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17. Transcription reactions were performed as described by H. L. Carter and C. P. Moran [*Proc. Natl. Acad. Sci. U.S.A.* **83**, 9438 (1986)] except that the polymerase was allowed to bind to template for 10 minutes at 37°C before the addition of nucleotides heparin (6 μg) was added 1 minute after the addition of RNA polymerase to prevent reinitiation, and, after the reactions were stopped, 30 μl of reaction mixture was subjected to electrophoresis. The plasmid containing the *cotD* promoter, pLRK100, was constructed by insertion of a 430-bp *B. subtilis* Eco RI–Hinc II DNA fragment from pBD156 (*5, 6*) into Eco RI– and Hinc II–digested pUC19 [C. Yanisch-Perron, J. Vieira, J. Messing, *Gene* **33**, 103 (1985)]. The in vivo transcription start site indicated for *cotD* at the top of the figure is based on unpublished results (*6*). The *spoIVCB* promoter-containing plasmid, pBK16, was constructed by insertion of an approximately 400-bp Pst I–Sac I *B. subtilis* DNA fragment from pBK9 (*3*) that had been made blunt-ended at the Sac I site by digestion with mung bean nuclease into Pst I– and Hinc II–digested pUC19. The indicated in vivo *spoIVCB* transcription start site results from (*3*).
18. The RNA polymerase used in the experiment of Fig. 1 was a fraction that eluted at high-salt concentrations from a DNA-cellulose column (provided by C. W. Cummings and W. G. Haldenwang). For Fig. 2 we used the following modification of their gradient elution procedure (*8*). *Bacillus subtilis* strain SC104 (S. Cutting and R. Losick, unpublished data) containing a *cotA-lacZ* translational fusion (which is similarly regulated to *cotD*) was harvested during sporulation when the gene fusion was substantially induced as monitored by assaying *cotA*-directed β-galactosidase synthesis. Cells were washed with harvest buffer [T. Linn, A. L. Greenleaf, R. Losick, *J. Biol. Chem.* **250**, 9256 (1975)] supplemented with 10 percent glycerol and then with buffer I (*19*) supplemented with 5 percent (v/v) phenylmethylsulfonyl fluoride (PMSF) (6 mg/ml in 95 percent ethanol) and stored at −70°C. Cells (56 g) were resuspended in 140 ml of buffer I containing PMSF, passaged twice through a French Pressure Cell (15,000 psi), sonicated for 1 minute (450 watts), and centrifuged for 90 minutes at 120,000*g* and 4°C. The clarified supernatant was subjected to heparin-agarose and DNA-cellulose column chromatography as described (*8*) except that the DNA-cellulose column (15 ml) was washed with 15 ml of buffer C containing 0.1*M* KCl and then with 15 ml of buffer C containing 0.5*M* KCl prior to elution with a linear gradient (120 ml) of 0.5*M* to 1.3*M* KCl in buffer C. Fraction 14 (Fig. 2) represents the beginning of the salt gradient. Fractions (4 ml) were collected and dialyzed into storage buffer (*19*) modified to contain 0.1*M* KCl and were stored at −20°C.
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22. Core RNA polymerase was prepared from *B. subtilis* strain ML21, which contains deletion mutations of the sigma factor encoding-genes *sigB* and *sigH* (M. Lampe and R. Losick, unpublished), as described (*19*) except that a Bio-Rex 70 column was substituted [R. R. Burgess and J. J. Jendrisak, *Biochemistry* **14**, 4634 (1975)] for the phosphocellulose column.
23. We thank W. G. Haldenwang for invaluable assistance during the early stages of this work and P. Stragier for pointing out that the σ^K amino acid sequence matches that deduced for the *spoIVCB* product and for stimulating suggestions during the final stages of the project. Supported by NIH grant GM18568 (R.L.), a postdoctoral fellowship from the Helen Hay Whitney Foundation (L.K.), and a predoctoral fellowship from NSF (B.K.).

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Amplification and Molecular Cloning of HTLV-I Sequences from DNA of Multiple Sclerosis Patients

E. PREMKUMAR REDDY,* MAGNHILD SANDBERG-WOLLHEIM, RICHARD V. METTUS, PHILLIP E. RAY, ELAINE DEFREITAS, HILARY KOPROWSKI

Techniques of gene amplification, molecular cloning, and sequence analysis were used to test for the presence of sequences related to human T-lymphotropic virus type I (HTLV-I) in peripheral blood mononuclear cells of six patients with multiple sclerosis (MS) and 20 normal individuals. HTLV-I sequences were detected in all six MS patients and in one individual from the control group by DNA blot analysis and molecular cloning of amplified DNAs. The viral sequences in MS patients were associated with adherent cell populations consisting predominantly of monocytes and macrophages. Molecular cloning and nucleotide sequence analysis indicated that these amplified viral sequences were related to the HTLV-I proviral genome.

ALTHOUGH EARLY DATA INDICATED that neurologic disorders of wild mice are caused by mouse retrovirus infection (*1*), no direct correlation has been established between human neurological disorders and retroviruses. However, recent evidence suggests that the human immunodeficiency virus (HIV-1) as well as HTLV-I and HTLV-II are associated with neurological disorders (*2–11*).

We previously (*4*) reported the presence of antibodies that react with the HTLV-I *gag* (p24) protein in samples of serum and cerebrospinal fluid (CSF) from patients with multiple sclerosis (MS) in Sweden and in Florida. Sequences of HTLV-I were detected by in situ hybridization analysis in the lymphocytes of cultures of CSF cells obtained from one-third of the patients (*4*). In three patients with progressive chronic encephalomyelopathy, we noted three different patterns of reactivity in relation to HTLV-I infection (*8, 9*): (i) the presence in serum of HTLV-I antibodies together with viral sequences, as determined by in situ hybridization and by the detection of viral antigen in lymphocytes; (ii) the absence of HTLV-I antibodies in the presence of viral sequences in CSF cells; and (iii) the presence of HTLV-I antibodies in the absence of viral sequences in peripheral blood lymphocytes (PBL) and CSF cells (*8, 9*). Subsequently, HTLV-I was isolated from lymphocyte culture (*10*) of one of these patients, and the proviral genomes were cloned (*11*) from these cells.

Since HTLV-I sequences can be detected by in situ hybridization assays in very few lymphocytes of MS patients, never in more than one in 10^4 or one in 10^5 cells (*4*), and since the absence of detectable antibodies to HTLV-I does not exclude HTLV-I infection, we used the polymerase chain reaction (*12, 13*) coupled with forced cloning of the amplified DNA to look for the presence of HTLV-I sequences in three males and three females with MS as defined according to McDonald and Halliday (*14*). The patients were from Sweden and all were diagnosed and treated at the Department of Neurology, University Hospital, University of Lund, Sweden. The age at onset of disease ranged from 11 to 34 years, and the duration, from 2 to 12 years (Table 1). All patients had a moderate mononuclear pleocytosis in CSF and five had oligoclonal immunoglobulin on isoelectrofocusing. The patient with no oligoclonal bands had multiple bilateral lesions on magnetic resonance imaging compatible with MS. At the time of the study, three patients had exacerbations and one of these was treated with corticosteroids. None of the patients had ever received a blood transfusion. Negative controls were ten healthy subjects from Sweden who had no antibodies for HTLV-I and ten healthy blood donors from Philadelphia, Pennsylvania.

Blood samples were obtained from the cubital vein, and the peripheral blood mononuclear cells (PBMC) were processed (*15*). Results of ELISA assays with the use of disrupted HTLV-I virions revealed the presence of antibodies in serum samples from two of the six patients at the time their PBMC were tested for the presence of HTLV-I sequences (Table 1). To amplify HTLV-I sequences in the cellular DNA extracted from PBMC, we used primer pairs from the *gag* and *env* region (*16*). These were 25 bases long and rich in G-C content to allow stable hybridization. The *gag* primers included the recognition sequence for Sma I or Pst I and the *env* primers contained the

E. P. Reddy, R. V. Mettus, P. E. Ray, E. DeFreitas, H. Koprowski, The Wistar Institute of Anatomy and Biology, 36th Street at Spruce, Philadelphia, PA 19104.
M. Sandberg-Wollheim, Department of Neurology, University of Lund, S-22185 Lund, Sweden.

*To whom correspondence should be addressed.

recognition sequence for Sal I and Kpn I, so that after amplification the DNA fragments could be cleaved with these restriction enzymes and cloned into a plasmid vector. The region of amplification for *gag* region contained a unique Nco I site, while the amplified *env* region contained a Cla I site for further diagnostic purposes. Thus, amplification of HTLV-I sequences with *gag* and *env* primers was expected to yield sequences of 530 and 467 bp, respectively. The amplified *gag* sequences, when cleaved with Nco I, would yield two fragments of 390 and 140 bp long while the cleavage of *env* sequences with Cla I would yield fragments of 263 and 204 bp. To detect amplified sequences, we used either a nick-translated probe derived from the HTLV-I proviral genome that spanned the amplified regions or oligonucleotide probes (*16*) that were labeled at the 5′ end with $^{32}PO_4$ and that were complementary to the sequences that were amplified.

We tested our strategy with four DNA samples: (i) a plasmid DNA containing the HTLV-I proviral genome; (ii) total cell DNA isolated from the MT-2 cell line, which is infected with HTLV-I (*17*); (iii) cell DNA derived from PBMC of a patient with chronic encephalomyelopathy initially diagnosed as tropical spastic paraparesis, or TSP (*10, 11*), from which we previously cloned a proviral genome (*11*); and (iv) normal cell DNA (Fig. 1). Distinct amplification of *gag* and *env* sequences occurred in the three cell DNAs that contained the HTLV-I proviral genome (see Fig. 1A, lanes 1, 4, and 6, and Fig. 1C, lanes 2 and 4). The band with the highest intensity was from the plasmid DNA that contained the cloned proviral genome; that with the least intensity was from the "TSP" patient DNA. No nonspecific amplification of DNA sequences occurred when the primers were used in combination with normal cell DNA (Fig. 1A, lanes 8 and 9, and Fig. 1C, lanes 6 and 7). When the amplified *gag* sequences were cleaved with Nco I, all three samples yielded two restriction fragments of 390 bp and 140 bp, further demonstrating the specificity of amplification (Fig. 1A, lanes 2, 5, and 7). Similar results were obtained with *env* sequences where cleavage with Cla I showed two bands of 260 and 200 bp long (Fig. 1C, lanes 3 and 5). Blot hybridization with *gag*- and *env*-specific oligonucleotide probes (*16*) revealed hybridization to the amplified sequence (Fig. 1B, lanes 1, 2, and 4 to 7, and Fig. 1D, lanes 2 to 5). These results showed that amplification of the proviral sequences could be achieved by using the primers we had chosen, that this amplification was specific for HTLV-I sequences, and that no amplification of nonspecific or endogenous viral sequences occurred under the conditions used.

We next tested DNA samples derived from PBMC and peripheral blood lymphocytes of 26 individuals; the six patients with MS (Table 1) and 20 controls. Amplification with *gag* primers of HTLV-I sequences occurred in all six samples from MS patients, and the amplified 530-bp DNA fragment was visually detectable. The intensity of the amplified DNA band was much lower than that of the positive controls from MT-2 cells and amplified DNA from cells of the patient with TSP. In general, analysis of samples from all patients showed bands visualized readily after ethidium bromide staining and

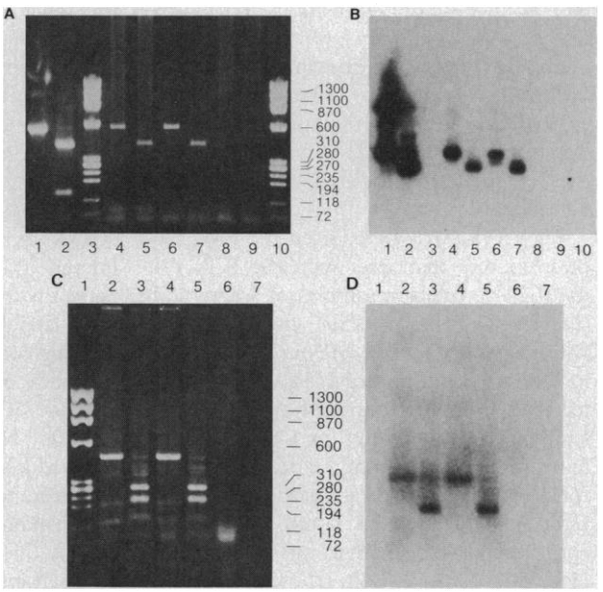

Fig. 1. HTLV-I *gag* and *env* gene amplification analysis of DNAs isolated from bacterial plasmid (A and B, lanes 1 and 2); MT-2 cell line infected with HTLV-I (A and B, lanes 4 and 5; C and D, lanes 2 and 3); peripheral blood lymphocytes of a TSP patient 3-19-3 (A and B, lanes 6 and 7; C and D, lanes 4 and 5); and a human cell line ML-2 known to be negative for HTLV-I sequences (A and B, lanes 8 and 9; C and D, lanes 6 and 7). Lanes 3 and 10 of (A) and (B) and lane 1 of (C) and (D) contain molecular weight markers (ΨX DNA digested with Hae III). (**A**) Ethidium bromide staining pattern and (**B**) hybridization pattern with the labeled oligonucleotide probe of *gag* primer amplified sequences (*16*). Lanes 1, 4, 6, and 8 contain undigested DNA while lanes 2, 5, 7, and 9 contain DNAs digested with Nco I. (**C**) Ethidium bromide staining pattern and (**D**) hybridization pattern of *env* primer amplified sequences (*16*). Lanes 2, 4, and 6 contain undigested DNA, while lanes 3, 5, and 7 contain DNAs digested with Cla I.

Table 1. Presence of HTLV-I sequences in PBMC of MS patients.

Patient code	Age/ sex	Duration of disease (years)	Initial symptoms	Clinical status at sampling	HTLV-I* antibody	HTLV-I sequences in mononuclear cells		PCR	
						PCR/ DNA	Molecular cloning sequence analysis	Adherent cell DNA	Non-adherent cell DNA
01-12	44/M	10	Sensory	Remission	+	+	+	++++	±
13-10	23/M	12	Diplopia	Remission	−	±	+	++++	±
10-02	21/M	3	Sensory	Remission	?	+	+		
01-14	29/F	8	Diplopia	Exacerbation	+	±	+	++++	±
09-1920	36/F	12	Optic neuritis	Progression	−	+	+		
05-08	23/F	2	Useless hand	Exacerbation	−	+	+	++++	−
Controls					−	1/20	−	0/13	1/13†

*Binding to disrupted HTLV-I virions. †All patients had undergone exacerbation and remissions since the onset of disease; patient 09-1920 has had chronic progressive disease for the last 4 years. Patient 05-08 was treated with corticosteroids and ACTH; none of the other patients or controls had been treated.

hybridization with the probe. However, in some experiments (two out of four) these bands could not be visualized readily and the hybridization intensity was very low, making it virtually undetectable (see Fig. 2A, lanes 9 and 11). Similar results were obtained with the amplified *env* sequences (Fig. 2B) where, in two out of four experiments, we could clearly see an amplified band that could be hybridized to the envelope-specific probes (Fig. 2B, lanes 5, 7, and 9), whereas in the other two experiments such clear amplification could not be detected by amplification and DNA hybridization (Fig. 2B, lanes 4, 6, and 8). This was circumvented as shown below by molecular cloning of the amplified sequences, when, regardless of the results of DNA hybridization, HTLV-I positive clones were isolated from all patients. Plasmid and phage DNA contamination of the cellular DNA preparations could have posed a serious problem with the PCR technique and led to artifactual results. We ruled out this possibility by also carrying out the reaction with primers derived from plasmid and phage sequences.

Of the ten amplified control DNA samples from individuals of Swedish origin, one contained detectable HTLV-I sequences. The other nine samples were negative. All ten amplified control DNAs from the American population were negative. The appearance of HTLV-I–related sequences in a normal individual with no signs of leukemia or MS was unexpected and is being further investigated.

We next molecularly cloned the amplified sequences from the MS patients. The DNAs were amplified with the two primers, cleaved with the appropriate restriction enzymes (Pst I and Xma I for *gag* sequences and Sal I and Kpn I for *env* sequences), and ligated to Bluescript vector DNA (Stratagene) that was also cleaved with Xma I and Pst I or Sal I and Kpn I. Bacteria were transformed with the ligated DNA and positive colonies were selected by hybridization with a nick-translated probe of HTLV-I proviral DNA or a labeled oligonucleotide probe (*16*). All six samples from all experiments had several positive clones whether amplification was seen by the DNA blotting technique or not. No positive clones were observed from control DNA ligations. Four to six positive colonies from each sample were colony-purified and the plasmid DNA was isolated. Restriction enzyme analysis of the purified DNA (Fig. 3) showed that each

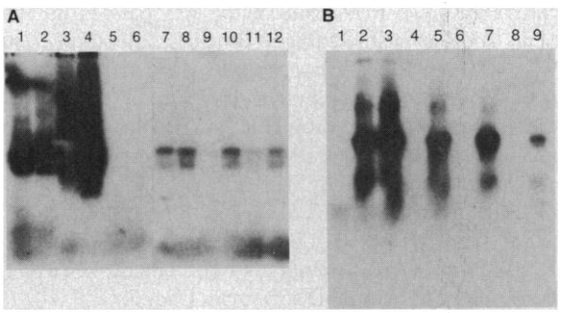

Fig. 2. (**A**) Representative *gag* sequence amplification analysis of DNA isolated from lane 1, MT-2 cell line infected with HTLV-I; lane 2, peripheral blood lymphocytes of a TSP patient 3-19-3; lane 3, plasmid containing HTLV-I proviral genomes of HTLV-I; lane 4, plasmid containing HTLV-I proviral genome isolated from the TSP patient 3-19-3; lane 5, ML-2 cells free of retroviral infection; lane 6, peripheral blood cells of a normal individual; and lanes 7 to 12, peripheral blood cells derived from the six MS patients under study. Lane 7, patient 09-1920; lane 8, patient 01-12; lane 9, patient 01-14; lane 10, patient 05-08; lane 11, patient 13-10; and lane 12, patient 10-02. (**B**) Envelope gene amplification of DNA isolated from lane 1, ML-2 cell line; lane 2, MT-2 cell line; lane 3, peripheral lymphocytes of a TSP patient 3-19-3; lane 4, patient 09-1920; lane 5, patient 01-12; lane 6, patient 01-14; lane 7, patient 05-08; lane 8, patient 13-10; and lane 9, patient 10-02. The DNAs were used in amplification analysis, electrophoresed on 2% agarose gels, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled oligonucleotide probe (*16*).

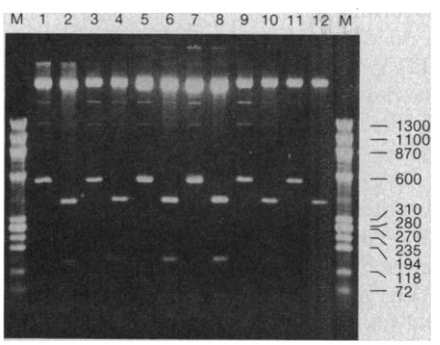

Fig. 3. Restriction enzyme cleavage analysis of plasmid clones derived from the amplified DNAs (using *gag* primers) of the six MS patients. Lanes 1 and 2, clones from DNA of patient 09-1920; lanes 3 and 4, clones from DNA of patient 01-12; lanes 5 and 6, clones from DNA of patient 01-14; lanes 7 and 8, clones from DNA of patient 05-08; lanes 9 and 10, clones from DNA of patient 13-10; and lanes 11 and 12, clones from DNA of patient 10-02. Lanes 1, 3, 5, 7, 9, and 11 contain plasmid DNA cleaved with Pst I and Sma I while lanes 2, 4, 6, 8, and 10 contain the same DNAs cleaved with Pst I, Sma I, and Nco I. Lanes marked M contain molecular weight markers (ΨX DNA digested with Hae III).

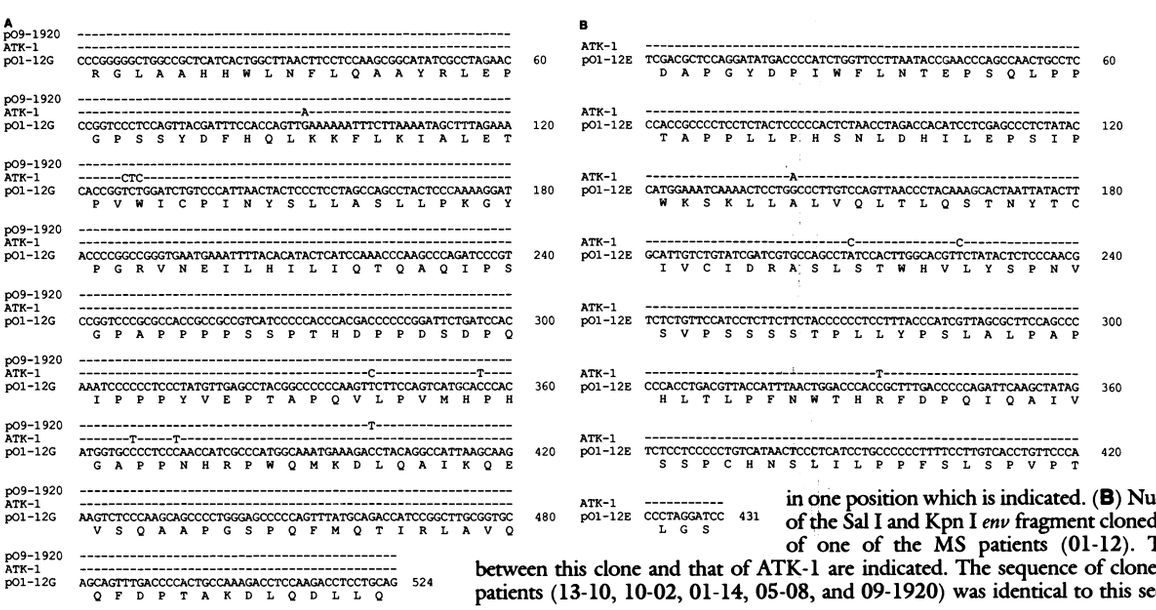

Fig. 4. (**A**) Nucleotide sequence analysis of the Sma I–Pst I *gag* fragment cloned from the amplified DNA of one of the MS patients (01-12). The sequence differences between this clone and that of ATK-1 [see (*16*)] are indicated on top of the sequence; the deduced amino acid sequence is given below. The sequence of clones from four other patients (13-10, 10-02, 01-14, and 05-08) was identical to this sequence. The sequence of the clone from patient 09-1920 differed in one position which is indicated. (**B**) Nucleotide sequence analysis of the Sal I and Kpn I *env* fragment cloned from the amplified DNA of one of the MS patients (01-12). The sequence differences between this clone and that of ATK-1 are indicated. The sequence of clones derived from five other patients (13-10, 10-02, 01-14, 05-08, and 09-1920) was identical to this sequence.

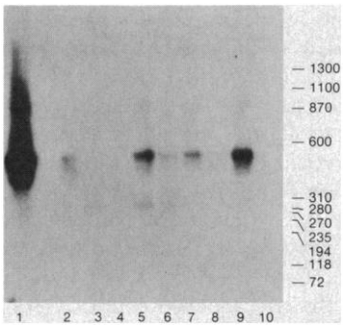

Fig. 5. DNA amplification analysis with *gag* primers of DNA derived from: lane 1, plasmid containing HTLV-I proviral genome; lane 2, MT-2 cells infected with HTLV-I; lane 3, adherent cells from a healthy individual; lane 4, nonadherent cells from a healthy individual; lanes 5, 7, and 9, adherent cells from peripheral blood cells of MS patients 01-12, 01-14, and 05-08, respectively; and lanes 6, 8, and 10, nonadherent cells derived from the same MS patients as in lanes 5, 7, and 9.

gag clone released an insert of 530 bp when cleaved with Pst I and Xma I; this fragment could be further cleaved with Nco I into two fragments of 390 and 140 bp. Similarly, all envelope clones released an insert of 460 bp with Sal I and Kpn I, which could be further cleaved with Cla I into two fragments of 260 and 200 bp.

We analyzed the sequence of the *gag* and *env* inserts using a combination of the methods of Sanger *et al.* (*18*) and Maxam and Gilbert (*19*) (Fig. 4, A and B). Five of the clones derived from the MS patient DNAs had identical *gag* sequences while a sixth clone differed in one position from the others (Fig. 4A). All six clones differed from the published sequence of a Japanese isolate (*16*) at six positions. The sequence of the HTLV-I clone derived from the MT-2 cell line (*17*) was identical to clone p01-12. However, the *env* inserts (Fig. 4B) contained at least two point mutations that differed from the ATK-1 sequence (*16*) as well as from the clone from the MT-2 cell line. These mutations suggest that the HTLV-I provirus in the six MS patients, though similar in many respects to the classical HTLV-I clone, differs at least in some positions in the envelope region. Such similarity between clones from MS patients is not surprising because of the high degree of sequence conservation between various isolates of HTLV-I. Recently, Tsujimoto *et al.* (*20*) cloned and sequenced the HTLV-I proviral genome isolated from a HAM (HTLV-I–associated myelopathy) patient of Japanese origin and found 99 to 100% nucleotide sequence homology in the coding of regions with the published sequence of Seiki *et al.* (*16*). If one considers that all six MS patients were from the same geographical location, and that highly conserved regions of HTLV-I were chosen for amplification, few differences in sequence would be expected.

Studies of HIV-I–infected individuals indicate that monocyte/macrophage cell populations are targets for virus infection (*21, 22*), and in some patients these infected cells occur in brain tissue (*21*). To test whether the HTLV-I–related sequences were present in lymphocytes or nonlymphocytic cells, we cultured PBMC from four of the MS patients and 13 healthy subjects in tissue culture flasks for 18 to 72 hours, and separated the adherent and nonadherent populations. When the DNA from these cells was amplified, the HTLV-I–related sequences were associated predominantly with adherent cell DNAs (Fig. 5, lanes 5, 7, and 9) in samples from the MS patients; nonadherent cell DNAs from these patients also contained low levels of hybridizable sequences (Fig. 5, lanes 6 and 8). When amplified sequences were cloned, both adherent and nonadherent cell DNAs were found to be positive. In contrast, the DNAs from the 13 normal subjects showed no hybridizing sequences under similar conditions (Fig. 5, lanes 3 and 4). HTLV-I–related sequences in cells of the one healthy subject from Sweden were found only in the nonadherent lymphocyte population.

An association between the occurrence of HTLV-I–related sequences in MS patients and the development of disease is implicated by our studies. It is possible that low-grade infection with HTLV-I or a related virus is more prevalent than previously suspected, particularly in those areas of the world where there is a high incidence of MS, such as the Shetland and Orkney islands. Since HTLV-I primarily infects T lymphocytes, and since the HTLV-I–related sequences in the MS patients were found mainly in adherent blood cells, some individuals may harbor a variant of HTLV-I that preferentially infects monocytes and macrophages. It would be interesting to determine whether these same cell populations in TSP and HAM patients contain sequences of HTLV-I or a related virus.

We found no correlation between the presence of antibodies reacting with disrupted HTLV-I virions and the presence of HTLV-I sequences at the time of blood collection. However, we cannot rule out the possibility that patients with positive sequences may have shown an immunological response to the virus at some time.

Recent data suggest that the HTLVs may exert their influence on a common set of cellular genes whose deregulation leads to CNS disorders. Henrichs *et al.* (*23*) found that transgenic mice containing the *tax* gene under the control of its own long terminal repeat developed tumors resembling human neurofibromatosis, a common single-gene disorder that affects the nervous system. HTLV-I–induced disorders of the nervous system in experimental animals might therefore prove useful in identifying cellular genes that act as targets for the action of genes such as *tax*. Prevention of neurological disorders in mice infected with murine retroviruses could be achieved by treatment with antiviral drugs such as azidothymidine (*24*), suggesting that similar treatments might be effective in some cases of chronic progressive myelopathies of humans.

Note added in proof: Since this study was conducted one of us [M.S.-W. (*25*)] has obtained data using immunoperoxidase staining techniques which suggest the presence of p19 *gag* protein in a small fraction of PBMC from the six patients studied here.

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16. The primers were derived from the *gag* and *env* regions of HTLV-I. The two *gag* primers were 5′-CGACCGCC CCGGGGGTGGCCGCT-3′ and 5′-GGTACTGCAGGAGGTCTTGGAGG-3′. These primers would be expected to amplify the region between nucleotides 842 and 1376 of the sequence described by Seiki *et al.* [M. Seiki, V. Hattori, M. Yoshida, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3618 (1983)]. The oligonucleotide probe was 5′-GATCCCGTCCCGTCCCGCGCCA-3′, which spans the region between nucleotide 1081 and 1102 of the published HTLV-I sequence. The two *env* primers were 5′-CTCCCTTCTAGTCGACGCTCCAGG-3′ and 5′-GCCACCGGTACCGCTCGGCGGGAG-3′. These primers would be expected to amplify the region between nucleotides 5684 and 6151 of Seiki *et al.* The oligonucleotide probe was 5′-GCCTCTCCACTTGGCACGTCC-3′, from nucleotide 5899 to 5919. Amplification of the DNA was performed with the Geneamp kit provided by Perkin-Elmer Cetus Corp. (Norwalk, CT). The reactions were carried out under the conditions specified by the manufacturer with 2 μg of DNA and 1.0 μmol of the primers. The reaction mixtures contained 25 m*M* TAPS-Cl, *p*H 9.3; 50 m*M* KCl, 2 m*M* $MgCl_2$, 1

mM DTT, 200 μM each of dATP, dGTP, TTP, and dCTP in a final volume of 50 μl; 2.5 units of *Taq* polymerase were used for each assay. Typically, for each cycle of amplification, the mixture was denatured at 94°C for 2 min, annealed at 55°C for 1 min, and then extended at 70°C for 2 min. From 36 to 40 cycles of amplification were performed and fresh enzyme (2 to 5 units) was added to each tube at the end of every tenth cycle.
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Germline Transmission of Exogenous Genes in the Chicken

ROBERT A. BOSSELMAN,* ROU-YIN HSU, TINA BOGGS, SYLVIA HU, JOAN BRUSZEWSKI, SUSAN OU, LEE KOZAR, FRANK MARTIN, CAL GREEN, FREDERICK JACOBSEN, MARGERY NICOLSON, JOSEPH A. SCHULTZ, KENNETH M. SEMON, WILLIAM RISHELL, R. GREGORY STEWART

Difficulties associated with in vitro manipulation and culture of the early chicken embryo have restricted generation of transgenic chickens to approaches that use replication-competent retroviruses. The need to produce transgenic chickens in the absence of replicating virus prompted development of a new method of gene transfer into the chicken. Microinjection of the replication-defective reticuloendotheliosis virus (REV) vector ME111 beneath unincubated chicken embryo blastoderms results in infection of germline stem cells. This vector contains genetic information exogenous to the chicken genome, including both the herpes simplex virus type 1 thymidine kinase gene and the Tn5 neomycin phosphotransferase gene. About 8 percent of male birds hatched from injected embryos contained vector DNA in their semen. All four positive males tested passed vector sequences onto their progeny. Analysis of G_1 offspring showed that gonads of G_0 male birds were mosaic with respect to insertion of vector provirus. Thus, primordial germ cells present in the unincubated chicken embryo blastoderm are susceptible to infection by defective REV vectors.

GENE TRANSFER INTO CHICKENS has usually depended on the use of replication-competent retroviral vectors (*1–4*), in part because it is difficult to manipulate the early avian embryo or to grow the embryo in vitro (*5, 6*). Attempts to alter the chicken germline by gene transfer into developing follicles (*7*) or in vitro cultured early embryos (*6*) have not been successful. Documented germline gene transfer results from injection of replicating virus into freshly laid unincubated eggs (*2–4*). Access to the embryo is easy just after ovaposition; however, the embryo has already reached a stage corresponding to a mammalian late blastula or early gastrula. At this time, the embryo consists of a thin layer of many pluripotent cells comprising the blastoderm (*8, 9*). In contrast, the ability to manipulate the early mouse embryo has led to success with a variety of approaches to gene transfer, including microinjection of DNA (*10, 11*), retroviral infection (*12, 13*), and the use of embryonic stem cells that can contribute to the germ-cell lineage of chimeric mice (*14–16*). To generate transgenic chickens in the absence of replicating virus, we have developed a new method of gene transfer based on microinjection of nonreplicating retroviral vectors into embryos of unincubated eggs.

We used the replication-defective reticuloendotheliosis virus (REV) vector ME111 (*17, 18*) to infect susceptible stem cells present in the unincubated chicken embryo. Chickens do not contain endogenous REV-related proviruses that might interfere with detection of newly acquired provirus, even though REV can infect at least some somatic stem cells of the early chicken embryo (*2, 7*). Primordial germ cells appear to reside in the outer layer or epiblast of the blastoderm (*19–22*). Since nonreplicating vectors would infect embryonic cells at about the time of injection, subsequent analysis of adult blood and semen would show whether or not susceptible somatic and germline stem cells were present at this time.

The ME111 vector lacks all viral structural genes and carries both the Tn5 neomycin resistance gene driven by the promoter of the REV long terminal repeat (LTR) and the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene (*17*). C3 helper cells generate stocks of ME111 with titers of about 10^4 TKTU per milliliter (TK transducing units) (*17*). Sequence comparison of the competent parental helper virus, packaging defective helper proviruses, and the vector provirus is shown in Fig. 1. Cells and vector were grown and harvested as previously described (*17, 18*). Ten-microliter volumes of vector-containing cell culture media were injected through the area pellucida into the subgerminal cavity of the blastoderm of unincubated eggs (*23*). Eggs were resealed and allowed to hatch (*24*). DNA from blood and semen of mature birds was analyzed for the presence of integrated proviral vector.

A total of 2599 eggs were injected, of which 38% hatched. DNA from the blood of 760 hatched chicks was analyzed by liquid hybridization with a vector-specific probe (*25*). Of these, 173 chicks contained vector sequences. Of 82 males whose blood was positive, 33 males also carried vector sequences in their semen. DNA blot analysis (*26*) of blood (*27*) and semen DNA confirmed integration of vector provirus. Restriction endonuclease fragments of DNA specific for replicating REV were not observed. Long-term culture assays (*28*), used to test for low levels of virus, detected competent REV in 2 of 14 G_0 birds with vector-positive blood. Sires and progeny described in this report were judged to be virus-negative by the same method. Vector-positive semen from four males was used to inseminate control females. All four transmitted vector sequences to G_1 progeny at a frequency that varied from ~2% to 8%. These results confirm vector-mediated infection of primordial germ cells present in the unincubated chicken embryo.

DNA blot analysis of proviral genome organization in G_0 vector-positive semen and in blood of vector-positive G_1 progeny is shown in Fig. 2. Lanes 3 to 5 in Fig. 2A

R. A. Bosselman, R.-Y. Hsu, T. Boggs, S. Hu, J. Bruszewski, L. Kozar, F. Martin, F. Jacobsen, M. Nicolson, Amgen Inc., 1900 Oak Terrace Lane, Thousand Oaks, CA 91320.
S. Ou, Department of Biology, California Institute of Technology, Pasadena, CA 91125.
C. Green, Vicam, Inc., 29 Mystic Avenue, Somerville, MA 02145.
J. A. Schultz, K. M. Semon, W. Rishell, Arbor Acres Farm, Inc., Marlborough Road, Glastonbury, CT 06033.
R. G. Stewart, Poultry Disease Research Center, University of Georgia, 953 College Station Road, Athens, GA 30601.

*To whom correspondence should be addressed.