JOURNAL OF VIROLOGY, Nov. 1986, p. 754–760
0022-538X/86/110754-07$02.00/0

Vol. 60, No. 2

Effects of Mutations within the 3′ *orf* Open Reading Frame Region of Human T-Cell Lymphotropic Virus Type III (HTLV-III/LAV) on Replication and Cytopathogenicity

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Received 27 May 1986/Accepted 8 July 1986

Mutations that eliminated the ability of the human T-cell lymphotropic virus type III to produce the 27-kilodalton 3′ *orf* product did not eliminate the ability of the virus to replicate in and kill T4$^+$ cells. A mutant carrying a mutation that deleted carboxy-terminal sequences from the envelope gene as well as the 3′ *orf* sequences retained the ability to kill T4$^+$ lymphocytes, but had a retarded replication rate.

The human T-cell lymphotropic virus type III (HTLV-III/LAV), a retrovirus, is the primary etiological agent of the acquired immune deficiency syndrome (AIDS) and related disorders (3, 14, 21). Infection with the virus can result in depletion of the T4$^+$ subset of helper T cells, producing a profound immunodeficiency (10, 22, 23). Cytopathic effects on T4$^+$ cells can also be observed in vitro, on cultured peripheral blood lymphocytes as well as on certain established T4$^+$ lymphoid cell lines (6–8). Infection by HTLV-III may also result in central nervous system disorders (9, 24).

The complete nucleotide sequence of several distinct strains of HTLV-III has been determined (13, 15, 20, 29). Like all other retroviruses, the genome of HTLV-III contains the *gag*, *pol*, and *env* genes. These genes encode, respectively, the viral capsid proteins, proteins required for replication of the virus, and the exterior glycoproteins. Unlike most other retroviruses, HTLV-III encodes four additional products using the *tat*, *art*, *sor*, and 3′ *orf* genes. Two of these genes, the *tat* and *art* genes, are essential for replication (18, 25). The third gene, *sor*, is not required for replication or cytopathic effect, as viruses deleted in this gene are capable of growing in and killing T4$^+$ cells, albeit at a reduced rate (11, 26). The fourth gene, 3′ *orf*, was recently shown to encode a 27-kilodalton (kDa) protein. Experiments that investigate the effects of mutations that eliminate the ability of the virus to produce this protein on the replication and cytopathogenicity of the virus are described here.

The structures of the virus genomes used for this analysis are pictured in Fig. 1. They include plasmid pHXB-*orf*, which encodes all the known viral proteins, including the 3′ *orf* gene. This plasmid was derived from the pHXBc2 plasmid, which has been shown to produce infectious virus that is cytopathic to lymphocytes (6, 26). The pHXBc2 plasmid contains a single termination codon within the 3′ *orf* gene that would truncate the 205-amino-acid reading frame of this gene at residue 122 (26). The pHXB-*orf* plasmid was produced by excising the sequences in pHXBc2 located between a unique *Xho*I restriction site in the 3′ *orf* open reading frame and a *Sac*I site within the 3′ long terminal repeat (LTR) and replacing them with the corresponding sequences from the BH8 clone. BH8 contains a complete 3′ *orf* open reading frame (15). The BH8 sequence differs from the pHXBc2 sequence at only a few nucleotides within this approximately 700-base-pair (bp) segment. pHXB-*orf* is identical to pHXBc2 in the sequences of all its other viral proteins.

Two deletions were introduced within the 3′ *orf* open reading frame, taking advantage of the *Xho*I restriction site. The deletions in plasmids pD*orf*-1 and pD*orf*-2 removed 11 and 50 amino acids, respectively, from the coding capacity of the 3′ *orf* open reading frame. Both deletions introduced a frameshift as well. An additional deletion was created which extended past the 5′ end of the 3′ *orf* gene, resulting in the removal of carboxy-terminal sequences from the envelope gene. This mutation (pD*orf*-3) removed the final 43 amino acids from the envelope gene product and substituted three incorrect amino acids. The polypurine tract located 5′ to the LTR was preserved in this deletion (Fig. 1).

The effects of these alterations in the 3′ *orf* gene on the infectious and cytopathogenic character of the virus were determined in a transfection assay. Two cell lines previously shown to be sensitive to the cytopathic effect of HTLV-III were used as recipients. These were the Jurkat-*tat* III cell line, a T4$^+$ cell line that constitutively expresses the functional *tat* III product (19), and the C8166-45 cell line, a T4$^+$ cell line transformed by HTLV-I. Proviral DNA was introduced directly into the Jurkat-*tat* III cell line as previously described (26). Infection of the C8166-45 cell line was accomplished by cocultivation with Jurkat-*tat* III cells that had been transfected with proviral DNA 72 h previously and then treated with mitomycin C as described (26). This cocultivation technique was used for the C8166-45 cells instead of direct transfection of proviral DNAs because of the extreme sensitivity of this cell line to the DEAE-dextran used in the transfection protocol.

The effects of virus infection were monitored in both cell lines by measurements of cell number, cell-free reverse transcriptase activity, HTLV-III-specific surface immunofluorescence, and syncytium formation. The profile of virus-specific proteins in infected cells was also determined by immunoprecipitation of ^{35}S-labeled protein, as was the structure of proviral DNA for some experiments. Transfection of the cells with the pD*sor*-3 plasmid, a deletion mutant of pHXBc2 that is not capable of replication (26), was used as a negative control. The results of these experiments are summarized in Fig. 2. Transfection of Jurkat-*tat* III cells with the plasmid that contained a complete 3′ *orf* gene resulted in marked cytopathic effect, as judged by both a

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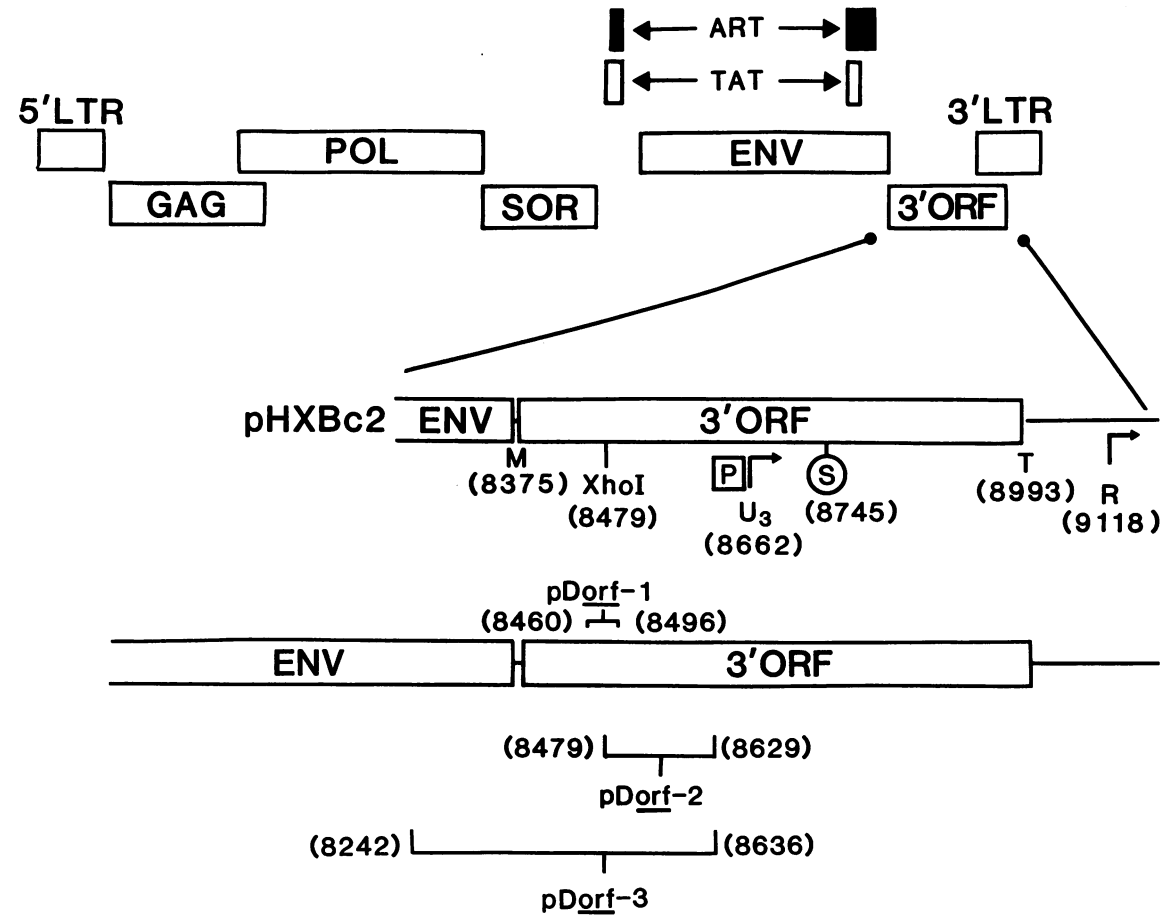


FIG. 1. Structure of the pHXBc2 proviral clone and 3' *orf* deletion mutations. The structure of the HTLV-III provirus in plasmid pHXBc2 is shown, with a detailed enlargement of the region near the 3' end encompassing the 3' *orf*. The beginnings of the U3 and R regions of the 3' LTR are indicated. The deletions present in pD*orf*-1, pD*orf*-2, and pD*orf*-3 are also represented. The numbers in parentheses refer to base pair numbers within the provirus. M, Methionine; T, termination codon; P, polypurine tract; S, premature stop codon within the pHXBc2 3' *orf* reading frame.

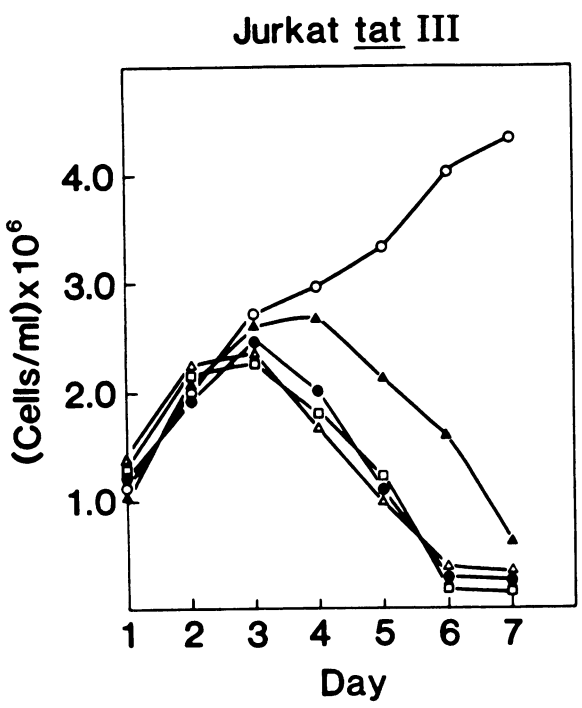
dramatic decrease in total cell number and the appearance of giant syncytia. Virus produced by the transfected Jurkat-*tat* III cells was also capable of killing the C8166-45 cells.

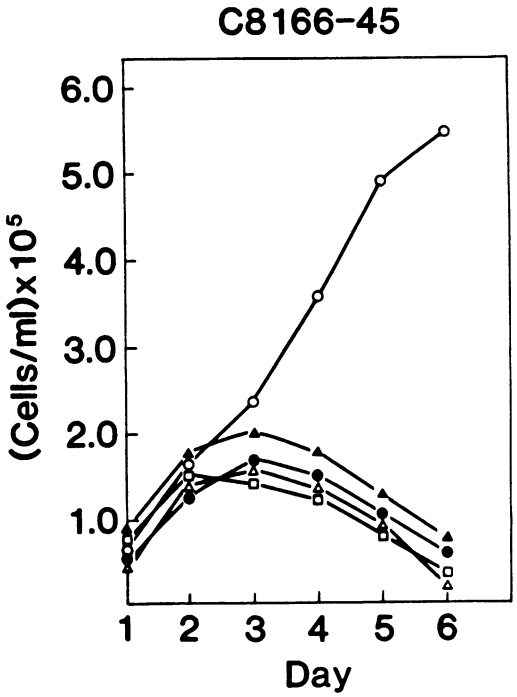
Transfection of the Jurkat-*tat* III cell line with proviruses that contained either a stop codon or deletions within the 3' *orf* gene also resulted in marked cytopathic effect (Fig. 2). In addition, the virus produced by these cell lines was also capable of replicating in and killing the C8166-45 cells.

No significant quantitative differences were observed in any of the paramenters measured between infections produced by pHXBc2, pD*orf*-1, and pD*orf*-2. However, in repeat experiments there was a small but consistent difference in the kinetics of cell death and indications of virus replication between cultures in which the pHXB-*orf* plasmid was used and those in which plasmids carrying mutations within the 3' *orf* gene were used. The onset of cell death, the formation of syncytia, the appearance of cells expressing virus-specific surface antigen, and the appearance of reverse transcriptase in the culture fluid were delayed in cells transfected or infected with the pHXB-*orf* virus compared with plasmids altered in the 3' *orf* gene. The profiles of virus-specific proteins synthesized in cells transfected with these plasmids and the structure of the provirus DNA were also investigated.

The virus-specific proteins produced following transfection of Jurkat-*tat* III cells with the proviral plasmids were analyzed by immunoprecipitation of ^{35}S-labeled proteins with an AIDS patient antiserum (Fig. 3). The ^{35}S-labeled extracts were prepared from each culture at the time of maximum immunofluorescence. Virus-specific proteins precipitated in these experiments included the envelope glycoproteins gp120, gp160, and gp41; the *gag* gene products p55, p38, p24, and p17; and the *pol* gene products p66 and p31. The p27 product of the 3' *orf* gene was evident only in the extract from cells transfected with the pHXB-*orf* plasmid. No such protein was evident in extracts prepared from cells transfected with the pHXBc2 plasmid or the pD*orf*-1 or p*Dorf*-2 plasmid. Southern blot analysis of DNA extracted from these cells revealed that the provirus in cells transfected with the pHXBc2 or pD*orf*-1 plasmid had the structure expected, with the *Xho*I site lost in the latter provirus (Fig. 4). Thus, the cytopathic effect cannot be attributed to reversion of the transfected plasmids, and virus defective for production of the 27-kDa 3' *orf* product can replicate in and kill T4$^+$ cells.

In contrast to the accelerated replication noted for viruses that contained mutations within the 3' *orf* gene, the replication of viruses containing a deletion of the 3' end of the envelope gene was noticeably retarded (Fig. 5). Cocultivation of C8166-45 cells with Jurkat-*tat* III cells transfected





Immunofluorescence (%)

Control Cells	0	0	0	0	0	0	0
pHXBc2	0	0	8	31	57	91	95
pDorf-1	0	0	10	28	61	95	96
pDorf-2	0	0	10	30	58	92	98
pHXB-orf	0	0	0	12	33	65	87

Immunofluorescence (%)

Control Cells	0	0	0	0	0	0
pHXBc2	0	0	10	35	74	99
pDorf-1	0	0	12	39	81	98
pDorf-2	0	0	13	40	78	99
pHXB-orf	0	0	0	16	45	83

Syncytia

Control Cells	−	−	−	−	−	−	−
pHXBc2	−	−	+	+	+	+	+
pDorf-1	−	−	+	+	+	+	+
pDorf-2	−	−	+	+	+	+	+
pHXB-orf	−	−	−	+	+	+	+

Syncytia

Control Cells	−	−	−	−	−	−
pHXBc2	−	−	+	+	+	+
pDorf-1	−	−	+	+	+	+
pDorf-2	−	−	+	+	+	+
pHXB-orf	−	−	−	+	+	+

Reverse Transcriptase

Control Cells	−	−	−	−	−	−	−
pHXBc2	−	−	−	+	+	+	+
pDorf-1	−	−	−	+	+	+	+
pDorf-2	−	−	−	+	+	+	+
pHXB-orf	−	−	−	−	+	+	+

Reverse Transcriptase

Control Cells	−	−	−	−	−	−
pHXBc2	−	−	+	+	+	+
pDorf-1	−	−	+	+	+	+
pDorf-2	−	−	+	+	+	+
pHXB-orf	−	−	−	+	+	+

FIG. 2. Transfection of Jurkat-*tat* III cells with HTLV-III/LAV proviral clones and infection of C8166-45 cells by cocultivation. DNA transfection and cocultivation were done as described previously (26). Membrane immunofluorescence was determined as described previously (5) with a 1:8 dilution of RV119 AIDS patient serum and a 1:30 dilution of fluorescein isothiocyanate-conjugated goat anti-human immunoglobin (Cappel Laboratories). Reverse transcriptase levels were measured as described previously (17) by using oligo(dT)-poly(A) template-primer and magnesium cofactor. Data were obtained from Jurkat-*tat* III cultures (left) and C8166-45 cultures (right). The percentage of cells exhibiting virus-specific membrane fluorescence was determined by scoring a randomly selected field of 200 cells. Symbols: ●, pHXBc2; ▲, pHXB-*orf*; □, p*Dorf*-1; △, p*Dorf*-2; ○, p*Dsor*3.

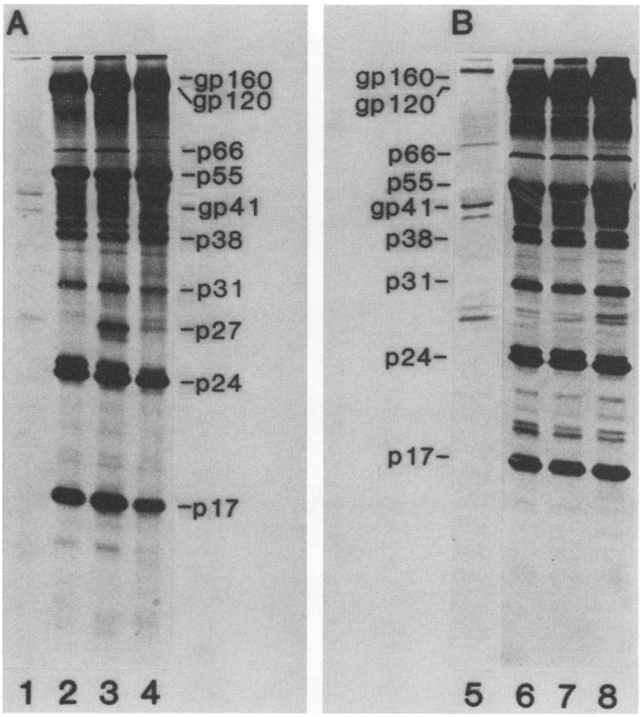


FIG. 3. Immunoprecipitation of Jurkat-*tat* III proteins. Jurkat-*tat* III cells transfected with pHXBc2 or altered proviral plasmids were labeled overnight at the time of maximum HTLV-III-specific membrane immunofluorescence with [^{35}S]cysteine as described previously (12). Labeled cell lysates were collected the next day and immunoprecipitated as described previously (12) with RV119 AIDS patient serum. Plasmids used for the transfections: (A) lane 1, pD*sor*-3; lane 2, pHXBc2; lane 3, pHXB-*orf*; lane 4, pD*orf*-3; (B) lane 5, pD*sor*-3; lane 6, pHXBc2; lane 7, pD*orf*-1; lane 8, pD*orf*-2.

with the pD*orf*-3 plasmid resulted in evidence of infection in the C8166-45 cells only in experiments in which cocultivation was initiated 8 days posttransfection, at a time when immunofluorescence was maximum in the Jurkat-*tat* III cells. No evidence of infection was obtained in the C8166-45 cultures when cocultivation was attempted 72 h following transfection of the Jurkat-*tat* III cells with this mutant plasmid. Infection of the C8166-45 cells with pD*orf*-3 occurred at a much slower rate in these experiments than was noted for either the virus that expressed the 3′ *orf* gene or viruses that contained mutations wholly within the 3′ *orf* gene.

It is also notable that the profile of ^{35}S-labeled proteins immunoprecipitated from extracts of cells infected with the pD*orf*-3 virus resembled that of cells infected with the pHXBc2 virus except in the region of the gp41 protein (Fig. 3A). gp41 was missing from the immunoprecipitated extract of pD*orf*-3-infected cells. The truncated protein encoded by this region in pD*orf*-3 was presumably comigrating with one of the several bands in the p38 region. Restriction enzyme analysis of proviral DNA isolated from the transfected cells confirmed that the replicating virus carried the pD*orf*-3 deletion (Fig. 4).

The experiments presented here indicate that the product of the 3′ *orf* gene is unnecessary for replication and cytopathic activity of the virus in T4$^+$ cell cultures. This result is puzzling, as this reading frame is conserved—except for the presence in some isolates of the stop codon at residue 122—in all HTLV-III/LAV strains investigated to date, even those in which the sequence diverges by more than 20% in some regions of the genome. Moreover, the 3′ *orf* protein is produced in the course of natural infections, as some AIDS patient antisera recognize the 27-kDa product of this gene (1). It is also noteworthy that a 27-kDa protein cross-reactive with the 3′ *orf* gene product is evident in extracts of cells infected with simian T-cell lymphotropic virus type III (2). These results extend the previous observation that the pHXBc2 plasmid that contains a termination codon can replicate by demonstrating that a virus that lacks almost the entire 3′ *orf* open reading frame can also replicate.

The conservation of the 3′ *orf* gene suggests that it plays an important role in the life cycle of the virus. Two possible functions for the gene may be envisioned. The 3′ *orf* gene may be required for the growth of HTLV-III/LAV in cell

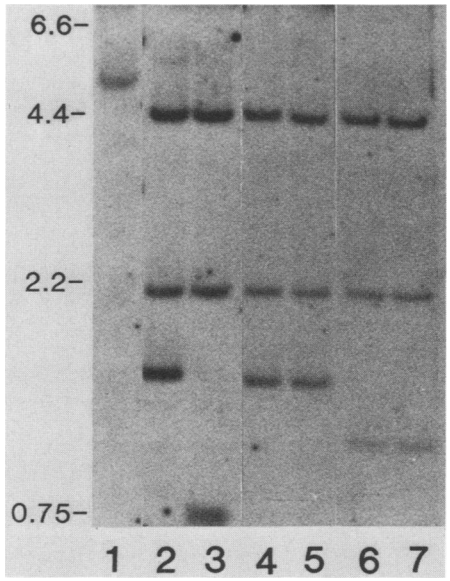
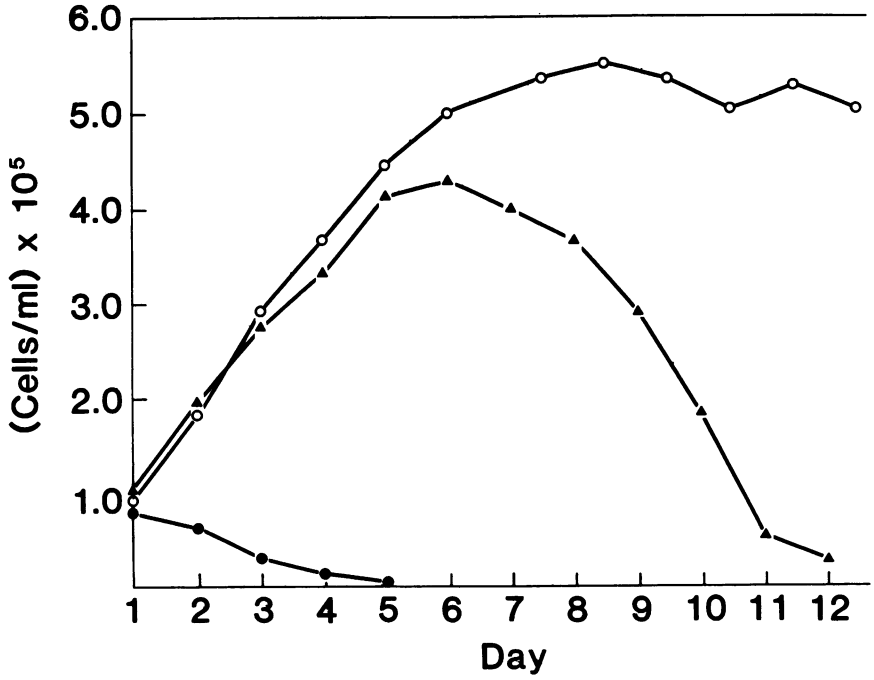


FIG. 4. Southern blot of high-molecular-weight DNA isolated from cultures of transfected Jurkat-*tat* III cells. Sizes (in kilobases) are indicated to the left. The DNA was collected from the cells at the time of maximum HTLV-III-specific membrane immunofluorescence. Portions (10 μg) of the DNAs were cut with restriction enzymes and blotted onto nitrocellulose paper as described previously (28). A pooled collection of gel-isolated *Bgl*II internal fragments derived from the pHXBc2 plasmid was used as a probe. The filter was washed for 2 h at 65°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) prior to autoradiography. pHXBc2-transfected cellular DNA was cut with *Xba*I (lane 1), *Hin*dIII (lane 2), or *Hin*dIII and *Xho*I (lane 3). *Xba*I did not cleave within the proviral DNA, and the only band observed corresponded to supercoiled proviral DNA. *Hin*dIII cleaved several times within the viral sequence to yield fragments of about 4, 2, and 1.5 kilobases (kb) from the pHXBc2 DNA. The *Xho*I site was contained within the 1.5-kb *Hin*dIII band, which disappeared when the DNA was cut with both *Hin*dIII and *Xho*I. Simultaneously a new band of about 700 bp appeared. pD*orf*-1-transfected cellular DNA was cut with *Hin*dIII (lane 4) or *Hin*dIII and *Xho*I (lane 5). The 1.5-kb band was present when the DNA was cut with *Hin*dIII but unchanged when the DNA was cut with both enzymes, confirming the absence of the *Xho*I site. pD*orf*-3-transfected cellular DNA was cut with *Hin*dIII (lane 6) or *Hin*dIII and *Xho*I (lane 7). In the *Hin*dIII-cut DNA the 1.5-kb band was no longer present. Instead there was a new band of about 1.0 kb, consistent with the large deletion contained within this fragment. The profile was unchanged when the DNA was cut with both *Hin*dIII and *Xho*I.



Immunofluorescence (%)

Control Cells	0	0	0	0	0	0	0	0	0	0	0	0
pHXBc2	17	32	56	84	99							
pDorf-3	0	0	0	0	0	10	22	41	67	86	91	95

Syncytia

Control Cells	−	−	−	−	−	−	−	−	−	−	−	−
pHXBc2	+	+	+	+	+							
pDorf-3	−	−	−	−	−	−	+	+	+	+	+	+

Reverse Transcriptase

Control Cells	−	−	−	−	−	−	−	−	−	−	−	−
pHXBc2	−	+	+	+	+							
pDorf-3	−	−	−	−	−	−	−	+	+	+	+	+

FIG. 5. Infection of C8166-45 cells with pHXBc2 or pD*orf*-3. DNA transfection of Jurkat-*tat* III cells and cocultivation with C8166-45 cells were performed as described previously (26), except that the cocultivation was not carried out until 8 days posttransfection. Membrane immunofluorescence and reverse transcriptase assays were carried out as described in the legend to Fig. 2. Symbols: ○, control cells; ●, pHXBc2; ▲, pD*orf*-3.

types other than $T4^+$ lymphocytes. Productive infection of monocytes and macrophages has recently been described (7a). HTLV-III/LAV has also been reported to propagate in the central nervous system (9, 16, 24). The 3′ *orf* gene may perform a function needed in these tissues. Alternatively, the 3′ *orf* gene may exert a regulatory function not readily measured in the assays used. The slightly retarded growth of the virus that expressed the 3′ *orf* gene compared with that of viruses carrying mutations within the 3′ *orf* gene raises the possibility that this gene acts to moderate the explosive replication of the virus, functioning as a repressor for specific virus functions. Others have reported that HTLV-III can establish a latent infection in lymphocytes, particularly in lymphocyte populations that have not been activated (30). It is possible that the 3′ *orf* gene plays a role in the establishment of such latent states.

The effects of the deletion that spans the 3′ end of the envelope gene and the 5′ end of the 3′ *orf* gene are probably

due to alterations in the *env* gene sequences, as viruses incapable of producing the 3′ *orf* gene product do not exhibit the retarded replication rate characteristic of this mutation. The deletion in pD*orf*-3 does not extend into the sequences encoding the *art* protein. The mutant virus also retains its cytopathogenic potential in both of the $T4^+$ indicator cell lines used. Nonetheless, sequences important for virus replication in $T4^+$ cells, possibly those that encode the 3′ end of the envelope gene, appear to be damaged by this mutation. One interpretation of these results is that the carboxy terminus of the *env* gene plays an important role in replication. This possibility is particularly intriguing, as HTLV-III and visna virus are unique in the exceptionally large size of their transmembrane envelope proteins (27).

We thank Wei Chun Goh and Kathleen Campbell for helpful discussion and Robert Gallo and Flossie Wong-Staal for the pHXBc2 plasmid.

The work was supported by grants to W.A.H. from the American Cancer Society and the State of Massachusetts. E.T. was supported by a National Institutes of Health Viral Oncology Training Grant. J.G.S. is a Special Fellow of the Leukemia Society of America and is the recipient of Public Health Service grants CA-40658 and CA-42597 from the National Institutes of Health. C.A.R. is the recipient of a postdoctoral fellowship from the National Institutes of Health (CA-07580).

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