chromosome of the transfected cells. Concatemers of the cloned viral DNA formed during calcium phosphate precipitation (16, 37) may function as pseudointegrated templates for AIDS RV RNA synthesis for relatively brief periods of time, after which they are degraded or otherwise eliminated. Another explanation for the transient expression of virus production after transfection is that cells are killed as a consequence of synthesizing and assembling viral gene products. Proving cell death after the introduction of viral DNA in the systems we used is technically very difficult. At best, about 20% of transfected cells (in the case of SW480) were synthesizing viral RNA during the first 48 h after transfection. Unlike experiments with $CD4^+$ lymphocytes in which virtually all of the cells are killed by waves of newly synthesized virions, nonlymphocytes, dying as a result of a productive virus infection, would be replaced in the monolayer by neighboring cells lacking the receptor(s) for secondary infection by the progeny, AIDS RV particles, and would thereby escape detection. Furthermore, the failure to obtain G418-resistant cells that produce infectious viral particles after cotransfection with pSV2NEO and pNL4-3 DNAs is certainly consistent with the death of nonlymphocytes expressing AIDS RV gene products.

Transfection of the cloned AIDS RV provirus resulted in virus production in 13 of the 16 non-T cells examined. The three instances in which no virus was detected should not be presently viewed as examples of intracellular restriction. Although no virus was generated after the introduction of the infectious clone into Raji cells, a second B-lymphocyte line (BJAB) synthesized replication-competent virions (Table 1). Factors associated with the transfection assay per se, such as the efficiency of DNA uptake, could very likely be responsible for the failure to detect virus particles in all of the cell lines examined.

A by-product of these investigations was the discovery of a human colon carcinoma cell line that was particularly sensitive to transfection. AIDS RV virions were demonstrable in cultures of SW480 cells 24 h posttransfection, thereby obviating the necessity for cocultivation with $CD4^+$ target cells. This susceptibility to transfection also extended to non-RV DNAs in experiments involving pSV2CAT DNA. At the present time, we have no explanation for this unique property of SW480 cells. Certainly, the augmented expression of transfected DNA is not due to increased DNA uptake (Table 2), nor is it a characteristic feature of colon carcinoma cells, since four other cell lines (Table 1) failed to exhibit this property of SW480 cells.

We (3) and others (17, 25, 32, 38) have noted that the genomic heterogeneity of the AIDS RV genome affects primarily *env* gene sequences. Because the studies described in this report demonstrate that the main determinant of cell tropism involves the interaction of the viral envelope and cell receptor(s), it is tempting, from an evolutionary viewpoint, to link *env* variability to the appearance of the AIDS virus as a human pathogen. Stripped of its capsid and envelope proteins, the cloned viral DNA was expressed as

TABLE 2. Relative CAT activity in cells transfected with pSV2CAT

Cell line	Description	% Conversion[a]	DNA uptake[b]	CAT activity[c]
SW480	Human colon cells	83.0	1.0	100
SK-CO-1	Human colon cells	6.8	1.2	7
SK-OV-3	Human ovarian cells	33.8	2.0	20
CAPAN-1	Human pancreatic cells	10.2	0.7	18
A-204	Human rhabdomyosarcoma cells	3.2	1.1	4
HMB2	Human melanoma cells	0.5	0.2	3
NIH 3T3	Mouse fibroblasts	0.7	0.8	1

[a] CAT assays were done with equivalent amounts of protein from each cell lysate (100 μg per assay). After analysis by ascending thin-layer chromotography, the rates of conversion were determined by scintillation counting. CAT activity of mock-transfected cell lysates was less than 0.1%.

[b] DNA uptake was determined by dot blot hybridization using ^{32}P-labeled pUC18 DNA. Membranes were subsequently monitored in a liquid scintillation counter, and DNA uptake was normalized relative to that of SW480 cells. The counts hybridized per minute varied from 2,338 (HMB2) to 29,700 (SK-OV-3); mock-transfected cultures contained less than 200 cpm.

[c] CAT activity was normalized for conversion and DNA uptake relative to that of SW480 cells.

infectious virus in virtually all cells tested. One could propose that an alteration more profound than is normally observed in the *env* region of different AIDS RV isolates drastically alters the cell and species target of a progenitor to the AIDS virus. In the process, such a virus could lose its original tropism and assume a new ecological niche: the helper and inducer $CD4^+$ lymphocytes of humans.

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