cells from the AIDS patients. If one assumes that the primary PBL had died after 3 weeks in coculture, which was when we detected the HBV DNA, this result implies that the H9 cells acquired the HBV from the patients' mononuclear blood cells.

Studies with five additional AIDS patients, two of whom were negative for conventional HBV markers, showed that HBV DNA sequences were present in cell populations derived from different lymphoid sources: bone marrow, semen, lymph node, as well as PBL. The concomitant infections of HBV-HTLV-III/LAV found in all of these patients, even in the absence of conventional serological HBV markers, are consistent with the possibility that HBV may be a cofactor in the development of AIDS, as previously suggested for CMV and EBV (*8*). Further studies are needed to assess this hypothesis, and it will be necessary to conduct similar studies with DNA probes for other viruses, such as EBV and CMV, and to determine the role of all of these viruses in the pathogenesis of AIDS.

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20. The work of F.L. on DNA sequences was performed at the Pasteur Institute (Unité de Recombinaison et Expression Génétique). Supported by grants from the Association pour la Recherche Contre le Cancer (Villejuif), Ligue Nationale Française contre le Cancer, Féderation André Maginot and Delegation de la Recherche et des Equipements Techniques. We thank V. Thiers and C. Pasquinelli and thank A. Cova for typing the manuscript.

27 March 1985; accepted 4 June 1985

Infection of HTLV-III/LAV in HTLV-I–Carrying Cells MT-2 and MT-4 and Application in a Plaque Assay

Abstract. *The human T-cell lines MT-2 and MT-4 carry the human T-cell leukemia virus type I (HTLV-I). When MT-2 and MT-4 were infected with HTLV-III, the probable etiologic agent of the acquired immune deficiency syndrome (AIDS), rapid cytopathogenic effects and cytotoxicity were observed that made it possible to titrate the biologically active virus in a plaque-forming assay. The cytopathogenic effects were preceded by the rapid induction and increase of HTLV-III antigens as revealed by immunofluorescence and immunoprecipitation. Activities of HTLV-III were neutralized by the human antibodies against the virus when immunofluorescence and plaque assays were used. Essentially the same results were obtained with the lymphadenopathy-associated virus (LAV_1).*

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In 1984, retroviruses termed HTLV-III and AIDS-related virus (ARV) were isolated in the United States from the peripheral blood lymphocytes of patients with AIDS or pre-AIDS (*1*). A similar virus, LAV_1, had been isolated in France from patients with lymphadenopathy syndrome (*2*). Determination of the complete nucleotide sequences of the genome of these viruses revealed that they were variants of the same virus (*3*). That this virus is the causative agent of AIDS is indicated by studies showing that patients with AIDS or AIDS-related complex (ARC) frequently possess serum antibodies against this virus (*4*) and that the virus is found with high frequency in AIDS and ARC patients (*5*), causes specific cytopathogenic changes in $OKT4^+$ cells (*6*), is transmissible through blood transfusions (*7*), and causes a similar disease in chimpanzees (*8*).

Transmission of HTLV-III to an established T-cell line, H9, was first achieved by Popovic *et al.* (*1*). The same group of investigators subsequently showed that several $OKT4^+$ cell lines were susceptible to HTLV-III infection (*9*). The discovery of cell lines that continuously grow and produce the virus after infection greatly facilitated further studies of this virus. It was also reported that LAV could be adapted to grow in Epstein-Barr virus–transformed B lymphoblastoid cell lines or in the CCRF-CEM T-cell line (*10*). However, viral replication in all of these cell lines, and in primary human lymphocytes, requires a considerable time lag after infection. (Growth of the virus in primary human lymphocytes usually results in cell lysis.) In H9 cells infected with concentrated HTLV-III, viral activity is detectable 6

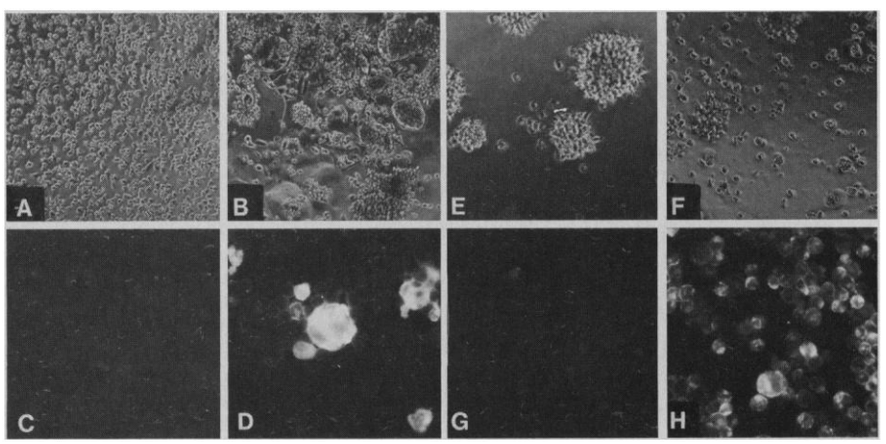

Fig. 1. HTLV-III–infected MT-2 (B and D) and MT-4 (F and H) cells and uninfected MT-2 (A and C) and MT-4 (E and G) cells. Virus was obtained from H9/HTLV-III culture medium after incubation for 4 days at 37°C. After removal of cells by centrifugation at 900*g* for 10 minutes, the supernatant was passed through a 0.22-μm Millipore membrane and stored at −80°C. Cells were infected with tenfold diluted virus preparation and photographed 3 days later by phase-contrast microscopy (A and B, ×60; E and F, ×120) or by immunofluorescent microscopy (C, D, G, and H, ×240) after staining.

Table 1. Inhibition of HTLV-III–induced plaque formation by the treatment of the virus with sera from two seropositive patients with hemophilia and two seronegative controls. Aliquots of HTLV-III (200 μl of 800 PFU/ml) derived from H9/HTLV-III cultures were incubated with 200 μl of twofold diluted sera from two patients and two controls for 2 hours at 4°C. Then 100 μl of each mixture was inoculated onto MT-4 monolayers. Triplicate plaque assays were performed (average ± standard error of the mean are given). When PBS was used instead of sera, 41.0 ± 12.2 plaques per plate were counted as the virus control. No plaque was observed without inoculation of the virus. The percentage reduction of the plaque is shown in parentheses and was calculated as follows: $[(P_c - P_t)/P_c] \times 100$, where P_c is the number of plaques in the virus control and P_t the number in the test. N.T.. not tested.

Donor	Antibody titer* against HTLV-III	Dilution of serum				
		1:10	1:20	1:40	1:80	1:160
Patient 1 (He-1)	1:4096	0 (100)	1.3 ±2.3 (96.8)	2.0 ± 1.0 (95.1)	9.0 ± 6.6 (78.0)	17.7 ± 10.5 (56.8)
Patient 2	1:512	7.3 ± 1.5 (82.2)	6.7 ± 1.5 (83.7)	9.0 ± 2.6 (78.0)	13.0 ± 6.6 (68.3)	18.0 ± 5.2 (56.1)
Healthy 1	<1:5	37.0 ± 7.9 (9.8)	37.0 ± 17.0 (9.8)	30.3 ± 6.7 (26.1)	N.T.	N.T.
Healthy 2	<1:5	36.7 ± 12.9 (10.5)	29.5 ± 4.9 (28.0)	29.0 ± 9.9 (29.3)	N.T.	N.T.

*Antibody titers were determined by indirect immunofluorescence of fixed H9/HTLV-III cells (*1*).

days after infection by the appearance of immunofluorescent antigens or the presence of reverse transcriptase (RT) (*1*).

We undertook these studies to find other cell culture systems that allow the efficient replication of HTLV-III. Sodroski *et al.* (*11*) had already shown that chloramphenicol acetyltransferase (CAT) expression from recombinants of the HTLV-I long terminal repeat (LTR) and the CAT gene is more enhanced in HOS cells infected with HTLV-I than in uninfected HOS cells. In this system, HTLV-I produced a transregulatory protein that influenced LTR-directed CAT expression. Chen *et al.* (*12*) obtained an analogous result with the HTLV-II–neo^{R}gene system. Moreover, Dalgleish *et al.* and Klatzmann *et al.* had shown that the OKT4 molecule is an essential component of the receptor for HTLV-III/LAV (*13*). On the basis of these observations we selected the MT-2 and MT-4 cell lines as the target for HTLV-III/LAV infection since both cell lines strongly express HTLV-I antigens and are positive for OKT4 surface antigens. We report here the efficient replication of HTLV-III/LAV in these cell lines and the use of these lines in a plaque assay.

Lines MT-2 and MT-4 were established from cord blood lymphocytes that had been cocultured with leukemic cells from patients with adult T-cell leukemia (ATL) (*14*). The cells in these T-cell lines resemble leukemic T cells of ATL in terms of cell surface markers. After infection with HTLV-III derived from H9/HTLV-III cells, the MT-2 and MT-4 cells showed differences in morphology, viability, and growth characteristics (Fig. 1 and 2A). MT-2 cells usually became attached to the plastic surface of the culture dish (Fig. 1A) after subculture in the fresh medium. Three days after exposure to a 1:10 dilution of culture fluid from H9/HTLV-III cells, the MT-2 cells showed cytopathic changes with the appearance of multinuclear giant cells (Fig. 1B), apparently due to cell fusion, that attached to the surface of the culture dish. Floating cells started to show swelling and the features of ghost cells. These cells, and many others that did not change in size, degenerated very rapidly. Cell multiplication was impaired and viability started to decrease rapidly 3 days after infection (Fig. 2A).

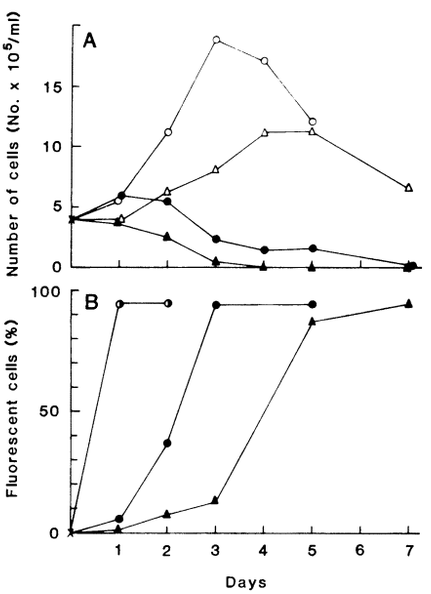

Fig. 2. Effect of HTLV-III infection on growth of cells and induction of HTLV-III–specific immunofluorescent antigens in MT-2 and MT-4 cells. MT-2 and MT-4 cells were infected with the various concentrations of HTLV-III. Virus suspension (0.5 ml, appropriately diluted) was added to the pellet of 2×10^6 cells of the target cells. The mixture was then incubated for 1 hour at 37°C for virus adsorption. Then, 4.5 ml of complete medium (RPMI 1640 with 10 percent fetal calf serum) was added to each tube, and the cells (1 ml per well) were inoculated into a 24-well plastic tray. After incubation at 37°C in a humidified atmosphere with CO_2, the cells were investigated for growth by the trypan blue dye exclusion method under the low-power microscope (A) and for HTLV-III antigen synthesis by indirect immunofluorescence (B) at the indicated times after infection. Symbols: △, uninfected MT-2 cells; ▲, MT-2 cells infected with HTLV-III (1:10); ○, uninfected MT-4 cells; ●, MT-4 cells infected with HTLV-III (1:10); and ◑, MT-4 cells infected with the undiluted HTLV-III.

The cytopathic changes in MT-4 cells infected with HTLV-III were somewhat weaker than those in the MT-2 cells. MT-4 cells are smaller than MT-2 cells and do not attach to the surface of the culture dish when placed in fresh medium (Fig. 1E). Three days after infection with tenfold dilutions of culture fluid from H9/HTLV-III cells, the MT-4 cells became rounded and lost their surface characteristics. Then they became dark and showed shrinkage of the nucleus but usually no swelling (Fig. 1F). When control H9 cells were exposed to tenfold dilutions of H9/HTLV-III culture fluids, multinuclear giant cells appeared after about 5 days; an additional 5 days were required for all the cells to become infected. In MT-2 and MT-4 cells, the time course of the appearance of these cytopathic effects depended on the viral dose.

The MT-2 and MT-4 cell cultures inoculated with the tenfold dilutions of virus culture fluids were examined by immunofluorescence (IF) with a standard antibody (A-1) to HTLV-III from a patient with AIDS (IF titer; 1:1280) as described (*1*) (Figs. 1 and 2B). The first antigen-positive cells appeared as early as 1 day after infection in both cell lines (Fig. 1, D and H) and increased with time. However, the proportion of cells with detectable antigen increased more rapidly in MT-4 cells than in MT-2 cells: about 5 percent at 1 day, 33 percent at 2 days, and nearly 100 percent after 3 days of culture (Fig. 2B). When the cells were infected with the undiluted culture fluid of H9/HTLV-III cells, the first antigen-positive cells appeared within 16 hours and almost all the cells became antigen-positive within 24 hours. In the MT-2 cultures, almost all the cells became antigen-positive 6 days after inoculation.

Under the same conditions, H9 cells started to show antigen positivity at 4 days, and the frequency of antigen-positive cells reached 100 percent about 10 days after infection. If the HTLV-III preparations were treated with human antibodies to HTLV-III obtained from an AIDS patient (A-1) or a hemophiliac boy (He-1; IF titer, 1:4096), the frequency of antigen-positive cells was greatly reduced and the cytopathogenic changes were blocked at lower dilutions of the sera.

When these experiments were repeated with LAV prepared from cultures of CEM/LAV$_1$ cells (*15*), we obtained essentially the same results. To ensure that the cytopathic effects and the appearance of immunofluorescent antigens were indeed caused by HTLV-III, we performed immunoprecipitation experiments (*16*). In MT-4 cells infected with the virus, eight dominant polypeptide bands were specifically precipitated with antisera to HTLV-III. The molecular weights of these polypeptides were approximately 120,000, 55,000, 46,000, 40,000, 36,000, 33,000, 24,000, and 17,000. In none of the experiments were HTLV-I antigens observed when the two HTLV-III antibodies were used.

We then tested whether MT-4 cells could be used as target cells for an HTLV-III–induced plaque assay (*17*). Since MT-4 cells were not adherent to the culture vessel, we used plates coated with poly-L-lysin (PLL) (molecular weight 90,000; Sigma) to make a monolayer of cells (*18*). The dilution experiment data (Fig. 3C) suggest that a single infectious virus particle is sufficient for infection and plaque formation (*17, 19*), but this test does not prove that every virion present is able to create a plaque. To determine whether plaques are formed by HTLV-III, we incubated portions [40 plaque-forming units (PFU)] of the virus preparation with various dilutions of sera from two seronegative controls and two patients with hemophilia A who were both seropositive to HTLV-III. Serum from patient 1, who had a high titer (1:4096) of antibody detected by IF (*1*), completely inhibited plaque at 1:10 and 1:20 dilutions (Table 1). The titer of neutralizing antibody to HTLV-III in the sera from both patients was almost 1:160, judged by the 50 percent reduction in plaque formation. In contrast, sera from two healthy donors (negative IF) did not remarkably reduce plaque formation, although some reduction in the number of plaques was observed. This result may reflect experimental variation due to the small number of PFU used as virus controls or the sensitivity of the assay. It may be necessary to improve the technique to minimize the standard deviation of the number of plaques. To confirm that plaque formation was specific to HTLV-III infection, we compared PFU, median tissue culture infectious doses (TCID$_{50}$), and the activity of RT from different preparations of virus (Table 2). There was a parallel relation between PFU titer and RT activity, suggesting that PFU reflected the amount of the retrovirus. Also, PFU was well correlated with TCID$_{50}$, as determined by the CPE of infected MT-4 cell cultures. Thus the values obtained were the same within the range of experimental error. Plaque formation was also observed when HTLV-

Table 2. Plaque-forming units (PFU), TCID$_{50}$, and RT activity of virus preparations from various sources. Portions (15 ml) of filtrated supernatants from MT-2 cell, H9/HTLV-III cells, HTLV-III–infected MT-4 cells, and MT-4 cell cultures were tested for PFU, TCID$_{50}$, and RT activity. Plaque formation was performed as described in Fig. 3. In order to determine the TCID$_{50}$, 100 μl of 60 × 10^4 per milliliter of MT-4 cells was plated into each flat well of microtiter plate (Terumo, Tokyo). The same volume of tenfold diluted supernatants was then inoculated into each well. Quadruplicate experiments were performed for each dilution. Half of the medium (RPMI 1640 with 10 percent fetal calf serum and antibiotics) was changed twice a week. Plates were incubated at 37°C for 4 weeks. Remarkable *p*H change of the medium and cytopathic effects of infected cells were counted. The Reed and Muench method (*20*) was used for calculation of the 50 percent end point. RT activity was detected as described by Poiesz *et al.* (*21*).

Viral source	Time in culture (days)	Quantitation of the virus		
		PFU/ml	TCID$_{50}$/ml	RT (count/min)
Medium		0	0	1.5 × 10^3
MT-2 cells	4	0	0	2.3 × 10^3
H9/HTLV-III cells	1	2.7 × 10	10$^{1.2}$	6.3 × 10^3
H9/HTLV-III cells	4	4.0 × 10^3	10$^{3.3}$	2.3 × 10^4
HTLV-III–infected MT-4 cells	4	2.2 × 10^5	10$^{5.5}$	1.7 × 10^6
MT-4 cells	4	0	0	3.8 × 10^3

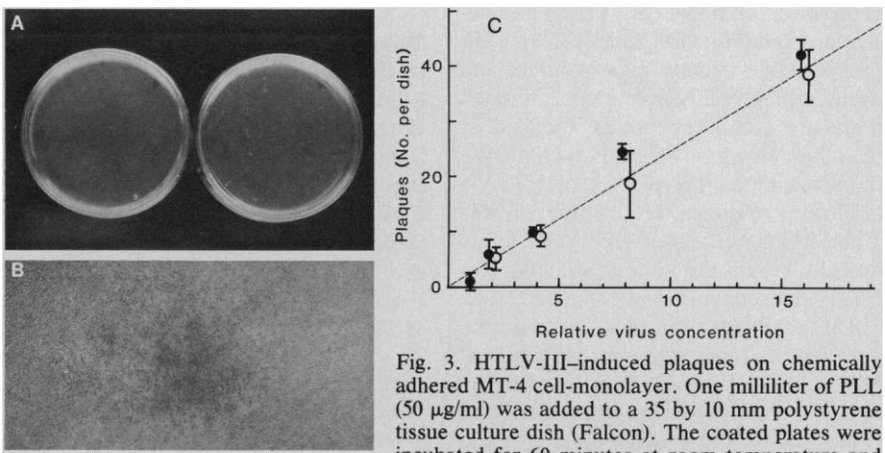

Fig. 3. HTLV-III–induced plaques on chemically adhered MT-4 cell-monolayer. One milliliter of PLL (50 μg/ml) was added to a 35 by 10 mm polystyrene tissue culture dish (Falcon). The coated plates were incubated for 60 minutes at room temperature and rinsed three times in phosphate-buffered saline (PBS). Portions (1.5 ml) of MT-4 cells (150 × 10^4 per milliliter) were washed three times in PBS and added to each PLL-coated plate. After incubation for another 30 minutes the unbound cells were removed by gently washing the plates three times with PBS. The MT-4 cell-monolayer was prepared, and 100 μl of diluted virus was slowly dropped onto cells. The plates were then incubated for 60 minutes at 4°C. After adsorption of the virus, 1 ml of the agarose medium was overlayed [Sea Plaque Agarose, Marine Colloid Corporation: RPMI 1640 medium supplemented with 10 percent fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 0.6 percent agarose]. The plates were incubated (CO_2 atmosphere) at 37°C for 3 days; 1 ml of agarose overlay medium containing neutral red (1:50,000) was added; and incubation was continued for another 3 days in the dark. On day 6, plaque production was observed (A) with the naked eye (right, infected; left, uninfected) and (B) microscopically. At this time, plaque diameter was 1 to 2 mm. HTLV-III–specific antigen detected by IF was expressed in the cells picked from the plaques. (C) Linear relation between number of plaques and virus concentration. Values for the relative virus concentration were chosen arbitrarily; however, a concentration of 16 corresponds to a 1:100 dilution of filtrated supernatant (4 × 10^3 PFU/ml) from H9/HTLV-III cell culture. Then, a twofold dilution of the virus was made. The dashed line represents the theoretical dose response of plaque formation according to the one-particle hypothesis (*19*). Open and solid circles display two independent experiments. The number of plaques per dish was expressed as the mean value of three dishes. The bars reveal the standard deviations (n = 3).

III/LAV–infected cells were used instead of cell-free virus (*22*).

Sodroski *et al.* reported that gene expression directed by the LTR sequence of HTLV-III is stimulated only in cell lines infected with HTLV-III and not with HTLV-I or -II (*23*). Gallo and his colleagues showed that an HTLV-I–positive cell line, C91/PL, was susceptible to HTLV-III infection although H9 cells were more efficient in terms of viral replication (*9*). Using a large panel of HTLV-I–positive cell lines of human and monkey origin, we determined whether the efficient replication of HTLV-III observed in MT-2 and MT-4 cells is a general phenomenon in HTLV-I–positive cell lines. Thirteen of 21 cell lines showed more rapid appearance and increase of viral antigens after HTLV-III infection than the H9 cells used as a control. However, HTLV-III replication efficiency did not appear to be correlated with the frequency of the cells positive for the HTLV-I antigens detectable by IF (*24*). Thus it is not known whether HTLV-III replication after infection is correlated with LOR gene expression in these HTLV-I–carrying cells.

It is also not known whether the virus produced from HTLV-I–positive cell lines after HTLV-III infection are phenotypically altered [an interaction has been observed between HTLV-I and vesicular stomatitis virus (VSV) (*25*)]. Cell-free HTLV-I usually fails to infect and transform normal lymphocytes, although there are some exceptions ascribed to the labile envelope of the virus (*26*). We found no biological activity of HTLV-I in filtrates of the culture medium of MT-2 and MT-4 cells after HTLV-III infection; however, the biological activity of HTLV-III seemed to be enhanced (*27*).

The establishment of a system that permits rapid and efficient replication of HTLV-III and cell death opens the way to the routine detection and isolation of HTLV-III from the infected patients and may facilitate studies of the AIDS virus.

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28. We thank R. C. Gallo and J. C. Chermann for supplying H9 and H9/HTLV-III and CEM/LAV cells, respectively, and Y. Koyanagi for typing the manuscript. Supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture, Japan.

25 March 1985; accepted 6 June 1985

T-Cell Receptor β-Chain Expression: Dependence on Relatively Few Variable Region Genes

Abstract. *Fifteen independently isolated complementary DNA clones that contain T-cell receptor (TCR) V_β genes were sequenced and found to represent 11 different V_β genes. When compared with known sequences, 14 different V_β genes could be defined from a total of 25 complementary DNA's; 11 clones therefore involved repeated usage of previously identified V_β's. Based on these data, we calculate a maximum likelihood estimate of the number of expressed germline V_β genes to be 18 with an upper 95 percent confidence bound of 30 genes. Southern blot analysis has shown that most of these genes belong to single element subfamilies which show very limited interstrain polymorphism. The TCR β-chain diversity appears to be generated from a limited V_β gene pool primarily by extensive variability at the variable-diversity-joining (V-D-J) junctional site, with no evidence for the involvement of somatic hypermutation.*

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The vertebrate immune system characteristically responds to a broad range of foreign antigens with great specificity. The mechanisms through which diversity and clonal specificity are generated have been described in great detail for the B-cell receptor, or antibody molecule. Antibody diversity is generated by (i) somatic recombination between a large pool of germline variable (V) elements and germline diversity (D) and joining (J) elements to produce a functionally rearranged immunoglobulin gene; (ii) variation in the precise sites of this joining event; (iii) insertion of random N region nucleotides at the junctional sites; and (iv) somatic hypermutation (*1*).

Recent findings have shown that the T-cell receptor (TCR) is a heterodimer (*2–4*), each chain of which is the product of a somatic DNA rearrangement similar to that described for immunoglobulin genes (*5–8*). The germline organization of the murine D_β, J_β, and C_β elements has been described (*5, 9–12*). Two closely linked constant region (C_β) elements exist, both of which are preceded by six functional J_β elements; two D_β elements have been localized thus far, with one D_β preceding each of the two J_β clusters. An unknown number of V_β genes are presumed to lie 5′ to the D_β, J_β, and C_β gene clusters.

In order to define the repertoire of V_β genes and assess their role in generating diversity in the T-cell receptor β chain, we examined a series of V_β containing complimentary DNA (cDNA) clones. Sequence analysis of these clones leads us to conclude that the number of functionally expressed germline V_β genes is relatively small, and that the generation of β chain diversity primarily involves variability introduced by the V-D-J rearrangement events.

To assess available diversity among mouse TCR V_β chains, we isolated and sequenced 15 independent V_β-containing clones from cDNA libraries screened with a TCR C_β-specific probe, including four from BALB/c thymus, nine from