the nuclear transport of proteins during interphase.

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Kinetic Characterization of Ribonuclease-Resistant 2′-Modified Hammerhead Ribozymes

WOLFGANG A. PIEKEN, DAVID B. OLSEN, FRITZ BENSELER, HELLE AURUP, FRITZ ECKSTEIN*

The incorporation of 2′-fluoro- and 2′-aminonucleotides into a hammerhead ribozyme was accomplished by automated chemical synthesis. The presence of 2′-fluorouridines, 2′-fluorocytidines, or 2′-aminouridines did not appreciably decrease catalytic efficiency. Incorporation of 2′-aminocytidines decreased ribozyme activity approximately by a factor of 20. The replacement of all adenosines with 2′-fluoroadenosines abolished catalysis in the presence of $MgCl_2$ within the limits of detection, but some activity was retained in the presence of $MnCl_2$. This effect on catalysis was localized to a specific group of adenines within the conserved single-stranded region of the ribozyme. The decrease in catalytic efficiency was caused by a decrease in the rate constant; the Michaelis constant was unaltered. The 2′-fluoro and 2′-amino modifications conferred resistance toward ribonuclease degradation. Ribozymes containing 2′-fluoro- or 2′-aminonucleotides at all uridine and cytidine positions were stabilized against degradation in rabbit serum by a factor of at least 10^3 compared to unmodified ribozyme.

THE HIGH SPECIFICITY OF ANtisense oligonucleotides and ribozymes has made them potential therapeutic agents for the treatment of diseases such as acquired immunodeficiency syndrome (*1, 2*). However, the instability of these molecules stands in the way of their practical application (*1, 3*). In this report we describe the kinetic characterization of a number of hammerhead ribozymes (*4*) that contain conservative nucleotide replacements within the RNA. These modifications render the RNA oligomers resistant to alkaline hydrolysis and ribonuclease degradation without causing major disruptions in the catalytic ability of the RNA enzymes.

RNA exists predominantly in the A-form, in which the ribose moiety adopts the 3′-endo (N) conformation, whereas B-DNA usually has the sugar in the 2′-endo conformation (S) (*5*). It was therefore not surprising that ribozymes that were partially substituted with 2′-deoxynucleotides exhibited large decreases in catalytic efficiency compared to an all-ribonucleic ribozyme (*6*). Structural studies of the 2′-deoxy-2′-fluoro analogs of the common nucleosides have revealed that they adopt an N conformation and polymers containing these analogs exhibit circular dichroism and nuclear magnetic resonance spectral properties that indicate they have an N conformation and an A-form structure [for a summary, see Williams and co-workers (*7*)]. The partition ratio of 2′-amino-2′-oligodeoxynucleosides between N and S conformations shows a slight preference for the S conformer compared to unmodified nucleosides (*8*). Therefore, hammerhead ribozymes in which the 2′-hydroxyl group was replaced by a fluorine atom or an amino group were prepared and kinetically characterized. The 34-nucleotide ribozyme described by Fedor and Uhlenbeck (*9*) (Fig. 1) was chosen for this systematic study because of its high turnover number.

The catalytic efficiency of hammerhead ribozymes has been thought to depend on the presence of a 2′-hydroxyl group at the site of cleavage and at specific positions throughout the ribozyme structure. The introduction of 2′-deoxynucleotides at the conserved positions E 13, 14, and 27 to 29 (Fig. 1) within the ribozyme sequence resulted in a 96% decrease of catalytic efficien-

Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, W-3400 Göttingen, Federal Republic of Germany.

*To whom correspondence should be addressed.

cy (*6*). This effect was due to a decrease in maximum rate; the Michaelis constant (K_m) remained unchanged. The kinetic constants of the 2′-fluoro– and 2′-amino–modified ribozymes examined in this study, in the presence of $MgCl_2$ as metal cofactor, are summarized in Table 1 (*10*).

Initially, we characterized ribozymes containing 2′-fluoro and 2′-amino modifications of uridine. The seven uridine residues are widely scattered over the ribozyme strand, and only one uridine (U9) is part of the conserved core sequence (Fig. 1) (*4*). The ribozyme in which all uridines were replaced by 2′-fluorouridine (E-FU_{total}) displayed a slight reduction in turnover number, whereas the K_m remained unaltered compared to $E_{unmodified}$ (Table 1). Even though the catalytic efficiency was about fivefold reduced by the introduction of seven 2′-fluorouridines into the hammerhead, this effect was small compared to the variance in catalytic efficiencies observed for hammerheads with different base sequences (*9*). Surprisingly, complete substitution of 2′-aminouridine for uridine (E-NU_{total}) did not drastically perturb the catalytic constants of the ribozyme reaction (Table 1).

The ten cytidine residues in the ribozyme are more clustered in the sequence than the seven uridines. In addition, C8 and C31 are part of the conserved region. All cytidines except C34 were replaced with 2′-fluorocytidine (E-FC_{total}). The C34 at the 3′ end could not be modified readily by automated synthesis because it is attached to the synthesis column. The K_m of E-FC_{total} was fivefold lower and the rate constant (k_{cat}) was somewhat reduced compared to $E_{unmodified}$, which resulted in a twofold increase in catalytic efficiency. However, replacement of all cytidines except C34 with 2′-aminocytidine (E-NC_{total}) resulted in a nearly 20-fold decrease in catalytic efficiency (k_{cat}/K_m = 1 μM^{-1} min^{-1}) compared to $E_{unmodified}$ (Table 1). This effect can be ascribed to a decrease in k_{cat}; the K_m was not affected (Table 1). However, the catalytic efficiency could be raised to the level of $E_{unmodified}$ with a ribozyme that was partially modified with 2′-aminocytidine (E-$NC_{17,18,25,26}$) (Table 1). Thus, the introduction of 2′-aminocytidines does not appear to prevent formation of the secondary structure in stem II (Fig. 1).

The degree of substitution was increased even more by incorporation of either 2′-aminonucleotides (E-NU_{total},NC_{total}) or 2′-fluoronucleotides (E-FU_{total},FC_{total}) at all uridines and all cytidines except C34. The catalytic efficiency of E-NU_{total},NC_{total} was nearly 50-fold reduced compared to $E_{unmodified}$, whereas the analogous 2′-fluoronucleotide–modified ribozyme (E-FU_{total},FC_{total}) gave an approximately 20-fold reduction compared to $E_{unmodified}$. However, these reductions in kinetic competence were more than offset by the gained stability toward ribonucleases (RNases), as discussed below.

Previous experiments involving ribozymes containing phosphorothioate internucleotidic linkages 5′ to residues A14, A28, and A29 showed a dramatic reduction in the catalytic efficiency of these RNA enzymes (*11*). The replacement of all six adenosines with 2′-fluoroadenosines (E-FA_{total}) completely abolished substrate cleavage within the limits of detection when $MgCl_2$ was used as metal cofactor. Changing the metal cofactor from $MgCl_2$ to $MnCl_2$ is known to increase k_{cat} by an order of magnitude (Tables 1 and 2) (*12*). Thus, the introduction of $MnCl_2$ raised the rate of reaction of E-FA_{total} above the limits of detection, so the kinetic analysis became possible. Compared to the cleavage rate of $E_{unmodified}$ in the presence of 10 mM $MnCl_2$, the cleavage rate of E-FA_{total} was slower by more than two orders of magnitude (Table 2). Two ribozymes that were partially modified with 2′-fluoroadenosine were designed to localize critical sites. E-$FA_{22,28–30}$ carried 2′-fluoroadenosines at positions 22 and 28 to 30, and E-FA_{14} carried a 2′-fluoroadenosine at position 14. E-FA_{14}, modified 3′ to a reported critical phosphate (*11*), displayed kinetic constants virtually identical to $E_{unmodified}$, not only in the presence of Mn^{2+} (Table 2), but also with Mg^{2+} as cofactor. Thus, the critical phosphate site does not correspond to a critical 2′-hydroxyl at the neighboring adenosine. The cleavage rate of E-$FA_{22,28–30}$, on the other hand, was only slightly higher than that of FA_{total} (Table 2). Therefore, the replacement of the 2′-hydroxyl with fluorine or of the phosphates with phosphorothioates at positions A_{28} and A_{29} (*11*) may involve a structural perturbation of the ribozyme resulting in a decrease in activity.

Cleavage by a hammerhead ribozyme requires a 2′-hydroxyl group at the site of strand scission (*6*). We were not surprised, therefore, to find that RNA substrates containing a 2′-fluorocytidine at the site of cleavage were completely resistant to ribozyme-catalyzed hydrolysis. A substrate carrying 2′-aminocytidine at the cleavage site also was not cleaved.

In light of the interest in hammerhead

Table 1. Kinetic constants of ribozymes containing 2′-modified uridine and 2′-modified cytidine in the presence of 10 mM $MgCl_2$. Kinetic constants were determined from Eadie-Hofstee plots obtained from initial velocity studies carried out with 5′-^{32}P–labeled substrate. The cleavage reactions were performed on a 20- to 40-μl scale in the presence of 10 mM $MgCl_2$ in 50 mM tris buffer (pH 7.5) at 25°C with a 5- to 10-nM concentration of ribozyme and concentrations of substrate between 50 and 1000 nM. Reactions were initiated by addition of substrate to enzyme. Initial rates were determined by transferring of 10-μl aliquots into 10 μl of water and 10 μl of urea stop mix (7 M urea, 50 mM EDTA, 0.04% bromophenol blue, 0.4% xylene cyanol) between 0.5- and 60-min reaction time before PAGE. The degree of product formation was determined by scanning laser densitometry (Pharmacia LKB Ultroscan XL equipped with Gelscan XL software) after autoradiography.

Enzyme	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m (μM^{-1} min^{-1})
$E_{unmodified}$	4.8	300	16
E-FU_{total}	0.8	300	3
E-NU_{total}	1.4	174	8
E-FC_{total}	1.8	62	30
E-NC_{total}	0.3	303	1
E-$NC_{17,18,25,26}$	3.2	247	13
E-NU_{total},NC_{total}	0.1	321	0.3
E-FU_{total},FC_{total}	0.2	229	0.6

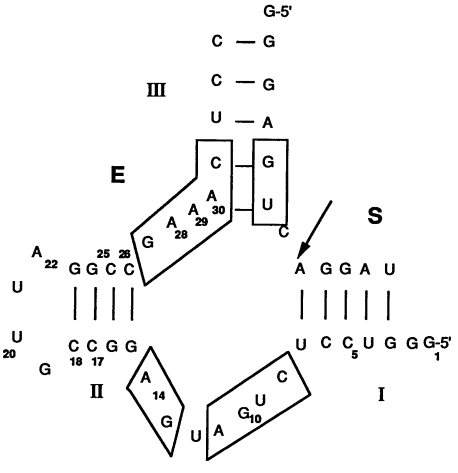

Fig. 1. Structure of the hammerhead ribozyme. Conserved sequences are shown within framed regions. The arrow denotes the site of RNA-catalyzed cleavage. The enzyme strand is marked E, the substrate strand S. The duplex domains are indicated by roman numerals.

Table 2. Kinetic constants of ribozymes containing 2′-fluoroadenosine in the presence of 10 mM $MnCl_2$. The reaction conditions used are as described in the legend of Table 1, with the exception that 10 mM $MnCl_2$ was used as metal cofactor.

Enzyme	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m (μM^{-1} min^{-1})
$E_{unmodified}$	41	220	186
E-FA_{total}	0.2	276	0.7
E-$FA_{22,28–30}$	0.75	125	6
E-FA_{14}	37.6	269	140

ribozymes as therapeutic agents for the inhibition of mRNA translation (*1, 3*), we tested the stability of the ribozymes modified with 2′-fluoro- and 2′-aminonucleotides to RNases. Homooligomers of 2′-fluorouridine are stable against degradation by RNases (*13*). The presence of 2′-amino groups also protects against degradation by RNase and alkaline cleavage (*14*). This stability is also observed for the ribozymes containing 2′-aminouridine. The partial digestion of E-NU_{total} with RNase A, which cleaves 3′ to U and C residues, is shown in Fig. 2. The control reaction of $E_{unmodified}$ with RNase A (lanes "2′-rUMP" in Fig. 2) showed cleavage at every U and C position (circled numbers). Partial digestion of E-NU_{total} with RNase A showed cleavage only at the C positions (lanes "2′-NH_2-UMP"); the 2′-aminouridine sites (arrows) remained protected. An analogous digestion of E-FC_{total},E-FU_{total}, and E-NC_{total} with RNase A and E-FA_{total} with RNase PhyM also revealed that modified positions are stable to RNase digestion (*15*). The incubation of E-FU_{total},FC_{total} or E-NU_{total}, NC_{total} with RNase A at a range of concentrations revealed a 10^6-fold stabilization compared to $E_{unmodified}$ as a lower limit (*16*). In a more realistic test, E-NU_{total}, NC_{total} and E-FU_{total},FC_{total} were found to be more stable in freshly prepared rabbit serum than $E_{unmodified}$ by a factor of approximately 1200 (*16*). Thus, even in this system, modification of the pyrimidine sites alone conferred stability to a degree that outweighed the reduction in catalytic function.

The protection of 2′-fluoro and 2′-amino groups against alkaline hydrolysis (Fig. 3) may be exploited for the rapid confirmation of the presence of the modified nucleotides within the RNA. The partial degradation of $E_{unmodified}$ is shown in lane 1. The partial degradation of E-NC_{total} (lane 2) revealed that the 2′-aminocytidine sites (boxed numbers) are protected from cleavage, albeit not completely. Faint cleavage-product bands are visible at position 5 and 6, with intensities of 7 to 10% of normal cleavage. Longer cleavage fragments displayed a different electrophoretic mobility than those from $E_{unmodified}$. Whereas the fragments from degradation of E-NC_{total} ran faster than those from $E_{unmodified}$, those from E-FC_{total} ran slower (lane 3). The fragments resulting from partial alkaline hydrolysis of E-NC_{total}, NU_{total} (lane 4) showed a similar running behavior to those from E-NC_{total}. Most 2′-aminonucleotide sites in lane 4 showed a small amount of hydrolysis product.

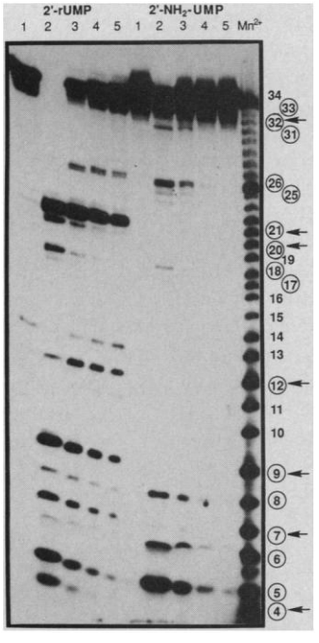

Fig. 2. Autoradiograph of partial RNase A cleavage of 5′-labeled $E_{unmodified}$ and E-NU_{total} separated by PAGE. The oligoribonucleotides $E_{unmodified}$ and E-NU_{total} were subjected to RNase A digestion according to the procedure of Donis-Keller and co-workers (*28*) and analyzed by 20% PAGE. Lane 1, no enzyme added; lane 2, 2×10^{-4} units of RNase A; lane 3, 4×10^{-5} units of RNase A; lane 4, 8×10^{-6} units of RNase A; lane 5, 16×10^{-7} units of RNase A. Base numbering was facilitated by counting of the bands of a Mn^{2+}-mediated cleavage of the unmodified transcript (10 μmol of RNA heated to 90°C for 3 min in 10 mM $MnCl_2$). The circled numbers indicate the bands expected from RNase A–susceptible cleavage positions. Arrows indicate the bands that arise from cleavage 3′ to uridine.

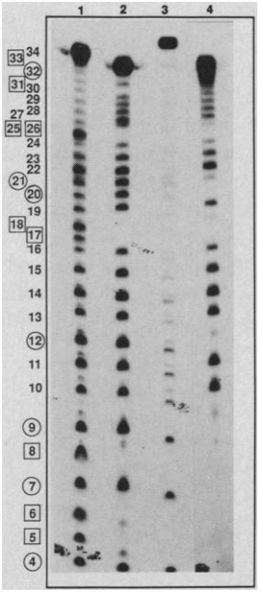

Fig. 3. Autoradiograph of partial alkaline hydrolysis of 5′-labeled oligoribonucleotides separated by PAGE. The [5′-^{32}P]oligoribonucleotides (5 pmol) were subjected to alkaline hydrolysis (*29*) and applied to 20% PAGE after cooling to 25°C. The numbered lanes correspond to the hydrolysis patterns of $E_{unmodified}$ (lane 1), E-NC_{total} (lane 2), E-FC_{total} (lane 3), and E-NC_{total},NU_{total} (lane 4). Boxed and circled numbers denote C and U sites, respectively. The slightly slower moving faint band that accompanies most cleavage sites is indicative of the initial formation of a 2′-3′ cyclic phosphate end by alkaline cleavage. Subsequent further hydrolysis of this cyclic phosphate to the open form gives rise to the more prominent, faster moving strong band.

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10. The unmodified synthetic ribozyme showed a catalytic efficiency of k_{cat}/K_m = 16 μM^{-1} min^{-1}. An unmodified ribozyme transcribed from a DNA template by T7 RNA polymerase gave a catalytic efficiency of k_{cat}/K_m = 28 μM^{-1} min^{-1}, which compares to the value determined by Fedor and Uhlenbeck (k_{cat}/K_m = 32 μM^{-1} min^{-1}) for the same system (*9*). The observed experimental error for k_{cat}/K_m values is a factor of 2, so the difference in cleavage efficiency between the synthetic and transcribed ribozymes falls within this error range. The k_{cat} and K_m values increase or decrease by the same factor between experiments, so the k_{cat}/K_m values remain constant. The comparison of k_{cat}/K_m values of different ribozymes therefore gives the most reliable assessment of their catalytic properties [A. Fersht, *Enzyme Structure and Mechanism* (Freeman, New York ed. 2, 1985), p. 98].
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16. The 5′-^{32}P–labeled oligoribonucleotides, E-FC_{total}, FU_{total} and E-NC_{total},NU_{total}, and $E_{unmodified}$ were separately incubated with different concentrations of RNase A (12-μl reaction volume, 5 μM RNA, five concentrations of RNase A between 8.3×10^{-2} U/μl and 8.3×10^{-10} U/μl) at 50°C for 5 min and analyzed by polyacrylamide gel electrophoresis (PAGE). Whereas $E_{unmodified}$ was degraded to 98% even at an RNase A concentration of 8.3×10^{-10} U/μl, E-FC_{total},FU_{total} and E-NC_{total},NU_{total} were not degraded at all even at the highest RNase A concentration. Therefore, the ribozymes in which the pyrimidines were modified were stable to RNase A by a lower limit of 10^6 compared to $E_{unmodified}$. Incubation of the ribozymes with different concentrations of freshly prepared rabbit blood serum (between 1.2- and 1.2×10^4-fold dilution) showed 17% degradation of $E_{unmodified}$ at 1000-fold dilution of serum, whereas E-FC_{total},FU_{total} and E-NC_{total},NU_{total} remained stable to even the highest serum concentration.
17. All of the nucleoside analogs used in this work have been described previously [2′-deoxy-2′-fluorocytidine (*18, 19*), 2′-deoxy-2′-fluoroadenosine (*20*), 2′-deoxy-2′-fluorouridine (*21*), 2′-deoxy-2′-aminouridine (*21*), and 2′-deoxy-2′-aminocytidine (*21*)]. They were prepared by improved methods (*23*). The 2′-deoxy-2′-fluoronucleoside 3′-phosphoramidites for automated oligonucleotide synthesis were prepared by the method of Sinha and co-workers (*24*).

For the synthesis of the corresponding 2′-amino derivatives, the amino group was protected by trifluoroacetylation (*25*). The oligoribonucleotides were prepared by automated oligoribonucleotide synthesis on an Applied Biosystems 380B DNA Synthesizer on a 1-μmol scale with the monomeric ribonucleotide phosphoramidites supplied by Milli-Gen/Biosearch. The oligomers were worked up according to the specifications of the supplier with the following modification: after removal of the protecting groups, the oligoribonucleotides were worked up according to Scaringe and co-workers (*26*). The dried pellets were taken up in 50 μl of water and subjected to PAGE. Bands were visualized by ultraviolet shadowing or, in the case of [5′-^{32}P]oligoribonucleotides (*27*), autoradiography, and were cut out. The RNA was isolated by freezing and then crushing the gel piece and eluting at 37°C overnight in sodium acetate (3 M, pH 6.0). The eluate can be concentrated either by SepPak cartridge reverse-phase chromatography or by ethanol precipitation. Concentrations were determined with an extinction coefficient per nucleotide of 6600 M^{-1} cm^{-1} (*9*). Aqueous solutions of the oligoribonucleotides were stored at −20°C.

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Deleted HTLV-I Provirus in Blood and Cutaneous Lesions of Patients with Mycosis Fungoides

WILLIAM W. HALL,* CHUNREN R. LIU, OLAF SCHNEEWIND, HIDEHIRO TAKAHASHI, MARK H. KAPLAN, GOSTA RÖUPE, ANDERS VAHLNE

Mycosis fungoides, a rare form of cutaneous T cell leukemia/lymphoma, is suspected of having a viral etiology on the basis of certain similarities to adult T cell leukemia, which is associated with human T cell leukemia/lymphoma virus type I (HTLV-I) infection. Cell lines were established from peripheral blood mononuclear cells (PBMC) of an HTLV-I–seronegative patient with mycosis fungoides. DNA hybridization analysis revealed the presence of HTLV-I–related sequences with unusual restriction endonuclease sites. Sequence analysis of subcloned fragments demonstrated the presence of a monoclonally integrated provirus with a 5.5-kilobase deletion involving large regions of *gag* and *env* and all of *pol*. Additional evidence for the presence of deleted proviruses was found by polymerase chain reaction (PCR) amplification of DNA from cutaneous lesions of five other HTLV-I–seronegative patients. The findings suggest that HTLV-I infection may be involved in the etiology of at least certain cases of mycosis fungoides.

THE CUTANEOUS T CELL LYMPHOMAS (CTCLs) are a group of rare disorders that include mycosis fungoides, Sézary syndrome, Woringer-Kolopp disease, and adult T cell leukemia/lymphoma (ATL) (*1*). ATL is an aggressive disorder with characteristic early visceral spread and poor prognosis and has been shown to be associated with infection by HTLV-I (*2, 3*). Phenotypically, the proliferating leukemic cells are predominantly $CD4^+$ and Tac^+. The HTLV-I provirus is monoclonally integrated into the abnormal cell population, and the large majority of patients are seropositive for HTLV-I (*4*). In contrast, mycosis fungoides and its variant Sézary syndrome are relatively indolent disorders. Although cutaneous involvement may be extensive, the disease progresses slowly, and visceral involvement may not occur for many years (*5*). In contrast to ATL, the leukemic cells of these disorders are $CD4^+$ and Tac^-.

The etiology remains unknown, and studies to implicate HTLV-I or a related retrovirus have yielded ambiguous results. Serological studies by enzyme-linked immunosorbent assay (ELISA) on more than 200 American patients with CTCL showed that fewer than 1% were HTLV-I–seropositive (*6*), whereas several studies of European patients yielded seropositivity rates of up to 12% (*7*). More recently, Manzari and co-workers (*8*) reported the isolation of a HTLV-I–related retrovirus, HTLV-V, from a cell line derived from an Italian patient with mycosis fungoides. Southern hybridization analysis showed that the provirus, although similar to HTLV-I, was unique, and it was proposed that HTLV-V may be involved in the etiology of some CTCLs in Italy.

To study a possible viral etiology in this disease, we grew lymphocytes from an American patient with mycosis fungoides in leukemic phase in RPMI medium containing 20% fetal bovine serum and 10% interleukin-2 (v/v) after an initial 72-hour stimulation with phytohemagglutinin (PHA). The patient was seronegative for HTLV-I in both ELISA and Western blot assays after being repeatedly tested over a 2-year period (*9*). After 10 days, cells were cocultivated with three different samples of cord blood lymphocytes stimulated with PHA. The lines (MF-B1, MF-B3, and MF-B6) proliferated spontaneously and grew in large clumps. Although no syncytia formation was evident, many of the cells were polynucleated. Comparative phenotypic analysis showed them to be predominantly B cells, which is in contrast to findings in the peripheral blood where the majority of cells were $CD4^+$ Tac^-. The B cells expressed surface heavy (immunoglobulin M, immunoglobulin D) and light (kappa) chains, and the phenotypic markers have remained constant in more than a year of culture. The cells have been shown to be Epstein-Barr virus (EBV)–infected by Southern hybridization and clonal on the basis of immunoglobulin heavy and light chain gene rearrangements (*10*). The proliferation and establishment of B cell lines from this patient, who had a $CD4^+$ leukemia, was not considered unusual because B cell lines with concomitant EBV and HTLV-I infections are often established from patients with ATL (*11*).

Although structures morphologically similar to C-type particles were infrequently observed by electron microscopy, no budding particles or evidence of virus assembly or formation at the cell membranes was ever observed (*12*). No significant levels of either Mg^{2+}- or Mn^{2+}-dependent reverse transcriptase (RT) activity were detected in concentrated supernatant fluids (*13*), and as such there was no evidence that the cell lines were productively infected with a retrovirus.

A probe containing the entire HTLV-I provirus hybridized specifically and under high stringency washing conditions [0.1× saline sodium citrate (SSC) containing 0.1%

W. W. Hall, C. R. Liu, H. Takahashi, M. H. Kaplan, Division of Infectious Disease, North Shore University Hospital–Cornell University Medical College, Manhasset, NY 11030.
O. Schneewind, The Rockefeller University, New York, NY 10021.
G. Röupe, Department of Dermatology, Sahlgrens Hospital, Göteborg, Sweden.
A. Vahlne, Department of Clinical Virology, University of Göteborg, Göteborg, Sweden.

*To whom correspondence should be addressed.