Infectious Mutants of HTLV-III with Changes in the 3′ Region and Markedly Reduced Cytopathic Effects

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A variant of human T-lymphotropic virus type III (HTLV-III) is described that replicates but does not kill normal human T cells in vitro. This variant, designated X10-1, was derived from the genome of a cytopathic HTLV-III clone (pHXB2D) by excision of a 200–base pair segment in the 3′ region of the virus, spanning the *env* and 3′-*orf* genes. Comparable variants with 55 to 109 base pairs deleted exclusively in 3′-*orf* produced, in contrast, virus that was extremely cytopathic. On the basis of these findings it is concluded that the 3′-*orf* gene is not required for cytopathogenicity or replication of HTLV-III. In addition, the results suggest that virus replication and cytotoxicity are not intrinsically coupled. Furthermore, since clone X10-1 retains the ability to *trans*-activate genes linked to the viral long terminal repeats, *trans*-activation per se is not responsible for T-cell killing by HTLV-III. These results also raise the possibility that the carboxyl terminus of the envelope gene of HTLV-III has a direct role in T-cell killing by this virus.

HUMAN T-LYMPHOTROPIC VIRUS type III (HTLV-III) is causally linked to the acquired immune deficiency syndrome (AIDS) (*1*). Its immunosuppressive effects in vivo are largely paralleled by its ability to selectively infect and kill OKT4 helper/inducer cells in vitro (*2*). A molecular clone of HTLV-III that can generate infectious virions and exert cytopathic effects on normal T lymphocytes in vitro was recently described (*3*). This clone provides direct evidence that a product of the HTLV-III genome mediates cell killing and also provides a means to dissect the viral determinants of pathogenicity. The HTLV-III genome has, in addition to the replicative genes *gag*, *pol*, and *env* that are found in other retroviruses, at least four additional genes: *sor*, *tat*, 3′-*orf*, and a new gene variously named *art* or *trs* (*4–6*). Of these, the *trans*-activator gene of HTLV-III (tat_{III}) has been localized (*7*) and shown to be critical for virus replication (*8*). The *art* gene also appears to be required for HTLV-III replication, modulating the expression of HTLV-III *gag* and *env* proteins (*5, 6*). The *sor* and 3′-*orf* genes, originally identified as open reading frames, have been shown to encode proteins that are immunogenic in vivo (*9*), but the functions of these gene products are unknown.

To investigate the possible role of 3′-*orf* in virus replication and cytopathogenicity, we constructed a series of deletion mutants of the biologically active molecular clone pHXB2D (*3*). Making use of a unique Xho I restriction site in the 3′-*orf* region of the viral element of plasmid pHXB2gpt, and the Bal 31 digestion technique, we prepared six variant genomes containing deletions around the Xho I site (Fig. 1). In the case of plasmids ΔX-A, ΔX-B, ΔX-C, and ΔX-D, the deleted segments of 55, 109, 85, and 100 base pairs, respectively, were confined to the 3′-*orf* gene. The sequences deleted from clones X10-1 and X9-3 were more extensive, encompassing the last 14 bp of the envelope gene, the initiation codon for 3′-*orf*, and the first 159 (X9-3) or 182 (X10-1) bp of this gene. The capacity of these variant genomes to generate HTLV-III virions was investigated by transfecting the plasmids into the human T-lymphoid cell line H9. Each of the variants, in addition to the parental pHXB2gpt clone, gave rise to cultures expressing HTLV-III *gag* (p17 and p24) and *env* (p41 and syncytia) proteins and HTLV-III virions (Table 1). In all but one case, virus expression was evident 1 to 2 weeks after transfection. In the case of X9-3, virus was recovered slightly later (after 3 to 4 weeks), suggesting either that the transfection efficiency of this clone was slightly lower than that of the others or that this clone produced virus with a mildly reduced capacity for replication.

Southern blot analysis of DNA prepared from transfected cultures and probed for HTLV-III sequences showed a predominant 9.6-kb band in cultures transfected with pHXB2gpt, ΔX-A, ΔX-B, ΔX-C, ΔX-D, X9-3, and X10-1, corresponding to the presence of unintegrated virus (Table 1). Northern blot analysis of RNA from transfected cells showed that the efficiency of the deletion genomes in generating viral transcripts was comparable with that of the wild-type clone pHXB2gpt. Figure 2 shows typical results obtained with pHXB2gpt (lane 1) and the clone bearing the largest of the deletions, X10-1 (lane 2). In these samples, bands corresponding to viral transcripts of approximately 9.5, 4.5, and 2.0 to 2.3 kb were detected, representing genomic

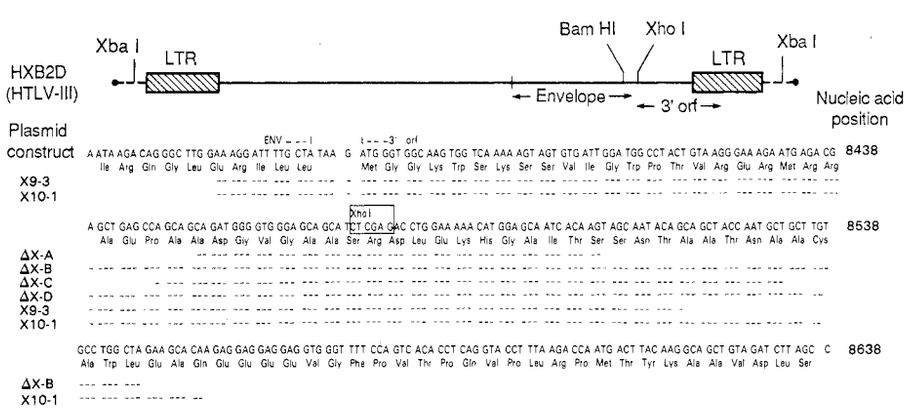

Fig. 1. Construction of HTLV-III clones with deletions in the 3′ region of the virus. The molecular clone pHXB2D contains a full-length, biologically active HTLV-III genome (9.6 kb) flanked by long terminal repeat (LTR) sequences (cross-hatched boxes) contained within the plasmid vector pSP62 (*3*). This genome has been extensively characterized and contains a single Xho I and Bam HI site in the 3′-*orf* and *env* genes, respectively (*17*). Plasmid pHXB2gpt, a derivative of this clone in which the proviral fragment of pHXB2D is inserted into pSP65gpt (a vector carrying the xanthine guanine phosphoribosyltransferase gene but devoid of HTLV-III sequences) was generated and used to construct variants bearing deletions at the Xho I site in the viral genome; briefly, pHXB2gpt was digested to completion with Xho I, treated with Bal 31, blunt-ended by using T4 DNA polymerase, and then self-ligated with T4 DNA ligase. The precise nature of each variant was analyzed by nucleotide sequencing. The position and extent of the deleted segments (shown as ---) relative to the nucleotide and amino acid sequence of pHXB2gpt are shown.

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messenger RNA (mRNA), *env* mRNA, and a heterogeneous population of mRNA's (including those for *tat* and 3′-*orf*), respectively. A slightly faster migration of the viral RNA species in the case of X10-1 is consistent with the removal of a 200-bp segment from the genome of this variant. No viral RNA was detected in cells transfected with control plasmid (pSP65gpt) devoid of HTLV-III sequences (lane 3).

These results show that the 3′-*orf* gene is not required for HTLV-III replication. Additional studies indicated that virus derived from clones ΔX-A, ΔX-B, ΔX-C, and ΔX-D, which contain deletions exclusively in the 3′-*orf* gene, were cytopathic for human T lymphocytes. In contrast, virus derived from clones X10-1 and X9-3, which contain additional deletions in the envelope gene, were severely limited in their ability to kill human lymphocytes.

The infectivity of virus produced from clones ΔX-C and X10-1 was initially analyzed by titration against H9 cells in standard assays. Concentrated virus stocks were prepared by direct centrifugation of supernatants harvested 6 to 8 weeks after transfection of H9 cells with pHXB2D, ΔX-C, or X10-1. Two different preparations of HXB2D virus ($HXB2D^A$ and $HXB2D^B$) were prepared from independently transfected cultures. These preparations were used to estimate the extent of "batch to batch" variation between virus stocks. All virus preparations were diluted to give multiplicities of infection of 10^3 or 10^5 virions per cell and used to infect "virgin" H9 cultures [cells that are susceptible to productive HTLV-III infection without succumbing to the cytopathic effect of the virus (*10*)]. Growth and expression of HTLV-III in these cultures were monitored at 2- to 4-day intervals after infection. As shown in Fig. 3A, all cultures grew well after infection with pHXB2D–, ΔX-C–, or X10-1–derived virus. The proportion of infected cells in the cultures, estimated as the percentage of cells expressing HTLV-III RNA, was analyzed by in situ hybridization (*11*) (see Fig. 3B). Virus generated from ΔX-C was highly infectious, yielding 30% to 50% HTLV-III–positive cells in cultures after the introduction of 10^5 particles per cell (days 2 through 14). It is possible that the initial high values include passively adsorbed virus at the cell surface and hence overestimate the number of infected cells. However, the increased incidence of cells expressing HTLV-III 2 to 7 days after infection with 10^3 ΔX-C virions per cell (2% to 60%) demonstrates the infectivity of the ΔX-C genome. Likewise, the proportion of HTLV-III expressing cells after infection with X10-1 virions increased from 5% to 33% (10^5 virions per cell) over 8 days and from <0.01% to 35% (10^3 virions per cell), over a 12-day period, similar to the increase in control cultures ($HXB2D^A$ and $HXB2D^B$). These results

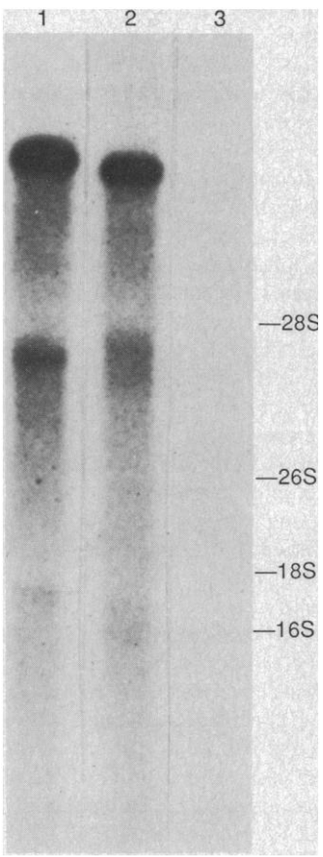
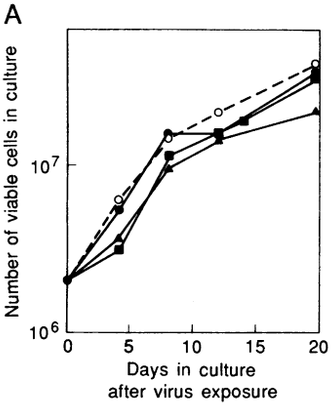


Fig. 2. Northern blot analysis of HTLV-III RNA expression after transfection. RNA was extracted from H9 cells 2 weeks after transfection with pHXB2gpt (lane 1), X10-1 (lane 2), or pSP65gpt (lane 3) by means of the hot phenol approach of Queen and Baltimore (*19*). Northern blotting was performed by standard methods with the use of 10 μg of RNA per channel (*20*). Nitrocellulose filters were hybridized to a ^{32}P-labeled probe prepared by nick translation of an Sst I–Sst I viral fragment of λBH10. The filters were washed with 0.5× standard saline citrate containing 0.1% sodium dodecyl sulfate at 65°C for several hours prior to exposure. Three bands corresponding to genomic mRNA (9.5 kb), envelope mRNA (4.5 kb), and a mixed population of lower molecular weight mRNA's (2.0 to 2.3 kb) were evident in samples from pHXB2gpt and X10-1 transfections but absent from pSP65gpt transfected preparations. Similar results were obtained with RNA extracted from parallel cultures by the guanidine isothiocyanate method of Chirgwin *et al.* (*21*).

A



B

Multiplicity of infection (virions/cell)	Days after virus exposure	Percentage of H9 cells expressing HTLV-III in cultures after exposure to virus			
		$HXB2D^A$	$HXB2D^B$	X10-1	ΔX-C
10^5	2	10	2	5	50
10^5	4	12	9	2	30
10^5	7	54	61	12	34
10^5	10	48	48	33	44
10^5	14	42	48	NT	36
10^3	2	<0.01	<0.01	<0.01	2
10^3	4	<0.01	<0.01	<0.01	7
10^3	7	30	<0.01	<0.02	60
10^3	10	56	45	19	33
10^3	14	39	40	35	31

Fig. 3. Infection of H9 cells by HTLV-III variants. Concentrated virus stocks prepared from X10-1, ΔX-C transfected H9 cultures and from two different cultures transfected with pHXB2D ($HXB2D^A$ and $HXB2D^B$) were used to infect H9 cells at different multiplicities. (A) The kinetics of cell growth of cultures infected with virus (10^5 virions per cell) derived from X10-1 (■), ΔX-C (●), and HXB2D (▲) were compared with the growth of uninfected cultures (○). The results shown for HXB2D represent the mean values obtained with preparations $HXB2D^A$ and $HXB2D^B$. (B) After exposure to virus (10^5 or 10^3 virions per cell), the proportion of cells expressing HTLV-III RNA (>20 grains per cell), expressed as a percentage of total, was determined by an in situ assay using the approach of Harper *et al.* (*11*) and a ^{35}S-labeled probe generated by transcription of clone pBH10-R3 (*11*). The results of a single experiment are shown, and the values represent the mean of three separate determinations. Virus stocks were prepared by harvesting 2 to 4 liters of culture supernatant from exponentially growing H9 transfected cultures, centrifuging the media at 100,000*g* for 1 hour at 4°C, and resuspending the pellet containing virus in 2 to 4 ml of RPMI 1640 medium containing fetal calf serum (FCS) and antibiotics. The concentration of virus particles per milliliter was assessed by electron microscopy and the stocks maintained under liquid nitrogen prior to use. Infection was achieved by incubation of 2×10^6 prewashed H9 cells in RPMI 1640 containing 2 μg/ml of Polybrene for 20 minutes at 37°C, washing the cells and resuspending them in 0.5 ml of medium containing 2×10^{11} or 2×10^9 virus particles, and incubating the suspension for 1 hour at 37°C with gentle agitation. The cells were returned to culture (37°C, 5% CO_2 in a humidified atmosphere) in 5 ml of RPMI 1640 containing 20% FCS and antibiotics and maintained at a density of 5×10^5 to 2×10^6 cells per milliliter for 2 to 4 weeks by addition of fresh medium.

show that virus generated from the deletion clones X10-1 and ΔX-C and the parental clone pHXB2D are comparable in terms of their ability to infect H9 cells. Thus the replication competence and infectivity of the HTLV-III genome do not appear to be compromised by the deletions contained in X10-1 and ΔX-C.

To compare the cytopathic potential of clones ΔX-C and X10-1, we introduced these clones, together with appropriate control plasmids, into cultures of phytohemagglutinin (PHA)-stimulated normal cord blood mononuclear cells using the protoplast fusion technique (*3*) (see Fig. 4). The results shown for each clone summarize data obtained from three (Fig. 4A) and two (Fig. 4B) transfection experiments, each performed independently with cells obtained from normal donors. As shown in Fig. 4, A and B, cultures transfected with full-length HTLV-III genomes (pHXB2D, pHXB2gpt, and pHXB2hygro) grew well for 10 to 12 days and then declined compared with cultures transfected with no HTLV-III sequences (pSP65gpt). These results confirm that the genome carried by pHXB2D is cytopathic for normal T cells when introduced alone, linked to the guanine xanthine phosphoribosyltransferase (gpt) gene, or a gene conferring resistance to hygromycin (hygro). Transfection of the deletion clone ΔX-C resulted in cell and virus growth kinetics that were indistinguishable from those obtained with the full-length genomes, falling within the range obtained for pHXB2gpt and pHXB2hygro (Fig. 4B). This is consistent with the ΔX-C genome being fully cytopathic for normal T cells. In contrast, the deletion clone X10-1 was markedly less cytopathic; the kinetics of cell growth of these cultures paralleled those of cultures transfected with control plasmid pSP65gpt (Fig. 4A). Infected cells, detected by in situ assay for HTLV-III RNA, were evident in X10-1–transfected cultures (4.2%, day 10) showing that this discrepancy is not the result of a failure of X10-1 to generate HTLV-III expressing cells. The proportion of HTLV-III–positive cells in these cultures was, on average, slightly less than in pHXB2D–transfected control cultures (7.2%, day 10), although the ranges of values obtained were clearly overlapping. The significance of this difference, if any, is not known.

Studies with concentrated virus stocks and an $OKT4^+$ immortalized cell line, ATH8, were performed to further test the cytopathic potential of the ΔX-C and X10-1 genomes. Virus stocks with comparable infectivity, as determined by titration against H9 cells (see Fig. 3), were used throughout these studies. The cell line ATH8, originally

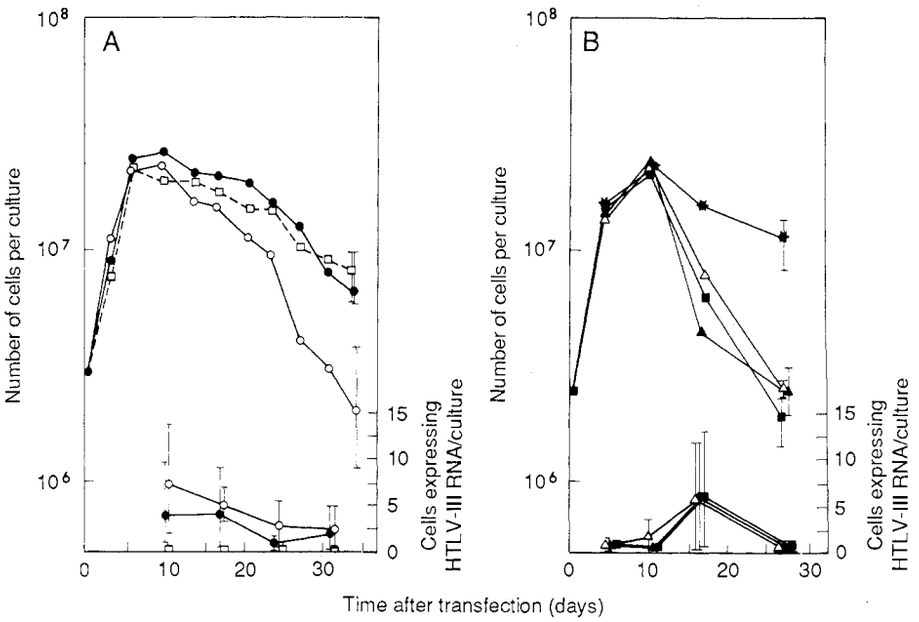


Fig. 4. Kinetics of growth and HTLV-III expression after transfection of cord blood cells with HTLV-III variant clones. Cord blood mononuclear cells were fused with bacterial protoplasts carrying the plasmids pHXB2D (○), X10-1 (●), pHXB2gpt (▲), pHXB2hygro (△), ΔX-C (■), and pSP65gpt [□ in (A) and * in (B)]. The results for each plasmid indicate the mean number of viable cells in culture. (A) Results obtained in three parallel experiments with cells from three normal donors. (B) Results obtained in parallel experiments with cells from two additional normal donors. In each case the variation within replicate experiments is shown by a bar. This range is shown only for the end point of each experiment. The percentage of cells expressing HTLV-III RNA (>20 grains per cell) assessed by in situ assay is indicated on the right hand ordinate of (A) and (B) and the range of values is shown as bars. Cord blood mononuclear cells obtained from normal donors were separated with the use of Ficoll-Triosil and cultured for 5 days in medium containing phytohemagglutinin (PHA). Protoplast fusion was performed according to the approach of Fisher *et al.* (*3*), with 10^{10} protoplasts and 3×10^6 cells. After transfection the cells were maintained at 5×10^5 per milliliter by addition of RPMI 1640 containing 20% FCS, antibiotics, and 10% interleukin-2. Cell viability was assessed by trypan blue exclusion or phase-contrast microscopy. In situ assays were performed on cytocentrifuge preparations of cells hybridized to a probe generated from pBH10-R3 (*11*).

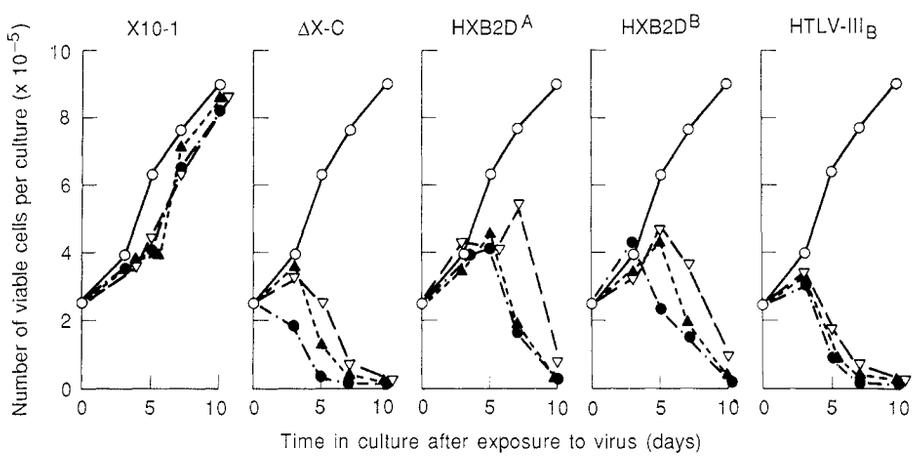


Fig. 5. Cytopathic effects of HTLV-III variants on ATH8 cells. Virus stocks prepared from X10-1, ΔX-C, and pHXB2D transfected H9 cultures or from the H9/HTLV-III$_B$ cell line (*10*) were used to infect ATH8 cells at the following multiplicities of infection: 10^4 virions per cell (●), 3×10^3 virions per cell (▲), and 500 virions per cell (▽). Control cultures, similarly treated but not exposed to virus (○), were also established. The results shown are for a single experiment. To check the reproducibility of these findings two additional preparations of both X10-1 virus and HXB2D virus were prepared and the assays against ATH8 cells were repeated. These assays gave results comparable to those shown here. Virus stocks were prepared as described in the legend to Fig. 3; 2.5×10^5 ATH8 cells (*12*) were treated with Polybrene (2 μg/ml for 30 minutes at 37°C), exposed to virus for 45 minutes, washed, and cultured in tubes containing RPMI 1640 with 15% FCS, 15% interleukin-2, and antibiotics. The total number of viable cells was assessed by the trypan blue exclusion method.

Table 1. Replication and cytopathic capacity of HTLV-III clones with deletions in the 3′ region of the genome. Plasmid clones ΔX-A, ΔX-B, ΔX-C, ΔX-D, X9-3, X10-1, pSP65gpt, and pHXB2gpt were introduced into H9 cells by means of a protoplast fusion technique (*3*). Briefly, 10^{10} plasmid-bearing bacterial protoplasts were combined with 3×10^6 H9 cells, treated for 1 minute with 48% polyethylene glycol, diluted, washed, and returned to culture in RPMI 1640 medium containing 20% fetal calf serum and antibiotics. The results shown summarize duplicate transfections performed independently. The appearance of multinucleated cells in culture (+, >1%) was assessed by examining Wright-Giemsa stained cytocentrifuge preparations of cells removed 3 to 5 days after transfection. Expression of HTLV-III *gag* proteins p17 and p24 and *env* protein p41 was determined 2 weeks after transfection by using a standard immunofluorescence technique and the monoclonal antibodies BT2, BT3, and M25, respectively (*18*) (+, expression by 10 to 90% of cells; −, no cells expressing virus). The presence (+) or absence (−) of HTLV-III virions in cultures 2 weeks after transfection was determined by electron microscopy.

Plasmid constructs	Number of base pairs deleted at Xho I site in 3′-*orf*	Position of deleted nucleotides	Expression of HTLV-III after transfection of H9 cells				Cytopathic effects on lymphocytes†
			Syncytia	Expression of p17, p24, or p41	HTLV-III virions	Unintegrated* HTLV-III DNA	
pHXB2gpt	0		+	+	+	+	+
ΔX-A	55	8453–8507	+	+	+	+	+
ΔX-B	109	8439–8547	+	+	+	+	+
ΔX-C	85	8448–8532	+	+	+	+	+
ΔX-D	100	8439–8538	+	+	+	+	+
X9-3‡§	177	8355–8531	+	+	+	+	−
X10-1§	200	8356–8555	+	+	+	+	−
pSP65gpt	No HTLV-III sequences		−	−	−	−	−

*The presence of unintegrated HTLV-III DNA in transfected cells was determined by Southern blotting using standard techniques (*19*) and a ^{32}P probe prepared by nick translation of an SstI to SstI fragment (HTLV-III insert) isolated from λBH10 (*17*). †The cytopathic effects of each clone were determined by the assays described in Figs. 4 and 5. Plus signs indicate results comparable with the parental clone pHXB2gpt, HXB2D virus and polyclonal virus derived from $HTLV\text{-}III_B$–infected cells; minus signs indicate results comparable with those obtained with the plasmid vector alone (pSP65gpt) and uninfected ATH8 cells. ‡In the case of X9-3, virus expression was detected in a single culture 3 to 4 weeks after transfection. §Clones with the last 14 bp of the envelope gene deleted.

derived from a cloned tetanus toxoid–specific T cell infected with HTLV-I, was used here as an indicator because of its extreme sensitivity to killing by HTLV-III (*12*). As shown in Fig. 5, when we used multiplicities of infection of 500, 3×10^3, and 10^4 virions per cell, virions from the deletion clone ΔX-C, the parental clone pHXB2D, and from the original $H9/HTLV\text{-}III_B$ line ("polyclonal" virus) were extremely cytopathic for ATH8 cells, killing the cultures in 10 days. The cytopathic effects of these viruses for ATH8 cells were, in all experiments, apparent 5 to 7 days after infection. At this point we estimated that 14 to 50%, 11 to 45%, 12 to 95%, and 16 to 95% of cells in cultures infected with $HXB2D^A$, $HXB2D^B$, ΔX-C, and $HTLV\text{-}III_B$, respectively, were virus-positive as judged by immunofluorescence and RNA in situ assays. In contrast, virus from the X10-1 genome did not kill or markedly suppress the growth of ATH8 cultures even when introduced at a high multiplicity (10^4 virions per cell). Ten days after infection 73%, 42%, and 2% of cells were positive for HTLV-III p17 (assessed by immunofluorescence with BT2 antibody) in cultures infected with 10^4, 3×10^3, and 500 X10-1 virions per cell, respectively. These results confirm the ability of X10-1 virus to infect but not kill T lymphocytes. Although at first sight the spread of virus in X10-1-infected ATH8 cultures appears less rapid than that in cultures infected with HXB2D, ΔX-C, and $HTLV\text{-}III_B$, this apparent reduction is probably due to differences in doubling times of the cell cultures rather than the infectivity of the virus preparations (Fig. 5).

Thus, by using a panel of variants created by deleting sequences in the 3′ region of the virus genome, we have shown that a major portion of the coding capacity of the 3′-*orf* gene is dispensable to the production of infectious cytopathic HTLV-III virions; deletions contained in the ΔX-A to ΔX-D series remove the coding capacity for amino acids 23 to 58 of 3′-*orf* and shift the remaining sequences of this gene out of frame. This conclusion is supported by the observation that the cytopathic genome HXB2D contains a termination codon midway through 3′-*orf* (*13*) (and thus would be predicted to be capable of generating only a truncated and possibly nonfunctional 3′-*orf* product) and that cells transfected with pHXB2D do not make detectable levels of 3′-*orf* protein (*14*). The conclusion that 3′-*orf* is not critical for virus replication is unexpected, because 3′-*orf* is not only a functional gene that directs the synthesis of a demonstrable viral protein (of approximate molecular size 27 kD) (*9*) but is also conserved across evolutionary lines, being found in the distantly related Old World monkey virus STLV-III (*15*).

Perhaps of greater interest is the observation that a variant genome (X10-1), with a deletion extending upstream to include the last 14 bp of the envelope gene, is fully competent for virus expression but is no longer able to kill human T cells. These data may help to elucidate the mechanisms by which HTLV-III kills susceptible T lymphocytes. First, HTLV-III replication and pathogenicity are not intrinsically coupled. Second, since studies of cells infected with X10-1 demonstrate (i) the accumulation of unintegrated viral DNA copies in the cell and (ii) syncytia formation, we conclude that these phenomena are not the sole causes of the cytopathic effects of HTLV-III. This is perhaps surprising because the presence of unintegrated viral DNA in infected cells is a property common to all cytopathic retroviruses and because syncytia formation [which probably involves the interaction of the major exterior glycoprotein (gp120) and the OKT4 cellular receptor] is associated with infection with every HTLV-III/LAV isolate analyzed to date and undoubtedly contributes to the cytotoxic effects of these viruses. Finally, since we have demonstrated that clone X10-1 *trans*-activates genes linked to HTLV-III long terminal repeats (LTR's) at comparable levels with the full-length cytopathic clone (*16*), and thus contains a functionally intact tat_{III} gene, we conclude that the *trans*-activator gene of HTLV-III is not directly responsible for the cytopathic properties of this virus. This provides evidence against the suggestion that tat_{III} alone kills infected cells either by modulating cellular genes leading to terminal differentiation of $OKT4^+$ cells or by simply overwhelming cells with virus.

Since immunosuppression in AIDS patients is probably attributable to HTLV-III infecting and killing helper/inducer lymphocytes, identification of the viral determinants of cytopathogenicity is of importance in revealing new avenues for the development of safe therapeutic and preventative approaches. Here we show that the cytopathic elements reside in the 3′ region of the HTLV-III genome, probably in that region encoding the small envelope protein (gp41). However, clone X10-1 does not contain the normal termination codon for *env* but instead terminates 15 codons downstream

from the deletion (see Fig. 1). This, we predict, adds 15 "new" amino acid residues (K-R-R-R-R-W-V-F-Q-S-H-L-R-Y-L) to the protein product of this gene and could conceivably affect the properties of this moiety. Likewise, clone X9-3, which also has a diminished cytopathic potential, has an "extended" envelope resulting from the substitution of the last five amino acids of *env* with the last 153 amino acids of 3′-*orf*. However, since we do not know precisely the replication competence of virus derived from X9-3, it is possible that reduced replication is, in this instance, the reason for its reduced cytopathic potential. Further studies will be necessary to determine precisely the genetic and biochemical changes that account for the loss of cytopathogenicity in clones X10-1 and X9-3. The present results suggest that caution should be used in selecting candidate immunogens for vaccination derived from the HTLV-III envelope region, since they might themselves be cytopathic for susceptible cells. Our data also suggest that novel therapeutic approaches such as those being developed specifically to block *tat* function may be ineffective unless they are designed to halt completely the expression of HTLV-III in infected cells.

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Probing Steric and Hydrophobic Effects on Enzyme-Substrate Interactions by Protein Engineering

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Steric and hydrophobic effects on substrate specificity were probed by protein engineering of subtilisin. Subtilisin has broad peptidase specificity and contains a large hydrophobic substrate binding cleft. A conserved glycine (Gly^{166}), located at the bottom of the substrate binding cleft, was replaced by 12 nonionic amino acids by the cassette mutagenesis method. Mutant enzymes showed large changes in specificity toward substrates of increasing size and hydrophobicity. In general, the catalytic efficiency (k_{cat}/K_m) toward small hydrophobic substrates was increased (up to 16 times) by hydrophobic substitutions at position 166 in the binding cleft. Exceeding the optimal binding volume of the cleft (~160 Å^3), by enlarging either the substrate side chain or the side chain at position 166, evoked precipitous drops in catalytic efficiency (k_{cat}/K_m) (up to 5000 times) as a result of steric hindrance.

BINDING SPECIFICITY IS A UBIQUITOUS feature of biological macromolecules; it is determined by chemical forces including hydrogen bonding and electrostatic, hydrophobic, and steric interactions (*1*, *2*). Substrate specificity studies of enzymes have been a traditional means of probing the relative importance of these binding forces. Although substrate analogs can be synthesized chemically, the production of complementary enzyme analogs has been extremely limited to natural variants (*3*) or chemically modified enzyme derivatives (*4*). Recently, through the technology of protein engineering, it has been possible to tailor a protein by site-directed mutagenesis of its cloned DNA sequence (*5*), and the mutant protein can be expressed in a heterologous host.

We have chosen subtilisin, a serine-class endopeptidase (M_r, 27,500), as a model to study the energetics of substrate specificity. The three-dimensional structure of subtilisin BPN′ (from *Bacillus amyloliquefaciens*) has been solved by x-ray crystallography to 2.5 Å resolution (*6*) and more recently to 1.8 Å resolution (*7*). Protein engineering has been applied to the cloned subtilisin gene (*8*) to create subtilisins with greater oxidative stability (*9*), to determine the importance of hydrogen bond formation in transition-state stabilization (*10*), to introduce disulfide bonds into subtilisin (*11*), and to alter the *p*H activity profile of the enzyme (*12*).

Our studies here are designed to probe steric and hydrophobic effects on substrate specificity of subtilisin. Steric and hydrophobic effects are among the most general and complicated of the chemical binding forces because they can embody the entire substrate. To analyze the contribution of these forces to substrate specificity, we have produced 12 noncharged mutations in the P1 (*13*) binding cleft of subtilisin (Fig. 1), and have determined the resulting specificities of mutant enzymes against P1 substrates of varying size and hydrophobicity. Mutant enzymes exhibit large changes in substrate specificity caused by a combination of steric hindrance and enhanced hydrophobic interactions.

To map the topology and chemical nature of the P1 binding cleft in subtilisin the kinetic parameters, k_{cat} (turnover number) and K_m (Michaelis constant), were determined for ten different noncharged substrates having the form succinyl-L-Ala-L-Ala-L-Pro-L-[X]-*p*-nitroanilide, where X is the P1 amino acid. The ratio of k_{cat}/K_m (also

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