The EMBO Journal vol.7 no.13 pp.4179-4184, 1988

Enzymatic amplification of exogenous and endogenous retroviral sequences from DNA of patients with tropical spastic paraparesis

Charles R.M.Bangham, Susan Daenke, Rodney E.Phillips, J.K.Cruickshank[1] and John I.Bell

Institute for Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU and [1]Northwick Park Hospital, Harrow, Middlesex, UK

Communicated by D.J.Weatherall

Using oligonucleotide primers that hybridize to conserved sequences in the reverse transcriptase (RT) gene, we have amplified by the polymerase chain reaction three sequence variants of HTLV-I from the genomic DNA of five patients with tropical spastic paraparesis (TSP), and a fourth sequence variant from a healthy carrier of HTLV-I. These results unequivocally identify the retrovirus associated with TSP as HTLV-I and suggest that no sequence variant is uniquely responsible for the condition. The same primers served to amplify two novel single-copy endogenous retroviral RT sequences related to the exogenous mammalian leukaemia viruses: and three *Kpn*I (LINE1) family DNA repeats. This strategy, combining the sensitivity of PCR with cross-reactive primers, may be useful in the search for known or novel retroviruses in other diseases of possible retroviral aetiology.

Key words: HTLV-I/neurological disease/PCR/retrovirus/sequence

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-I), the first clearly established human retrovirus, was originally isolated from a patient with a cutaneous T-cell lymphoma (Poiesz *et al.*, 1980). Epidemiological, haematological and molecular evidence indicates (Yoshida and Seiki, 1987) that HTLV-I is the cause of adult T-cell leukaemia/lymphoma (ATL; Uchiyama *et al.*, 1977), though the mechanism of leukaemogenesis is not yet understood (Yoshida and Seiki, 1987). HTLV-I is closely related to other mammalian leukaemia viruses (McClure *et al.*, 1988), particularly HTLV-2 (Kalyanaraman *et al.*, 1982) and bovine leukaemia virus (BLV) (Sagata *et al.*, 1985).

Tropical spastic paraparesis (TSP) is a chronic neurological disorder characterized by low back pain, bladder dysfunction, and a progressive spastic paraparesis which can leave the pateint severely disabled within a few months (Montgomery *et al.*, 1964). In 1985, Gessain *et al.* reported that 10 of 17 patients with this disease had antibodies, detected by ELISA and Western blot, which cross-reacted with HTLV-I antigens. This serological association has now been confirmed in many countries; over 75% of TSP patients are seropositive for HTLV-I (Lancet, 1988). A similar condition described in Japan (Osame *et al.*, 1987), called HTLV-I-associated myelopathy (HAM), has an apparently higher seropositivity rate for HTLV-I. Recently, Tsujimoto *et al.* (1988) have obtained DNA sequence evidence that the virus present in the cerebro-spinal fluid cells of a patient with HAM was indeed HTLV-I. Such evidence has not been obtained in the case of TSP, although recent studies (Bhagavati *et al.*, 1988; Jacobson *et al.*, 1988) indicated that a closely related virus was involved.

In order to identify the retrovirus associated with TSP, we have sequenced part of the polymerase gene from five TSP patients of West Indian origin living in the UK, after amplification of the genomic DNA by the polymerase chain reaction (PCR). Because there were indications (see Discussion) that the retrovirus involved might not be identical with HTLV-I, and to develop the technique for application to other diseases of known or possible retroviral aetiology, our strategy was to use amplification primers homologous to the region of the *pol* gene encoding reverse transcriptase, which has areas of strong sequence conservation between diverse retroviruses (Chiu *et al.*, 1985; McClure *et al.*, 1988), to enable the detection of both HTLV-I and other retroviruses. Using this strategy, we have identified three variant sequences of HTLV-I that differ from the sequence of the isolate (λATK-1) described by Seiki *et al.* (1983) by three, four and five nucleotides over the amplified fragment (352 bp excluding the primers). Two of the sequences, and a sequence identical with λATK-1, were identified in the five TSP patients. These results clearly identify the virus serologically associated with TSP as HTLV-I, and suggest that more than one sequence variant of the virus is associated with the disease. The third HTLV-I sequence was found in a healthy HTLV-I-seropositive West Indian patient with a history of transient idiopathic thrombocytopaenic purpura 18 months earlier.

The same oligonucleotide primers also amplified long interspersed repeats (LINE1 family; *Kpn*I repeats), which are known to contain reverse transcriptase (RT)-like nucleotide sequences (Hattori *et al.*, 1986; Loeb *et al.*, 1986), and two previously undescribed single-copy endogenous retroviruses closely related to the mammalian leukaemia viruses.

Results

Genomic DNAs were studied from six HTLV-I seropositive adults of West Indian origin resident in the UK, five with a diagnosis of TSP and one with a past episode of idiopathic thrombocytopaenic purpura, and from three healthy HTLV-I seronegative (caucasian) adults. PCR amplification was done under conditions of low stringency (see Materials and methods), to maximize the cross-reaction of the oligonucleotide primers with different retrovirus-related DNA sequences. Under these conditions a perfect match is not required between each primer and the sequence to be amplified; an HIV *pol*-containing plasmid was successfully amplified with an HIV-specific 5′ primer and primer no. 79, which contains HTLV-I sequence and which differs from

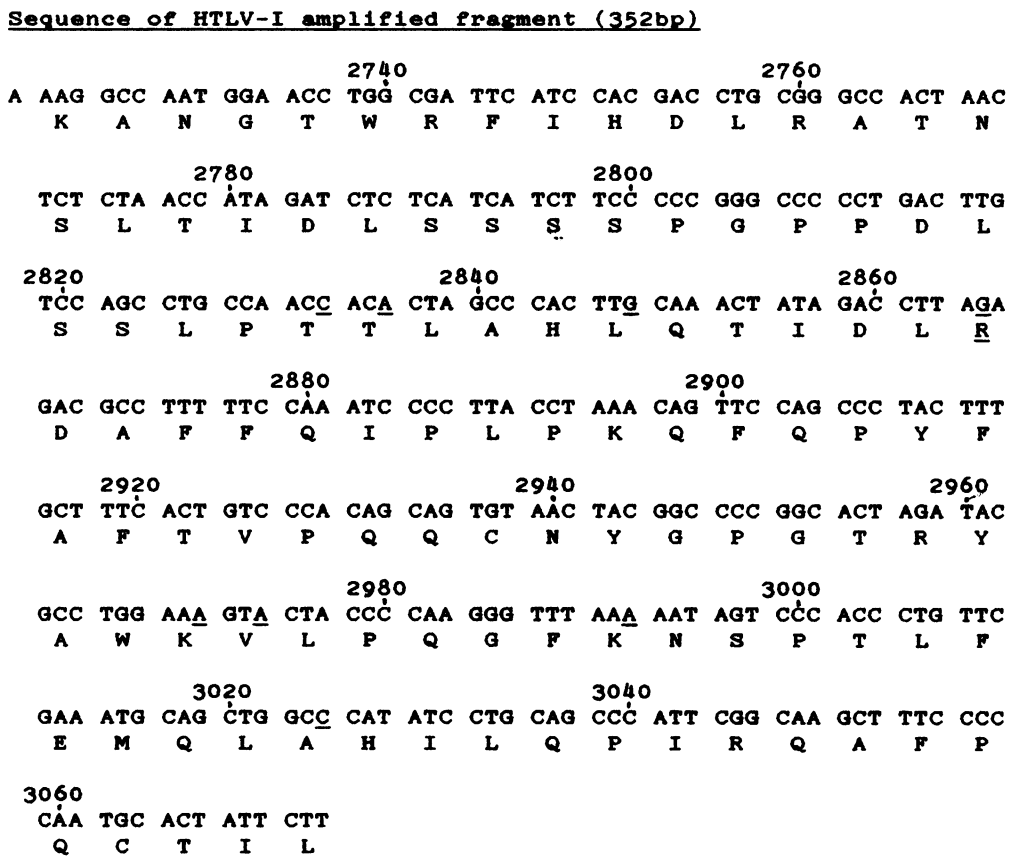

Fig. 1. Nucleotide and amino acid sequences of the amplified fragment of HTLV-I, omitting the amplification primers. The bases are numbered as in the EMBL database. Bases shown in both type differ between the sequence variants described (see Table I).

the corresponding HIV sequence at six out of 20 bases (results not shown). Amplification of 1 μg of genomic DNA [from $\sim 2 \times 10^4$ peripheral blood lymphocytes (PBL)] under these conditions produced, in both seronegative and seropositive cases, a band at or near the size (392 bp, including the primers) predicted from the base sequence of HTLV-I (Seiki *et al.*, 1983) (Figure 1).

Nucleotide sequences cloned from HTLV-I-seropositive individuals' genomic DNA

Southern blot hybridization of the DNA amplified from HTLV-I seropositive patients with an end-labelled HTLV-I-derived oligonucleotide probe (G: see Materials and methods) showed the presence of HTLV-I-like sequences in the band at the expected size in each case; seronegative controls gave no such signal (results not illustrated). M13 recombinant clones were detected by filter hybridization with the same oligonucleotide probe. In this way we obtained HTLV-I RT sequences in 0.5–8.0% of recombinant M13 clones from all six seropositive subjects. An equivalent number of plaques were screened from the amplification product of the three seronegative control DNAs: no HTLV-I sequence was isolated.

The results shown in Table I indicate the eight nucleotide positions in the three variant sequences which differ from the previously described sequence of HTLV-I. In each case the sequence was clearly HTLV-I: the most variant sequence (Jam) differed at five nucleotides over the amplified fragment. Compared with the HTLV-I sequence λATK-1 (Seiki *et al.*, 1983), all three variants (HTLV-I_{EJ}, HTLV-I_{ASm} and HTLV-I_{Jam}) had the same C to T transition at base 2833, and all had the same conservative amino acid change (Arg to Lys) resulting from a G to A transition at base 2865 (numbering as in EMBL database); no other nucleotide substitution changed the amino-acid sequence. No sequence was unique to all the patients with TSP: two of the three sequences, and the λATK-1 sequence, were isolated from these individuals. The relationship of these sequences to previously published HTLV-I RT sequences is discussed further below.

Table I. Variant nucleotide positions in the amplified segment of HTLV-I

Nucleotide position	λATK-1	EJ	ASm	Jam
2833	C	T	T	T
2836	A	·	·	T
2848	G	·	A	·
2865	G(Arg)	A(Lys)	A	A
2971	A	·	·	G
2974	A	·	G	·
2992	A	·	·	G
3025	C	T	·	·
No. of TSP patients	2	2	1	0

·denotes the same residue as in the λATK-1 clone in that position. Nucleotide positions are numbered according to the HTLV-I (λATK-1) sequence on the EMBL database.

Endogenous reverse transcriptase-related sequences

Two species of DNA predominated amongst the clones of

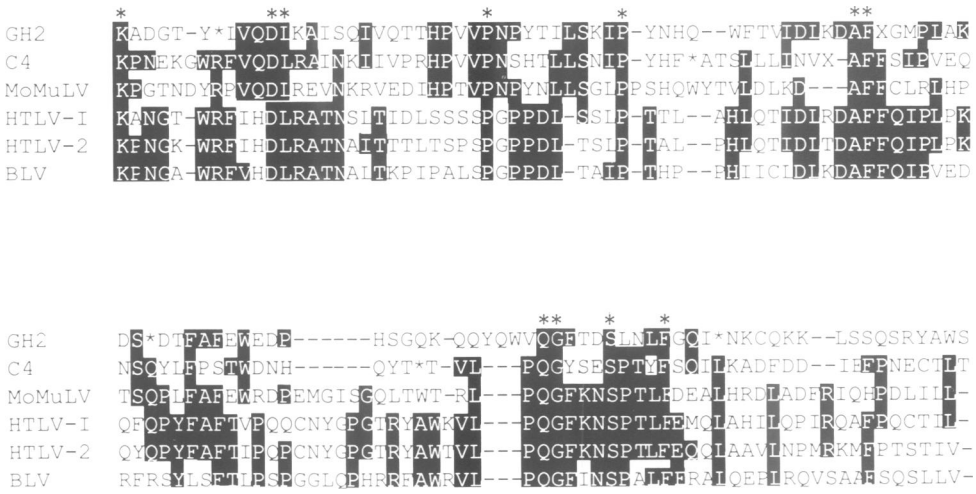

Fig. 2. Alignment of reverse transcriptase sequences of GH2, C4 and related mammalian leukaemia viruses. Amino acids shared by three or more of the sequences are boxed in black; those shared by all six sequences are marked above by an asterisk. The sequences of the amplification primers have been omitted. To maintain the reading frame in the endogenous species, one base was inserted in the C4 sequence (an X denotes the resulting amino acid); one base was deleted and one inserted in the GH2 sequence. An asterisk in the amino acid sequence denotes a stop codon. References for the leukaemic sequences are: Shinnick *et al.* (1981) (MoMuLV); Seiki *et al.* (1983) (HTLV-1); Shimotohno *et al.* (1985) (HTLV-2); Sagata *et al.* (1985) (BLV).

M13 sequenced from the genomic DNAs of the three HTLV-I seronegative and the six seropositive individuals. One, called GH2, accounted for >70% of the recombinant clones identified; the other, called C4, accounted for ~10% of the clones. Southern blotting analysis of genomic DNA, digested with *Eco*RI and probed with the cloned fragments of C4 and GH2 respectively, showed that in nine normal individuals each sequence was present in one restriction fragment of 12-kbp (C4) or 20-kbp (GH2) (results not illustrated). This is consistent with there being a single copy per genome of each endogenous species. The amino-acid sequences of C4 and GH2 are shown in Figure 2: the relationship of each species to Moloney murine leukaemia virus is apparent. GH2 is also closely related to a defective retrovirus element present in the haptoglobin pseudogene (Maeda, 1985) (49% amino-acid identity). C4 has significant amino acid similarity to a defective endogenous species reported by Mager and Freeman (1987) to be related to the HTLV family (49% identity).

The remaining 20% of DNA clones sequenced included three members of the long interspersed repeat (LINE1 or *Kpn*I) sequence family (not illustrated). However, several species could not be identified with certainty, in spite of having low-level (30−50%) nucleotide identity with sequences related to DNA-dependent RNA polymerases. One such species, with 52% identity to a yeast delta element over 159 nucleotides, was cloned from three unrelated individuals, but cannot yet be identified more precisely.

Fidelity of Taq polymerase

The identification of true variant nucleotide sequences in the PCR amplification products described here depends on the error rate of the Taq polymerase. To estimate this error rate, we compared the sequences of 18 clones of the GH2 endogenous species (see above) from six individuals. Over 210 nucleotides in each of these 18 clones (a total of 3780 bases), there was one clone with a single deletion of an A residue, and one with a T to C transition. This gives a net error rate of 1 in 1890 nucleotides. Note that this is the error rate in the final product, after 40 cycles of amplification; the error rate per generation will be considerably lower. It is highly unlikely that all the observed nucleotide differences in the HTLV-I isolates described here were due to mistakes made by the Taq polymerase, because of this low error rate and because two HTLV-I sequences (λATK-1 and HTLV-I_{EJ}) were each cloned in independent experiments from two different individuals.

Discussion

Amplification of DNA by the polymerase chain reaction has the advantage that detection of viruses does not depend on *in-vitro* propagation or replication competence of the virus. A further important advantage is that target sequence selection on the basis of primer specificity can be precisely controlled, since the oligonucleotide primers can be directed against different parts of the sequence to be detected, and the stringency of the hybridization conditions can be controlled. In this study we made use of the second advantage by designing amplification primers that recognize conserved sequences in the region encoding reverse transcriptase, which is the most highly conserved gene in retroviruses (Chiu *et al.*, 1985; McClure *et al.*, 1988). Using this strategy we have cloned and sequenced part of the RT gene of HTLV-I in six of six HTLV-I antibody-positive individuals, identifying four sequence variants of the virus; in the same reactions, the primers also amplified a range of other RT-related DNAs, including two single-copy endogenous retroviruses and three LINE1 repeats. The proportion of amplified clones containing HTLV-I can be increased by secondary amplification with an internal primer (oligonucleotide G; see Materials and methods) (results not shown). However, we chose to sequence the primary amplification products to increase the length of the characterized sequences, and to avoid further selection of particular sequences.

Any method of nucleic-acid sequence detection that depends on the replication of the detected sequence is likely to exert some selection for particular species of DNA. For example, recovery of viruses from prolonged *in-vitro* culture of cells will select for replication-competent viruses. Also, Overbaugh *et al.* (1988) have reported frequent recombination between feline leukaemia virus (FeLV) and endogenous FeLV-like sequences during *in-vitro* propagation of the virus. They concluded that the pathogenetic effects of retroviruses should be investigated as far as possible with direct procedures, such as *in-situ* hybridization, to minimize the chance of these spontaneous and rapid genetic changes.

Tsujimoto *et al.* (1988) have described partial DNA sequence of a retrovirus recovered from a lymphoid cell line derived from the CSF lymphocytes of a patient with HAM: the sequence had >97% nucleotide identity with λATK-1. The region encoding the RT was not sequenced by Tsujimoto *et al.* (1988): in the protease region, five substitutions were found, causing two coding changes, in 702 nucleotides, by comparison with λATK-1. Recently, Jacobson *et al.* (1988) similarly identified an 'HTLV-I-like' retrovirus in patients with TSP by immunoprecipitation and by restriction mapping. The growth requirements of T-cell lines derived from these patients differed from those of previously described HTLV-I-transformed lines; this difference, and the inability of one of the lines to inhibit anti-influenza cytotoxic T-cell function, led the authors to conclude that the viruses they isolated might be variants of HTLV-I, or another (related) retrovirus. Bhagavati *et al.* (1988) detected HTLV-I-related DNA in fresh PBL of 11 HTLV-I seropositive patients with chronic progressive myelopathy, using Southern blotting and dot-blot hybridization of DNA amplified from the *pol* gene (downstream of the RT encoding region amplified in the present study); no DNA sequence data were presented. There was no consistent clinical difference between the seronegative and seropositive cases, but no HTLV-I-like DNA was detected in seronegative patients. Reddy *et al.* (1988) used *in situ* hybridization with an HTLV-I probe to detect HTLV-I-related sequences in the PBL of a seropositive Haitian patient with chronic myelopathy, but again no DNA sequence data were available.

The sequence data we present here unequivocally identify the viruses isolated from the PBL of five of five patients with TSP as HTLV-I. Further, the presence of three distinct sequences of HTLV-I amongst five TSP patients suggests that no one sequence variant is associated with TSP. However, we cannot exclude the possibility that there are differences in other genes which are required to cause neurological disease. In this context it is interesting that minor sequence variations in the related murine leukaemia viruses radically change the tissue tropism of the virus, leading to paralytic disease (Rassart *et al.*, 1986; Szurek *et al.*, 1988). It will therefore be important to characterize the homologous (*env*) gene in HTLV-I isolates from TSP patients, preferably from neural tissue, to discover whether similar changes are responsible for causing TSP. The episode of idiopathic thrombocytopaenic purpura in one HTLV-I-seropositive patient (Jam), who did not have TSP, was probably incidental: there is no evidence that HTLV-I causes this disease.

We are confident that the nucleotide changes observed are genuine, because a comparison of the sequence of 18 clones of one of the endogenous retroviruses produced an estimate of the error rate of Taq polymerase of 1/1890 bases in the final amplification product. The isolation of one of the variant HTLV-I sequences (HTLV-I_{EJ}) from two unrelated individuals in different experiments strengthens the conclusion that these sequence differences are real. Also, the productive G to A transition at position 2865, seen in all three novel sequences described here, is not due to a particular repeated mistake made by the enzyme at this position: all clones amplified from the plasmid containing the HTLV-I λATK-1 sequence had the expected G residue at position 2865.

Only one full-length HTLV-I provirus sequence has been reported (Seiki *et al.*, 1983). Initial evidence, from restriction mapping (Wong-Staal *et al.*, 1983) suggested that there was little sequence variation among isolates of HTLV-I from areas as widely separated as Colombia and Japan. However, there is now evidence (Ratner *et al.*, 1985; Fukasawa *et al.*, 1987) of a distinct strain present in Africa, although isolates more closely related to the Japanese sequence λATK-1 are also present in Africa. Ratner *et al.* (1985) show that this strain, HTLV-Ib, lacks a *Pst*I restriction site present at position 3032 in λATK-1 (among other restriction site differences). The three variant HTLV-I RT sequences described here have all preserved this site.

In the human genome, endogenous retroviruses have been described that are related to MoMuLV (Martin *et al.*, 1981; Steele *et al.*, 1984); baboon endogenous virus (O'Connell *et al.*, 1984); mouse mammary tumour virus (Dean and Sweet, 1986; May and Westley, 1986; Ono *et al.*, 1986); and simian sarcoma-associated virus (Leib-Mösch *et al.*, 1986). McClure *et al.* (1988) have shown that the genes encoding enzymes, especially RT, are the most highly conserved retroviral genes. On the basis of its RT sequence, the endogenous RT sequence GH2 is much more closely related to MoMuLV (ALIGN score 14.1) and to Rous sarcoma virus (ALIGN score 11.2) than to other exogenous retroviruses. The other endogenous species we amplified (C4) is related to the HTLV branch as well as MoMuLV; the most homologous endogenous retrovirus compared is RTVL-H2, which was found by Mager and Freeman (1987) to be related to the HTLV group.

Hattori *et al.* (1986) and Loeb *et al.* (1986) have shown that the predicted amino-acid sequences of *Kpn*I repeats are homologous to RT, although all are highly defective genes, and no RT activity attributable to them has been found. It is therefore not surprising that our cross-reactive primers amplified *Kpn*I repeats as well as endogenous and exogenous retroviral sequences. Indeed, alignment of the amplified fragment of these *Kpn*I repeats with the *pol* gene of HTLV-I (not shown) demonstrated that this fragment corresponded to the region encoding RT.

Retroviruses may cause malignancies in humans other than ATL, such as hairy cell leukaemia, but none has yet been convincingly demonstrated. There is also controversial evidence (Koprowski *et al.*, 1985; Ohta *et al.*, 1986) that an HTLV-like retrovirus is associated with multiple sclerosis (MS). The strategy described here of using PCR gene amplification directed by primers that recognize 'islands' of conserved retroviral sequences may be useful in the identification of hitherto undescribed retroviruses in such

diseases, since the base sequence between the islands of conservation, even in *pol*, diverges considerably even between present-day retroviruses.

Materials and methods

Oligonucleotide preparation

Oligonucleotides were synthesized on an Applied Biosystems 381A DNA Synthesizer (using β-cyanoethylphosphoramidite derivatives), purified by 20% polyacrylamide gel electrophoresis, desalted on a C_{18} Sep−Pak cartridge (Millipore), resuspended in water to 200−500 μg/ml, and stored frozen. For use as probes, 100 ng of an oligonucleotide were end-labelled by T4 polynucleotide kinase (Amersham International) with high specific activity [^{32}P]dATP (150 mCi/ml) (Amersham International) and separated from unincorporated radiolabel on a Sephadex G-50 (Pharmacia) column. The amplification primers were: (i) no. 78, (5′-AACCCAGTATTCCC-AGTTAA-3′) (coding sense), which corresponds to bases 2702−2721 of the HTLV-I genome (numbered as in EMBL database); and (ii) no. 79, (5′-AGAATGTCATCCATGTACTG-3′) (anticoding sense), which anneals to bases 3074−3093 of the HTLV-I genome. The detection oligonucleotide 'G' was 5′-ACCCTTGGGGTAGTACTTT-3′ (anticoding sense); this hybridizes to the highly conserved base sequence (bases 2969−2987) between the amplification primers.

DNA extraction and amplification

Genomic DNA was extracted from 5−10 × 10^6 fresh peripheral blood lymphocytes by the conventional proteinase K technique (Maniatis *et al.*, 1982); 1 μg was used in each amplification reaction. PCR gene amplification (Saiki *et al.*, 1985) was carried out in a buffer containing 50 mmol/l KCl, 10 mmol/l Tris−HCl (pH 8.3 at 20°C), 1.5 mmol/l $MgCl_2$, 0.01% gelatin (Difco), 0.2 mmol/1 of each of dGTP, dATP, dCTP, dTTP (Pharmacia), 1 μmol/l of each oligonucleotide primer, and 4 units of Taq DNA polymerase (Cetus Corp.); the mixture was overlaid with 30 μl of liquid paraffin (B.P.C.). After an initial denaturation of 5 min at 93°C, 25 cycles were done with the following conditions: 93°C (1 min), 37°C (2 min) 72°C (3 min); the last extension reaction (72°C) was prolonged to 10 min, and the reaction mixture stored frozen until analysis.

The amplification products were examined on a 1% agarose (Sigma) minigel stained with ethidium bromide, and the band at the expected size was electro-eluted in a 1% low melting-point agarose (Bethesda Research Labs) gel on to NA45 membrane (Schleicher and Schuell), followed by three organic solvent extractions and two ethanol precipitations.

Cloning and sequencing of amplified DNA

Approximately 50 ng of amplified DNA were blunt-ended using Klenow fragment (Amersham International) in a 10 μl of reaction volume for 30 min at 37°C. After two ammonium acetate/ethanol precipitations and washing once with 70% ethanol, the dried DNA pellet was redissolved in 10 μl of H_2O, and half of this was used to ligate into M13 using T4 DNA ligase (Biolabs). The M13 vector used was the modified version of M13K8.2 used by Todd *et al.* (1987). The ligation product was transformed into *Escherichia coli* strain JM101 using the procedure described by Hanahan (1985).

Recombinant (clear) plaques were gridded on to a fresh lawn of JM101 cells, lifted on to a nylon filter (Amersham International no. RPN.82N) and hybridized with an end-labelled oligoprobe, for 1 h at 37°C, in a buffer containing 0.9 mol/l NaCl, 90 mmol/l Tris−HCl (pH 8.0), 6 mmol/l EDTA, 0.5% (v/v) NP40 (Sigma), 10% (w/v) dextran sulphate (Pharmacia) and 100 μg/ml salmon sperm DNA (Sigma). The filters were washed at 40°C (2 × 5 min) in 6 × SSC (0.9 mol/l NaCl, 0.09 mol/l Na_3 citrate) before exposure to Fuji RX film at −70°C for 2−6 h, with two intensifying screens.

The DNA sequence of these cloned fragments was determined by the dideoxy chain termination method, using either Klenow fragment or T7 DNA polymerase (Sequenase, US Biochemical Corp). These sequences, translated into amino acids and adjusted to maintain the reading frame (see Figure 2) were compared with retrovirus-related sequences in the National Biochemical Research Foundation (NBRF) database, using the NBRF ALIGN program (Dayhoff *et al.*, 1983). Optimal alignment scores are expressed as the number of standard deviations above the mean score obtained from 100 random permutations of the sequences. The sequences of the amplification primers, which are necessarily identical in every amplified clone, were omitted from the sequences compared by the ALIGN program.

Acknowledgements

We are indebted to M.Yoshida for providing HTLV-I-containing plasmids, J.K.Cruickshank for obtaining the TSP blood samples, and to M.Brozovic and P.Rudge for allowing us to study their patients. This study was supported by a grant from the Oxford University Research and Equipment Committee. C.R.M.B., S.D. and J.I.B. were supported by the Wellcome Trust.

References

Bhagavati,S., Ehrlich,G., Kula,R.W., Kwok,S., Sninsky,J., Udani,V. and Poiesz,B.J. (1988) *New Engl. J. Med.*, **318**, 1141−1147.

Chiu,I.-M., Yaniv,A., Dahlberg,J.E., Gazit,A., Skuntz,S.F., Tronick,S.R. and Aaronson,S.A. (1985) *Nature*, **317**, 366−368.

Dayhoff,M.O., Barker,W.C. and Hunt,L.T. (1983) *Methods Enzymol.*, **9**, 524−545.

Deen,K.C. and Sweet,R.W. (1986) *J. Virol.*, **57**, 422−432.

Fukasawa,M., Tsujimoto,H., Ishikawa,K., Miura,T., Ivanoff,B., Cooper,R.W., Frost,E., Delaporte,E., Mingle,J.A.A., Grant,F.C. and Hayami,M. (1987) *Virology*, **161**, 315−320.

Gessain,A., Barin,F., Vernant,J.C., Gout,O., Maurs,L., Calender,A. and de The,G. (1985) *Lancet*, **2**, 407−410.

Hanahan,D. (1985) In Glover,D.M. (ed.), *DNA Cloning—A Practical Approach*. IRL Press, Oxford, Vol. 1, pp. 109−135.

Hattori,M., Kuhara,S., Takenaka,O. and Sakaki,Y. (1986) *Nature*, **321**, 625−627.

Jacobson,S., Raine,C.S., Mingioli,E.S. and McFarlin,D.E. (1988) *Nature*, **331**, 540−543.

Kalyanaraman,V.S., Sarngadharan,M.G., Robert-Guroff,M., Miyoshi,I., Blayney,D., Golde,D. and Gallo,R.C. (1982) *Science*, **218**, 571−573.

Koprowski,H., DeFreitas,E.C., Harper,M.E., Sandberg-Wollheim,M., Sheremata,W.A., Robert-Guroff,M., Saxinger,C.W., Feinberg,M.B., Wong-Staal,F. and Gallo,R.C. (1985) *Nature*, **318**, 154−160.

Lancet (1988) (editorial) *Lancet*, **i**, 217−219.

Leib-Mosch,C., Brack,R., Werner,T., Erfle,V. and Hehlman,R. (1986) *Virology*, **155**, 666−677.

Loeb,D.D., Padgett,R.W., Hardies,S.C., Shehee,W.R., Comer,M.B., Edgell,M.H. and Hutchison,C.A. (1986) *Mol. Cell. Biol.*, **6**, 168−182.

Maeda,N. (1985) *J. Biol. Chem.*, **260**, 6698−6709.

Mager,D.L. and Freeman,J.D. (1987) *J. Virol.*, **61**, 4060−4066.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York.

Martin,M.A., Bryan,T., Rasheed,S. and Khan,A.S. (1981) *Proc. Natl. Acad. Sci. USA.*, **78**, 4892−4896.

May,F.E.B. and Westley,B.R. (1986) *J. Virol.*, **60**, 743−749.

McClure,M.A., Johnson,M.S., Feng,D.-F. and Doolittle,R.F. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 2469−2473.

Montgomery,R.D., Cruickshank,E.K., Robertson,W.B. and McMenemy,W.H. (1964) *Brain*, **87**, 425−462.

O'Connell,C., O'Brien,S., Nash,W.G. and Cohen,M. (1984) *Virology*, **138**, 225−235.

Ohta,M., Ohta,K., Mori,F., Nishitani,H. and Saida,T. (1986) *J. Immunol.*, **137**, 3440−3443.

Ono,M., Yasunaga,T., Miyata,T. and Ushikubo,H. (1986) *J. Virol.*, **60**, 589−598.

Osame,M., Matsumoto,M., Usuku,K., Izumo,S., Ijichi,N., Amitani,H., Tara,M. and Igata,A. (1987) *Ann. Neurol.*, **21**, 117−122.

Overbaugh,J., Riedel,N., Hoover,E.A. and Mullins,J.I. (1988) *Nature*, **332**, 731−734.

Poiesz,B.J., Ruscetti,F.W., Gazdar,A.F., Bunn,P.A., Minna,J.D. and Gallo,R.C. (1980) *Proc. Natl. Acad. Sci. USA.*, **77**, 7415−7419.

Rassart,E., Nelbach,L. and Jolicoeur,P. (1986) *J. Virol.*, **60**. 910−919.

Ratner,L., Josephs,S.F., Starcich,B., Hahn,B., Shaw,G.M., Gallo,R.C. and Wong-Staal,F. (1985) *J. Virol.*, **54**, 781−790.

Reddy,E.P., Mettus,R.V., DeFreitas,E., Wroblewska,Z., Cisco,M. and Koprowski,H. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 3599−3603.

Sagata,N., Yasunaga,T., Tsuzuku-Kawamura,J., Ohishi,K., Ogawa,Y. and Ikawa,Y. (1985) *Proc. Natl. Acad. Sci. USA.*, **82**, 677−681.

Saiki,R.K., Scharf,S., Faloona,F., Mullis,K.B., Horn,G.T., Erlich,H.A. and Arnheim,N. (1985) *Science*, **230**, 1350−1354.

Seiki,M., Hattori,S., Hirayama,Y. and Yoshida,M. (1983) *Proc. Natl. Acad. Sci. USA.*, **80**, 3618−3622.

Shimotohno,K., Takahashi,Y., Shimizu,N., Gojobori,T., Golde,D.W., Chen,I.S.Y., Miwa,M. and Sugimura,T. (1985) *Proc. Natl. Acad. Sci. USA.*, **82**, 3101−3105.

Shinnick,T.M., Lerner,R.A. and Sutcliffe,J.G. (1981) *Nature*, **293**, 543–548.
Steele,P.B., Rabson,A.B., Bryan,T. and Martin,M.A. (1984) *Science*, **225**, 943–947.
Szurek,P.F., Yuen,P.H., Jerzy,R. and Wong,P.K.Y. (1988) *J. Virol.*, **62**, 357–360.
Todd,J.A., Bell,J.I. and McDevitt,H.O. (1987) *Nature*, **329**, 599–604.
Tsujimoto,A., Teruuchi,T., Imamura,J., Shimotohno,K., Miyoshi,I. and Miwa,M. (1988) *Mol. Biol. Med.*, **5**, 29–42.
Uchiyama,T., Yodoi,J., Sagawa,K., Takatsuki,K. and Uchino,K. (1977) *Blood*, **50**, 481–491.
Wong-Staal,F., Hahn,B., Manzari,V., Colombini,S., Franchini,G., Gelmann,E.P. and Gallo,R.C. (1983) *Nature*, **302**, 626–628.
Yoshida,M. and Seiki,M. (1987) *Annu. Rev. Immunol.*, **5**, 541–559.

Received on August 22, 1988; revised on September 30, 1988

Note added in proof

Recently K.T.A.Malik *et al.* (*J. Gen. Virol.*, **69**, 1695–1710) have published the sequence of an HTLV-I isolate from a patient with adult T-cell leukaemia. This sequence has the same nucleotide changes as we observed at positions 2833, 2848 and 2865 (Table I).