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MECHANISM OF CYTOTOXICITY OF THE AIDS VIRUS, HTLV-III/LAV

ANNUAL REPORT

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Understanding the mechanism of human immunodeficiency virus (HIV) type 1 replication and cytopathicity is critical to determining the pathogenesis and treatment of the acquired immunodeficiency syndrome (AIDS) and associated diseases. Studies of related viruses, simian immunodeficiency virus (SIV) and HIV-1, complement these studies and allow additional animal model systems for testing hypothesis. We have studied three aspects of HIV pathogenesis. First, a mutagenic analysis of HIV-1, SIV, and HIV-2 genes (including gag, env, vif, vpr, nef, and vpx) has been performed to decifer their role in replication and cytopathic activity. Second, immunopathogenetic consequences have been studied with respect to the characterization of a lymphotoxin-like molecule which is secreted by HIV-1 infected cells. Third, the structure and function of HIV-1 env variation has been stuided in a clustered outbreak of HIV-1 infection to elucidate the role of envelope alterations in immune evasion and tissue tropism.

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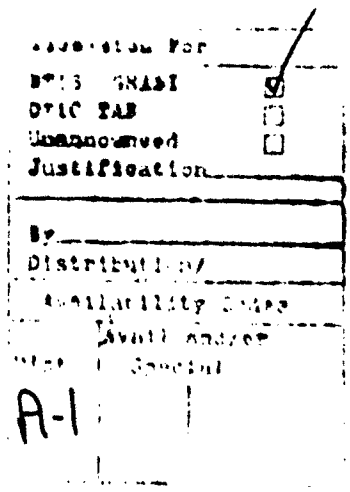
Foreword

1) Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

2) For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46.

3) The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.



Background and Significance

Human immunodeficiency virus (HIV) type 1 is a complex virus with at least three structural and five regulatory genes (1,2). The structural genes include gag, pol, and env. The 53 kilodalton (kd) gag precursor protein is encoded by the 9.0 kb full-length viral mRNA. It is cleaved by the viral protease to a 17 kd myristoylated protein, a 24 kd phosphorylated major capsid protein, a 9 kd nucleic acid binding protein, and a 7 kd proline-rich protein. A 180 kd gag-pol precursor is synthesized from the same mRNA by ribosome frame-shifting occurring in the region of overlap between the gag and pol genes. The pol proteins processed from this polyprotein include a 10 kd aspartyl protease, a 55 kd reverse transcriptase, a 61 kd reverse transcriptase-RNase H, and a 32 kd endonuclease or integrase. The 160 kd envelope protein is encoded from a 4.5 kilobase (kb) spliced mRNA. The primary translation product is 863 amino acids long, including the first 30 amino acid signal peptide which is cleaved off. It is therefore estimated that this glycoprotein is 40% carbohydrate. The envelope precursor is processed by a cellular protease to a 120 kd extra-cellular envelope protein with 481 amino acids, and a 41 kd transmembrane protein with 345 amino acids.

At least five genes encode regulatory proteins. The transactivator protein, tat, gene is encoded by a double or triple spliced 2.0 kb mRNA. It is a 14 kd protein which is both highly basic and includes an array of cysteine and histidine residues characteristic of "zinc fingers." It interacts with the tat-responsive region, tar, localized between nucleotide 1 and 34 within the R region of the long terminal repeat sequences (LTR) and/or the mRNA (3). Tat increases gene expression about 1000-fold. However, its mechanism of action is not clearly defined, and may include effects on the initiation or elongation of transcription, RNA stability or processing effects, and/or effects on translation (4,5).

The regulator of virion protein production (REV) is an 18 kd protein encoded from a 2.0 kb mRNA. It acts as a strong positive-feedback regulator of virus expression increasing protein synthesis about 100-fold. Its mechanism of action is not understood.

The virion infectivity factor gene (vif) is likely encoded by a 5.0 kb mRNA. It is translated into a 23 kd protein. This protein acts at a post-translational step to markedly increase the infectivity of the virus particle (6).

The negative factor gene (nef) is encoded by a double or triple spliced 2.0 kb mRNA. It encodes the synthesis of a myristylated and phosphorylated 27 kd protein (7). This protein acts as a negative regulator of virus replication (8).

The mechanism of expression of the vpr gene product is not well understood (9). The function of this protein is not yet defined.

Lastly additional potential genes exist, including one designated vpu which may be encoded from the minus strand of the proviral DNA, and another which may be encoded from the plus strand of the proviral DNA (10).

The mechanism of cell killing by HIV-1 is likely a critical event in the development of immunosuppression in vivo. Indirect experimental data suggests an important role for both the HIV-1 envelope and the cellular T4 antigen. The extracellular envelope domain is critical for interactions with T4 and the formation of syncytia (11). The transmembrane domain serves to anchor the extracellular envelope on the infected cell or virion, as well to have a role in cell fusion, and a separate role in cell killing. The latter activity determined by the carboxyl terminal domain of the transmembrane envelope protein has been explored in these studies (12). The possible role of interactions of T4 and envelope independent of syncytia formation have also been studied here with respect to their role in cell killing. The development of new assays for cell killing independent of virus replication have been critical to these studies.

The role of the cellular gene products in cell killing also remains to be determined. Though viral products might cause direct lysis of infected cells through membrane effects, other mechanisms are more consistent with experimental data. Induction of cytopathic cellular proteins may explain the cytopathic effects of the virus and are likely to be more consistent with data obtained from both in vitro and in vivo studies (13). Several of these possible mechanisms have been explored in the studies outlined below.

Lastly the interaction of the virus and the immune system is critical to the pathogenesis of HIV-1 infections in vivo, and to the development of vaccines for this disease. It is clear that HIV-1's are a heterogeneous collection of related viruses, which differ more in the extracellular envelope product than other viral proteins (14). However, the functional signficance of envelope sequence variation with respect to rate of virus replication, infectivity, tissue tropism, responsiveness to neutralizing antibodies and other immune responses remains to be determined.

Experimental Methods and Results

Analysis of HIV-1 Replication and Cytopathicity by Mutagenesis

Gag

We have been interested in the role of gag p17 myristylation. In the cases of Moloney murine leukemia virus and Mason Pfizer monkey virus, alteration of the myristoylation acceptor site in gag leads to a marked decrease in virus replication, and loss of assembly and release of mature virus particles (15,16). It is likely that myristylation of gag p17 is critical to HIV-1 replication and that methods of inhibiting myristylation in vivo may provide anti-viral therapies. Thus, to test the role of gag myristoylation in HIV-1 replication, we have constructed an M13 clone with this portion of the HIV-1 genome. It has been mutagenized by the method of Kunkel (17), to substitute the glycine codon at the second codon positior of gag to an alanine. The resultant mutant fails to give rise to infectious virus particles. The mechanism of this effect was studied. We have demonstrated that myristylation is required for tight association with the membrane of the gag precursor and for subsequent proteolytic cleavage

Furthermore, we have demonstrated that heteroatom substituted analogues of myristic acid block HIV-1 expression ~~(Appendix~~ 6) and gag precursor processing. Mechanistic studies have been explored in model systems that we developed or adapted utilizing 1) acute HIV-1 infection of lymphoid cells, 2) chronically infected lymphoid cells, 3) recombinant vaccinia gag-pol expression viruses, and 4) bacterial expression systems in the presence of N-myristoyl transferase expression.

Env

We have begun three types of analyses of specific domains of the envelope gene. The first set of studies examine the role of the carboxyl terminal domain of gp41 in virus replication and cytopathicity. Two previously constructed mutants demonstrated virus replication in the absence of detectable cytopathic effects (12). The low percentage (1-2%) of syncytia formed in cultures infected by these viruses was similar to that found in cultures infected by the parental viruses. These clones were designated X10-1 which had a deletion of 5 amino acids of envelope replaced by 15 amino acids, and X9-3 which had a deletion of 5 amino acids of envelope replaced by 153 amino acids. A clone with a large deletion of env (about 50 amino acids), designated delta-E demonstrated greatly attenuated virus replication. The reason for the diminished cytopathicity of these

viruses was not related to an alteration of interaction with T4 as demonstrated by the formation of syncytia using a number of different lymphoid cell types. A defect in envelope protein synthesis or processing was also not detected as demonstrated by immunoprecipitation analysis of 35S-methionine labeled cultures infected with these virus strains. Thus, we felt that it is likely that the carboxyl terminus of gp41 has a separate enzymatic function separate from the ability of envelope to interact with T4 or form syncytia, and that this activity was critical for cytopathicity.

Though T4-envelope interactions were shown not to be sufficient for this cytopathic activity, it could not be ruled out that such interactions are necessary for the activity. We next asked whether envelope-T4 interactions may occur on the same cell, since these interactions may be critical to cytopathic effects. Thus, we asked whether capping either envelope or T4 using monoclonal antibodies and cross-linking them with a second antibody, led to internalization of the other protein. For this purpose, we have determined the concentration dependence of antibody binding to these proteins to obtain saturating levels. These concentrations of antibodies are in use in the co-modulation experiments at this time.

We also asked what portions of the carboxyl terminus of gp41 were important for virus replication and cytopathic effects. Thus, additional mutants have been constructed by Bal 31 nuclease treatment as previously described with or without the addition of a termination codon Xba I linker, their structure determined, and their function studied after transfection of COS-1 cells and cocultivation with MOLT 3 or H9 cells Several interesting phenotypes are readily apparent. First, large deletions or additions (>10 amino acids) to gp41 lead to greatly attenuated virus replication in both Molt 3 and H9 cells. Several viruses, however, replicate well in H9 cells but not Molt 3 cells. Thus, differing modes of transmission, i.e. cell-to-cell versus free virus transmission, may be dependent on the conformation of gp41. All of the mutants with alterations of TM demonstrated reduced cytopathic activity

We have also constructed mutants in the N-terminal domain of envelope. These utilized Bal 31 deletions from a unique Nde I site in a vif-minus clone, dF/R (6). The structure of each mutant was determined by nucleotide sequencing, and the data is summarized in Fig. 1 (Appendix). Notably, 3 clones have in-frame deletions in envelope. Clone dF/R/E79 has a deletion of amino acides 10-102, and thus a loss of most of the signal peptide. Clones dF/R/E-22 and dF/R/E-76 have slightly different deletions of the first conserved domain without affecting the signal peptide, including amino acids 36-105 and 35-107, respectively. Other clones include frameshifts and deletions (dF/R/E-7, 10, 19,

70, 82, 87, 92, 100, and 122). Versions of these proviral clones in which vif was replaced, dE-76, dE-22, dE-19, and dE-10 were also analyzed. All of these clones produced non-infectious virus particles without envelope (Fig. 2 & 3, ~~Appendix 1~~). Sucrose gradient analysis demonstrated particles with a density of 1.12-1.14 which are trypsin-resistant but triton-sensitive, suggesting that the nucleocapsid is surrounded by a lipid membrane.

We have also analyzed two mechanisms of cell killing (single-cell killing and fusion) using viral mutants which separate these phenomena. A series of site-directed mutations in proviral clones have been constructed in the portion of env encoding the N-terminus of TM (Fig. 2, ~~Appendix 1~~). These include conservative amino acid substitutions, deletions, and insertions. Detailed studies have been performed with FI3, FI6, FI6B, and FI6D. In addition, Cys to Ser mutations have been constructed in the two well-conserved Cys residues, and Asn to Gln mutations in each of the potential N-linked oligosaccharide acceptor residues. Lastly, a mutation has been constructed in the ORF located on the oppostive DNA strand overlapping this region to rule out effects of the mutations on this potential product. Furthermore, the env gene of each of these mutants has been cloned into vaccinia virus for studies of envelope structure and function separate from other viral proteins.

The insertion mutants FI6B and FI6D are non-infectious, whereas FI3 and FI6 produced virus with mild or marked decreases in infectivity, respectively. Mutant FI6 is also markedly deficient in syncytia production both during acute infection assays, and upon cocultivating uninfected lymphocytes with chronically infected H9 cells. This suggests that virus-cell and cell-cell fusion are mechanistically similar. The alteration in the FI6 envelope function is due to qualitative not quantitative abnormalities in expression. In both HIV-1 and recombinant vaccinia virus infected cells, the FI6 and parental envelope proteins are synthesized, processed, and transported to the cell surface at a similar rate. These findings are based on pulse-chase studies with 35S-methionine and cysteine radiolabeling as well as studies of surface iodinated proteins. Despite these findings, when equivalent amounts of infectious particles of FI6 and parental virus are used, no difference is seen in replication rate or cell killing in a variety of cell types. These findings suggest that single cell killing is a predominant mechanism of cell killing which is at least partially mechanistically separable from fusion mechanisms.

Studies of the TM Cys mutations have shown that neither clone is able to give rise to infectious virus. Studies of the TM N-glycosylation mutants have shown no, minimal, or moderate impairment in virus infectiivity in each of the three cases. The nature of these defects is under analysis.

In additional studies of HIV-1 cytopathic effects, we

SIV-specific mRNAs in the absence of nef (Fig. 7), and nuclear run-off assays confirmed that the effect was on RNA initiation (Fig. 8). No effects were seen on RNA stability in actinomycin D treated cells (Fig. 9). Radioimmunoprecipitation analysis confirmed the synthesis of a 31 kd NEF protein from SIVmac-102 but not SIVmac-BA transfected cells, identical in size to the protein expressed from cells transfected with a SIVmac-nef expression plasmid (Fig. 10). Myristic acid labeling has been performed to further confirm the identity of the immunoprecipitated product.

We have also constructed a nef- and nef+ pair of clones of HIV-2-ROD which behave as do the SIVmac-BA and -102 clones. Furthermore, we have constructed a variant from the pathogenic SIVmac-239 clone in which we have corrected the nef mutation, and are examining its replication potential.

A myristylation minus mutant of nef has also been obtained by site directed mutagenesis for analysis of the role of this post-translational modification in nef activity. No effect is seen on NEF, GAG, or ENV protein synthesis or stability. Effects on phosphorylation, glycosylation, membrane binding, and cell release are under investigation. Furthermore, we have constructed 4 mutants in the presumptive GTP binding site of NEF, in the phosphorylation acceptor site at position 15, and 5 additional sites predicted to affect GTPase activity.

Vpx

HIV-2 and SIV differ from HIV-1 in that they contain an open reading frame encoding a 14 kd protein designated vpx (18). We have constructed functional clones of both HIV-2 and SIV, as demonstrated by the production of syncytia and reverse transcriptase in transfected H9 cells and in the case of HIV-2 in transfected CEM cells as well. Three mutations have been constructed in the vpx gene of HIV-2 (Appendix 4). These mutations do not affect the infectivity, replication rate, or cytopathicity of vpx in a wide range of lymphoid and monocytoid cell lines and primary human lymphocytes and monocytes. However, in a preliminary experiment differences in viral GAG and ENV protein production were seen in primary lymphocytes infected with vpx-mutant and parental viruses. This effect of vpx is being further evaluated.

Interaction of HIV-1 and the Immune System

We have undertaken two types of studies of the interaction of HIV-1 and the immune system. First, we are establishing a murine model for study of cytotoxic responses to HIV-1. For this purpose we have established an AKR-2B murine cell line expressing the HIV-1 envelope. This was performed by transfecting an env expression clone with a selectable marker into these cells, and selecting a clone with expressed

have established a new assay system for measuring cytopathic effects in the absence of virus replication. For this purpose we have constructed a T4 expressing HOS cell line, and have been provided the T4 expressing HeLa cell line constructed by Maddon and colleagues. Transfection of HIV-1 DNA clones which also contain the xanthine-guanine phosphoribosyltransferase gene into T4 negative HOS or HeLa cell lines led to the production of only 3-fold fewer gpt+ clones in the presence of mycophenolic acid than did the vector lacking HIV-1 sequences. We are currently assaying a number of gpt+ clones obtained after transfection of T4 positive cell lines as a measure of cytotoxicity from either full proviral clones or partial proviral clones. The transfection efficiency in this assay is standardized by measuring both the level of transfected DNA by slot blot hybridization and the level of expression of HIV-1 envelope by FACS.

Vpr

We have constructed 4 mutants in the vpr gene. These are summarized in Appendix 2. Virus has been obtained from each DNA clone. The R40 virus has been extensively studied, and found to replicate and kill lymphocyte similar to the parental virus. Cell lines used for these analyses included normal peripheral blood mononuclear cells and monocytes, as well as several lymphoid cell lines (Sup T1, CEM, Molt 3, HUT 102) and a monocytoid cell line (U937). Data with R31, R21, and R2 demonstrate that these clones also give rise to functional virus. These data would suggest that vpr is not critical to HIV-1 replication and cytopathicity in vitro. No difference in replication, infectivity, or cytopathicity was detected in CEM, Molt 3, or H9 cells. A vpr mutant was also constructed in HIV-2. This clone showed similar infectivity, replication, and cytopathicity in CEM, U937, and Sup T1 cells, as well as primary human lymphocytes. Recent data with MT4 cells, however, demonstrates impaired infectivity of the vpr mutant. The role of vpr in vivo remains to be determined.

We have raised a rabbit antiserum to the first 30 amino acids of the VPR product. Furthermore, we have obtained a goat antiserum to a recombinant HIV-1 VPR and a goat antiserum to SIV mac VPR from Dr. T-H. Lee (Harvard), and a rabbit antiserum to a recombinant form of HIV-1 VPR from Dr. J. Ghrayeb (Centocor). Experiments are underway to assess the ability of each antiserum to detect E. coli expressed VPR products and VPR products expressed in the presence or absence of other viral proteins in eukaryotic cells.

Furthermore, we have constructed a number of proviral clones to test the role of vpr in infection and replication assays in monocytes. These include a functional HIV-2-ROD clone, and monocytpe-tropic clones of HIV-1 including

HIV-1-JR-CSF (provided by I. Chen, UCLA), HIV-1-Yu-2 (provided by G. Shaw), HIV-1-ADA-M (Westervelt, Gendelman, & Ratner, manuscript in preparation), and HIV-1-JFBR (Appendix 7).

Nef

Our previous studies with mutants in the nef gene demonstrated that nef was not required for virus replication or cytopathic effects (12). We have now analyzed more critically a clone with an intact F gene, pHIV-F+, and one with a deletion in nef, pHIV-F- (Appendix 5). Transfection of these clones into several lymphoid cell lines, including Jurkat, H9, CEM, and Molt 3 demonstrated that virus derived from pHIV-F- replicated 10-30-fold faster than virus derived from the pHIV-F+ DNA clone over 20 days. Down-regulation of T4 was more notable in the pHIV-F- infected cells compared to pHIV-F+ infected cells, contrary to the predictions of Guy and colleagues who showed that overexpression of nef led to depression of T4 (7). Mixing cells infected with the F- and F+ virus gave a phenotype consistent with F- infected cultures, demonstrating that a factor down-regulating HIV-1 replication was not secreted from F+ infected cells. Transfection of the clones into COS-1 cells, similarly demonstrated a 3-5-fold difference in virus production as measured by reverse transcriptase or p24 antigen assays in a single cycle of virus replication. Similar results were obtained with HeLa and SW480 colon carcinoma cells. F- and F+ virus derived from COS-1 cells showed similar infectivity on H9 cells as measured by the production of viral DNA within 12 hrs after infection. We have demonstrated that nef is a trans-activating factor. Cell co-transfected with a nef- HIV-1 DNA clone and a nef expression plasmid have the same phentoype as nef+ transfected cells. Thus, these data demonstrate that the effects of nef in down-regulating virus production occur exclusively in the latter half of the virus replication cycle. Studies of gag p24 antigen production by immunoprecipitation studies of F- and F+ infected COS-1 cells again revealed a 3-5-fold difference, suggesting that nef operated at a translational or pre-translational step. Studies of HIV-LTR-CAT assays in the presence or absence of tat with or without nef demonstrated downregulation of LTR directed gene expression. The effects of nef have been localized to the level of transcription by Northern blot analysis and nuclear run-off experiments.

Similar analyses have been performed with SIVmac clone 102 (derived from SIVmac251 infected cells), and a nef-mutant clone SIV-BA constructed with a termination codon mutation at amino acid position 40 (Fig. 5). In each case, SIV antigen production in the medium was 2-5-fold higher from cells transfected with the nef mutant than the parental clone (Fig. 6). Northern blot analysis confirmed a higher level of

the HIV-1 envelope as detected by immunofluorescence studies with a specific antibody. These cells are being used as a target to study the cytotoxic responses of vaccinia-env vaccinated mice in collaboration with Dr. R. Markham (Johns Hopkins University).

Second, we have studied a clustered outbreak of HIV-1 infection in 3 children who received HIV-1 contaminated blood via transfusion from an asymptomatic HIV-1 infected donor (Appendix 7). We have cultured virus from the blood donor and three of the recipients. All three patients have now developed AIDS and two patients have died of their disease. Interestingly, the virus isolates from the lung and brain were capable of infection and replication in primary monocytes, whereas those from the blood failed to grow normal monocytes. DNA has been prepared from cultures infected with isolates from each of the patients, and libraries have been screened for HIV-1 env clones from blood isolates from these patients. Thirteen clones have been obtained and sequenced from isolates of brain, blood, and lung. We have detected only 2 amino acid changes in envelope, among the clones. No differences were identified in nef and LTR sequences among isolates from brain or blood. These data suggest that sequence heterogeneity is more restricted than that described in the literature. Second, these data suggest that monocyte-tropism is likely not to be based exclusively on sequences in env, nef, or LTR.

To further elucidate the basis for monocyte tropism, we have in collaboration with Dr. Howard Gendelman (WRAIR) obtained a 3' proviral clone from a monocyte tropic clone, designated ADA-M. The complete sequence of env, nef, and LTR has been determined. Genomic libraries have been constructed with DNA from the lymphocyte tropic virus counterpart of ADA.

To assess the biological basis of monocyte tropism, two studies have been initiated. First, we have cloned the LTR from the clone from the brain isolate, and the LTR of the ADA monocyte-tropic isolate 5' to the CAT gene. We are currently establishing the most efficient method of transfection of primary monocytes to examine whether tissue tropism may be due to LTR activity. Second, we have constructed chimeric proviruses between HXB2 (lymphocyte-tropic) and either env-nef-LTR of the clone of the brain isolate, env of the clone of brain isolate, or a full proviral clone from the brain isolate. For this purpose, two termination codons in env are being corrected to trp by site directed mutagenesis. Chimeric proviral clones have also been constructed with HXB2 using the env-nef-LTR sequence of ADA monocyte tropic clone. We have found that a determinant within envelope including the CD4 binding region is critical for monocyte infection. However, virus was detectable only upon cocultivation of infected monocytes with primary lymphocytes. In contrast, a molecular clone, Yu-2 (provided by G. Shaw) shows high level virus replication. Further chimeric clones have been

constructed between HXB2, Yu-2, and ADA-M to decipher the minimal sequences required for infection of monocytes, and to identify sequences required for high level virus production from monocytes.

Conclusions

Work performed during the last year under this contract has better defined viral determinants, cellular determinants, and interactions of viral and cellular determinants in HIV-1 induced cytopathicity.

In studies of viral determinants of HIV-1 replication and cytopathicity, we have produced a number of mutants to better characterize the function of particular gene products. A myristylation acceptor mutant of gag has demonstrated the requirement of myristoylation for membrane association of the gag precursor, proteolytic cleavage, and infectious virus production. Similar results have now been obtained with sulfur and oxygen substituted analogs of myristic acid.

We have constructed a number of mutants with an altered carboxyl terminus of gp41. These have demonstrated that additions or deletions of more than 15 amino acids attenuates infectivity and/or replication. Replication of certain mutants occurred in H9 cells but not Molt 3 cells suggesting that a region within the carboxyl terminus of gp41 may be critical to cell-to-cell transmission of virus but not transmission via free virus particles. Several of these mutants should be useful for characterizing regions in the carboxyl terminus of gp41 which affect cytopathicity without affecting virus replication. A number of mutations in the first conserved domain of gp120 have also been produced, which give rise to non-infectious virus particles, demonstrating that envelope-free virus particles can be produced. These particles may be useful as vaccine preparations. We have also constructed interesting mutations in the TM domain of envelope which dissect different mechanisms of cell killing. Interactions of envelope with T4 may be critical for cytopathicity. We have determined experimental conditions which are appropriate to study whether co-modulation of T4 and env occur with cross-linking antibodies which would suggest an interaction on the same cell. We have also established an assay for cytopathicity independent of virus replication using sub-genomic proviral clones transfected into HeLa or HOS cells expressing T4. This assay should be useful in defining viral determinants of cell killing and their mechanism of action.

Studies of vif have demonstrated that it acts at a post-translational level to increase the infectivity of the virus particle. A number of vpr mutants have also been constructed. Our data suggests that they all give rise to virus which replicates and kills lymphoid cells similar to

the parental virus. However, in MT4 cells, vpr is contributes to the infectivity of the virus.

Studies of nef have demonstrated that it is negative a regulator of virus replication acting at the level of viral RNA synthesis for both HIV and SIVmac. Additional studies should better characterize its mechanism of action. Site directed mutants in the myristylation acceptor codon, phosphorylation acceptor codon, and presumptive GTP binding and GTPase domains of nef have been constructed to assess the role of these activities in regulation of HIV-1 replication by nef.

HIV-2 and SIV have an additional gene, designated vpx. We have constructed several HIV-2 and SIV functional clones to test the effect of mutation of the vpx gene. We have constructed three HIV-2 X mutants. No alterations in infectivity, replication, or cytopathicity has been detected.

To examine interactions of viral and cellular determinants of cytopathicity, we have focused on the significance of HIV-1 envelope sequence heterogeneity. We have isolated virus from 3 children who were infected from a single blood donor. Virus was isolated at different times and from different tissues. Interestingly, virus isolates from the lung and brain of one child was monocyte-tropic whereas that isolated from the blood grew poorly in monocytes. Env clones have been obtained from lung and blood isolates from one child, lung, brain, and blood isolates of a second child, and from two blood isolates of the donor. Sequencing of 13 clones from these seven isolates has demonstrates <0.02% amino acid variation in envelope. The validity of these data have been confirmed by sequence analysis of sequences obtained by PCR directly from fresh tissue. Furthermore, no differences in env, nef, or LTR are apparent to explain monocyte tropism of certain isolates. A clone has also been obtained from the monocyte tropic ADA isolate and sequenced. LTR CAT clones have been constructed as well as chimeric proviral clones between clones from lymphocyte-tropic and monocyte-tropic clones. A determinant within the HIV-1 gp120 envelope protein was found to be critical for monocyte infection.

Recommendations

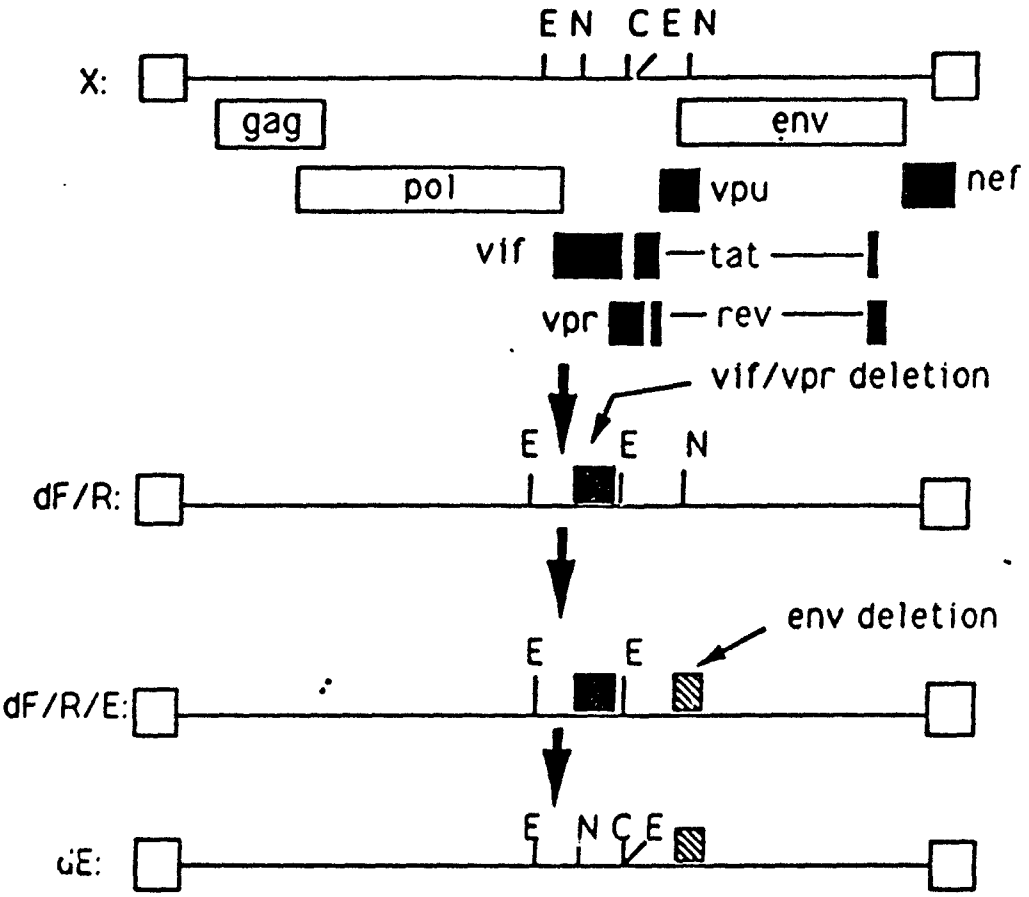
We recommend that in the last 3 months of this contract we complete the studies of SIV nef. We further recommend at the completion of this contract in 3 months that several studies by pursued. The interesting work on gag and env determinants required for virus assembly, replication in lymphocytes and monocytes, and cytopathicity will be continued through alternative funding sources.

We recommend that work supported under the continuation of this contract focus on regulatory gene function, vpr, nef, and vpx. These studies are outlined in detail in the

enclosed copy of the continuation application that is awaiting funding (Appendix 10). These studies are concerned with structure-function analyses of these regulatory proteins in HIV-1, HIV-2, and/or SIVmac, including both tissue culture and animal model system studies.

Fig. 1

Envelope Deletion Clones of HIV-1



Clone	Frame-shift	Deleted Nt	Number Deleted Nt	Amino Acids Deleted	Number Amino Acids Deleted	Intact Signal Peptide	Mature Envelope Product
X	-	-	-	-	-	+	
22	-	5907-6116	210	36-105	70	+	TEKLW/EDI...
76	-	5904-6122	219	35-107	73	+	TEKL / I...
79	+	5829-6107	279	10-102	93	-	
7	+	5853-6133	281				
10	+	5867-6134	268				
19	+	5885-6125	241				
70	+	5912-6086	175				
82	+	5910-6136	227				
87	+	5833-6116	274				
92	+	5910-6125	216				
100	+	5832-6114	283				
122	+	5811-6118	308				

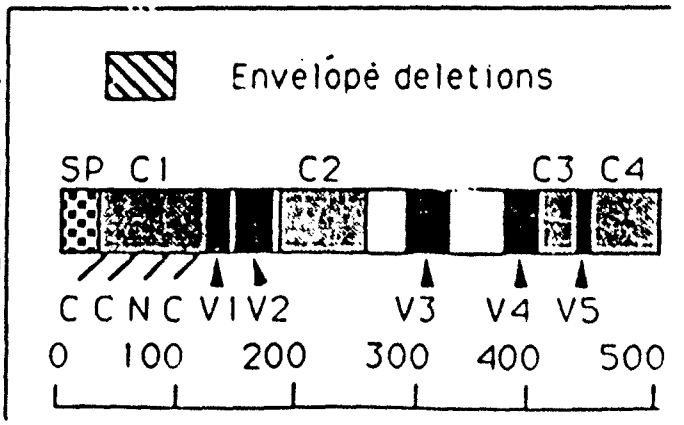
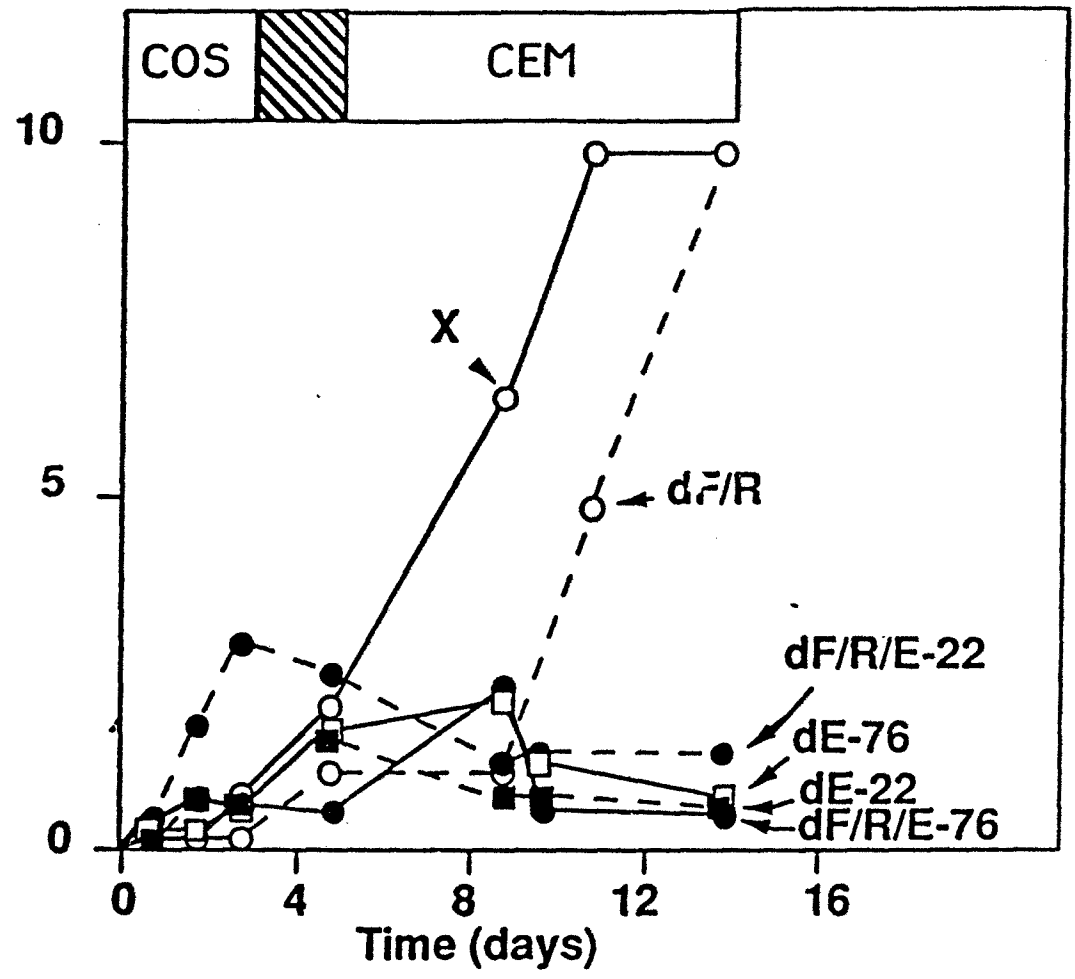


Fig. 2

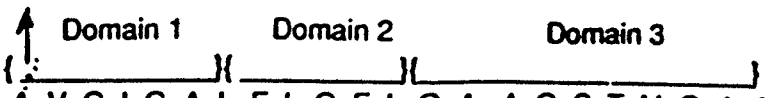
a)



b) p24 Antigen Production after Cocultivation with H9 Cells

Clone	p24 Antigen (ng/ml)
X	17.7
dF/R	0
dE-22	0
dF/R/E-22	0
dE-76	0
dF/R/E-76	0
dE-19	0.1
dF/R/E-19	0.2

RM4 L substitution mutant Fig. 4
FA3 G



A V G I G A L F L G F L G A A G S T M G A A S M

Substitution

normal infectivity F2 P
F4 P
F7 P
F8 I
F9 P
F10 V
F11 A
F19 P

loss of infectivity R2 R
R9 R
R15 R
FA1 W G
FA2 P G
FI2 A G
FPP P P

A V G I G A L F L G F L G A A G S T M G A A S M

FI3B A G F
FI3C A G I
FI3D A G I
FI3E A G I

DELETIONS

loss of infectivity - DEL2 V G I
DEL3 L F L
DEL4 V G I G A

INSERTIONS AT AMINO-TERMINUS

FI3A A V G
FI6 A G V A G A
FI6B A G A A A A
FI6D A T A G T M

Amino-terminus of the TM protein (shown at top with domains delineated) with the mutations made shown below.

Rotein Project Transfect cos-7, Assay gp120

?Alterations of Cleavage, Syncytia, Infectivity

?cos-7 ≠ CD4

Fig. 5

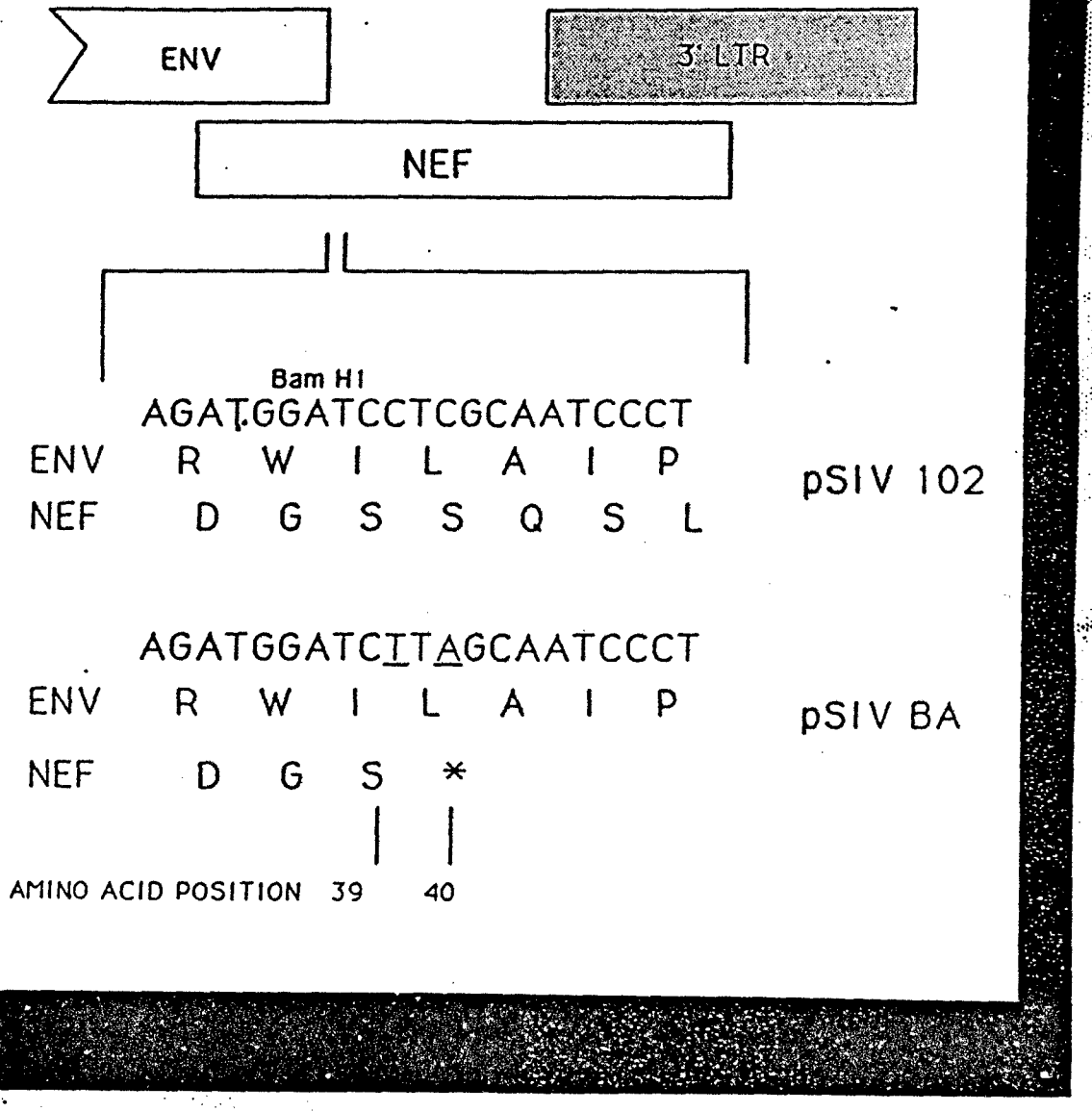

ENV
3' LTR
NEF
Bam HI
AGATGGATCCTCGCAATCCCT
ENV R W I L A I P
NEF D G S S Q S L
pSIV 102
AGATGGATCITAGCAATCCCT
ENV R W I L A I P
NEF D G S *
pSIV BA
AMINO ACID POSITION 39 40

Fig. 6

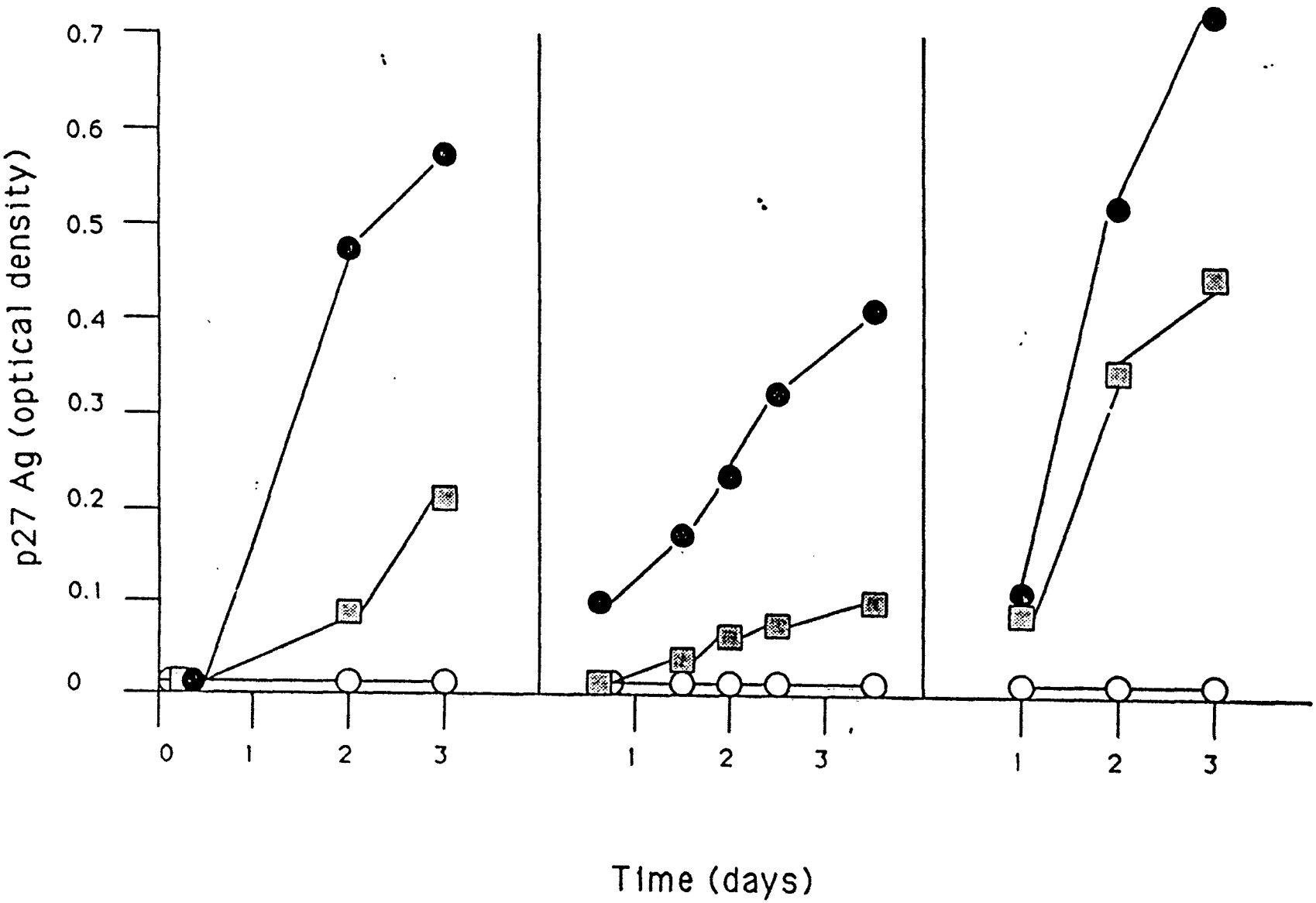

p27 Ag (optical density)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
1
2
3
1
2
3
Time (days)

Fig.. 7

SIV NEF DEPRESSES VIRAL mRNA ACCUMULATION

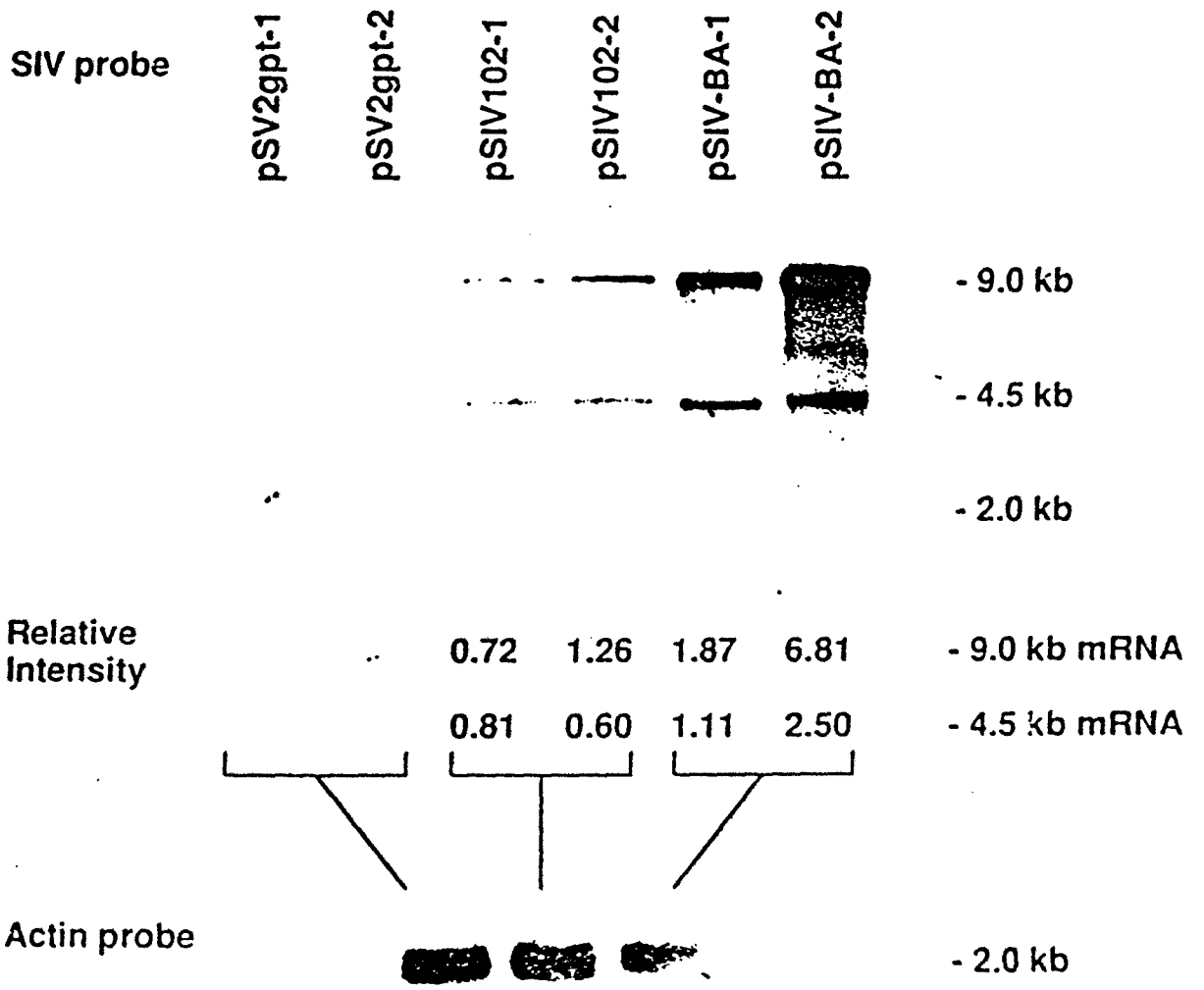


Fig. 8

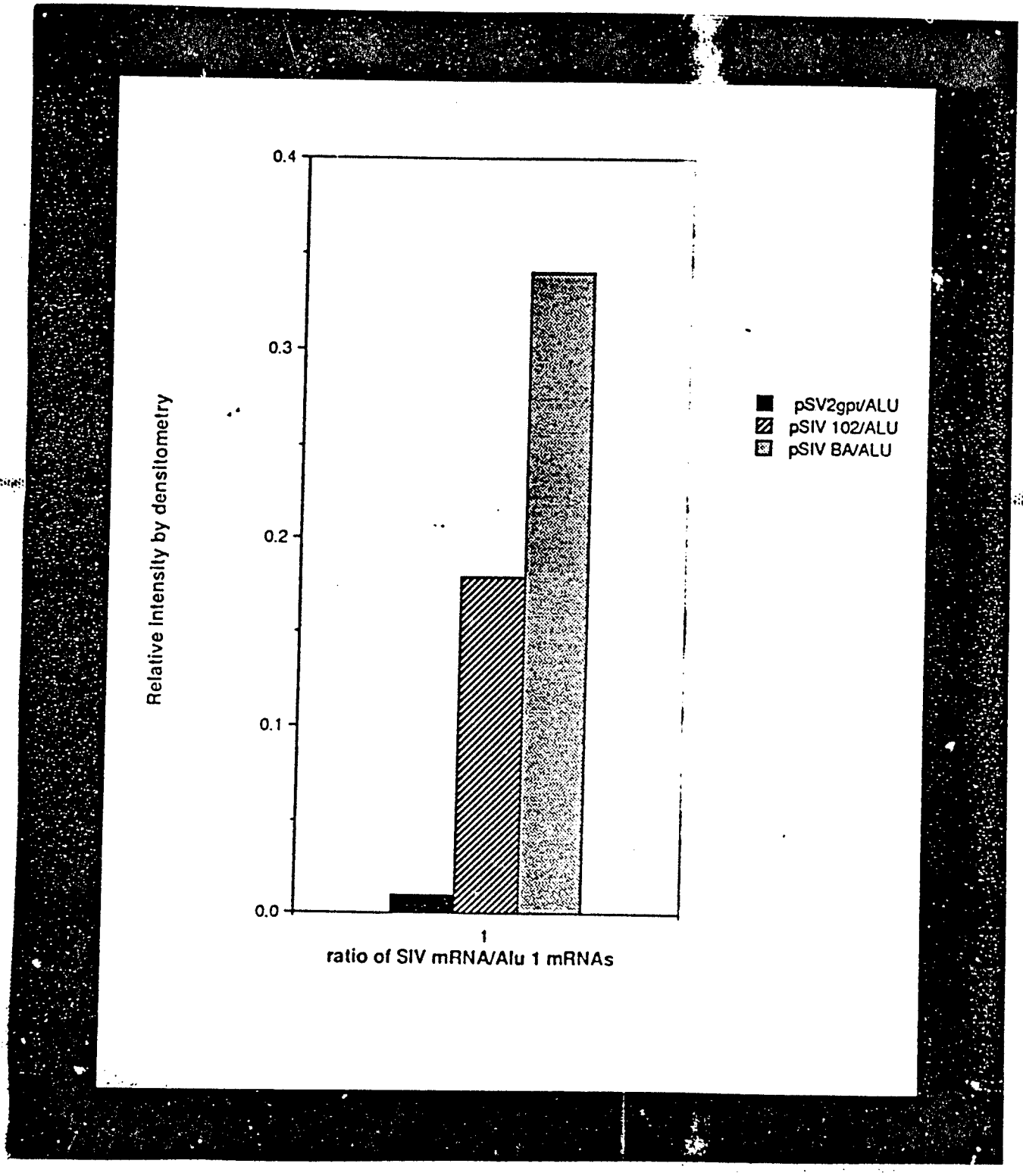
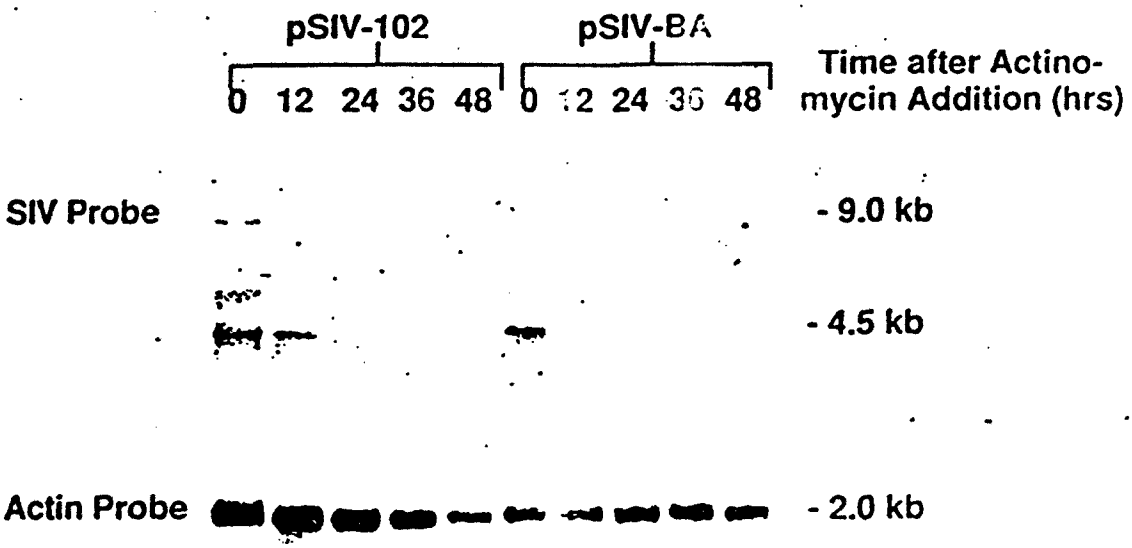

0.4
0.3
0.2
0.1
0.0
Relative Intensity by densitometry
pSV2gpt/ALU
pSIV 102/ALU
pSIV BA/ALU
1
ratio of SIV mRNA/Alu 1 mRNAs

Fig. 9

NEF DOES NOT DESTABILIZE SIV MRNAs



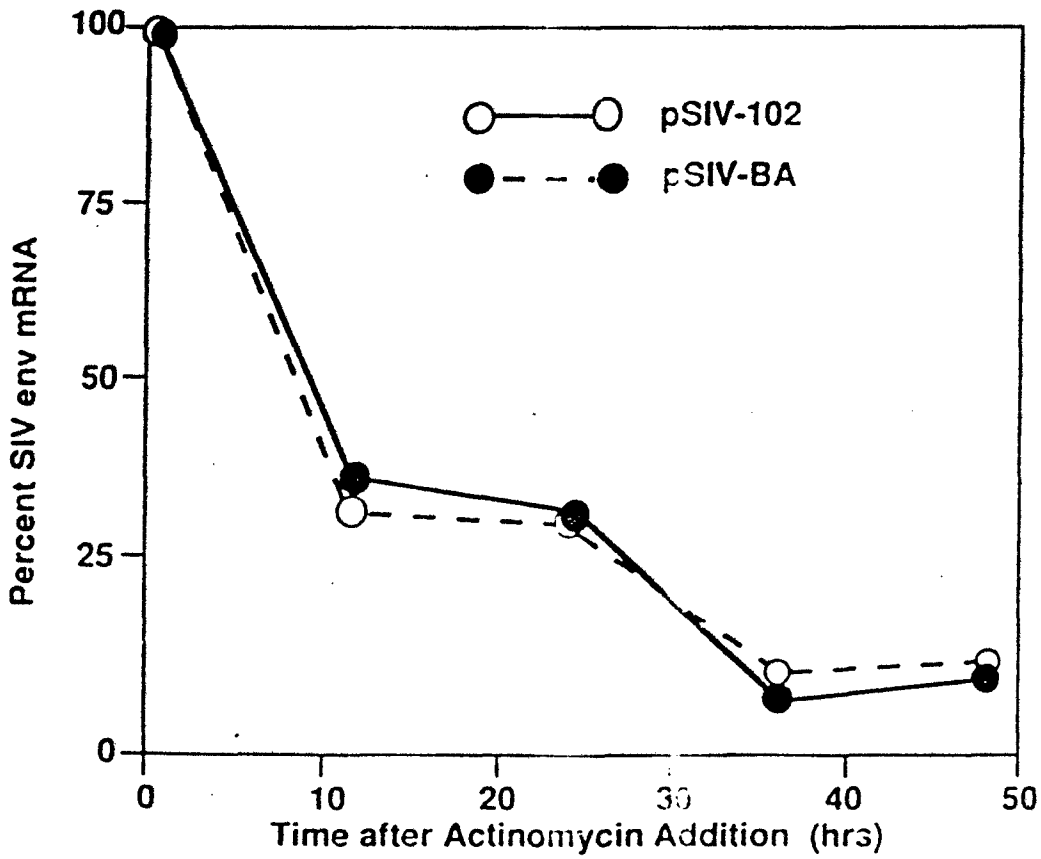
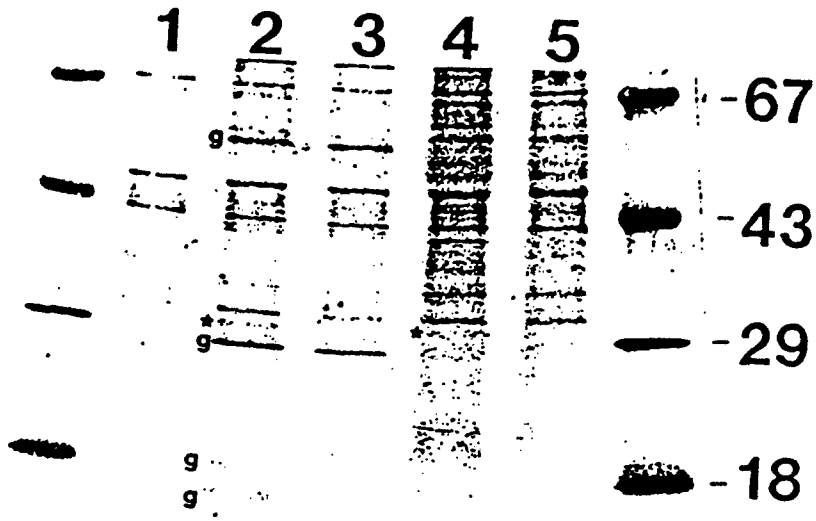


Fig. 10



Appendix 1. Figures

Fig. 1. Envelcpe deletion clones of HIV-1. The top portion of the figure demonstrates the method used for construction of envelope deletion clones of HIV-1 proviral clone, X. A clone with a vif/vpr deletion was constructed by exchanging the Eco RI fragment of X with that from a clone, in which an Nde I to Nco I blunt-ended deletion was constructed, producing clone dF/R. This clone was digested with Nde I, treated with Bal 31 and T4 DNA polymerase, and religated with T4 DNA ligase producing a set of clones with deletions in vif, vpr, and env designated dF/R/E. In some cases, the Eco RI fragment of clones dF/R/E was replaced with that derived from clone X, to produce a set of clones with deletions only in env, designated dE. The bottom half of the figure lists the clones, the presence or absence of a frameshift in envelope, the nucleotides deleted, the number of nucleotides deleted, the amino acids deleted for those clones with in-frame deletions, the number of amino acids deleted, the presence or absence of a signal peptide sequence, and the sequence of the mature envelope product after removal of the signal sequence with the / indicating sequences deleted. The insert shows a schematic of the HIV-1 envelope gp120 protein, indicating the conserved (C1-4) and variable (V1-5) domains, signal peptide (SP), and positions of cysteine (C) and potential N-linked oligosaccharide acceptor positions (N), and the residue number.

Fig. 2. Envelope deletion clones of HIV-1 are non-infectious. a) COS-1 cells transfected with each of the indicated clones were cocultivated with CEM cells, and p24 antigen production assessed. b) Similar results were obtained with H9 cells which were assayed for virus production 12 days after coculti[illegible]ation.

Fig. 3. Sucrose gradient analysis of HIV-1 particles from cells transfected with clones with deletion in env. a) Antigen assays were performed on sucrose gradient samples from virus particles of conditioned media of cells transfected with the indicated clones. In the middle panels sucrose gradients were performed with virus particles which were treated with trypsin, triton, or untreated, derived from cells transfected with clone b) dE-10, c) dE-19, and d) X. e) A Coomassie blue stained gel of proteins from virus samples of the dE-10 virus preparation treated with triton, trypsin, triton and trypsin, or untreated are shown.

Fig. 4. Mutations in the domains of the N-terminus of the transmembrane protein. Insertions, deletions, or substitutions are indicated in individual clones constructed by site directed mutagenesis.

Fig. 5. Mutagenesis of SIVmac102 nef gene. Site directed mutagenesis was used to introduce a termination codon at amino acid position 40 and eliminate a Bam HI site, without affecting the overlapping region of env. The structure of this clone was confirmed by restriction enzyme digestion and nucleotide sequencing.

Fig. 6. SIVmac102 nef depresses gag p24 antigen production in medium of transfected COS-1 cells. The results of three independent experiments are shown. Different DNA preparations were used in each experiment and transfection efficiency was found to be within 5% in each case using co-transfected RSV-CAT. The Abbott HIV-1 p24 antigen assay which had significant cross-reactivity with SIV gag proteins was utilized. Similar results were obtained in two additional experiments in which a Coulter SIVmac antigen ELISA assay kit was utilized.

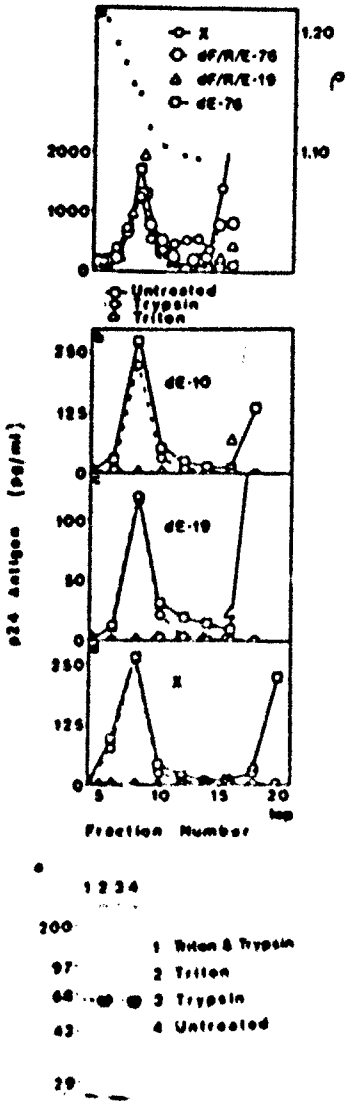
Fig. 7. SIVmac102 nef depresses steady-state viral mRNA levels. Duplicate plates of COS-7 cells were transfected with pSV2gpt, SIVmac102, or SIVmacBA. RNAs were isolated 60 hrs after tranfection and electrophoresed on a 1.0% agarose gel. RNAs were transfered to nitrocellulose and hybridized with a 3.5 kb Sac I fragment including 3' SIV sequences or an actin probe. Values obtained from integrated densitometry tracings are shown, as well as activity of cotransfected RSV-CAT.

Fig. 8. SIVmac102 down-regulates RNA initiation. Nuclei were isolated from COS-1 cells transfected with pSV2gpt, SIVmac102, or SIVmacBA, and run-off assays performed. [32P]-UTP labeled transcripts were hybridzed with the SIV and Alu I repeat sequence probes, and hyridization ratios determined from densitometry tracings of the autoradiogram.

Fig. 9. SIVmac102 nef does not destabilize SIV RNAs. Duplicate plates of COS-1 cells were transfected with pSV2gpt, SIVmac102, or SIVmacBA. After 48 hrs, the cultures were treated with actinomycin D for the indicated times prior to isolation of RNAs and Northern blot analysis with same SIV probe described in Fig. 7 as well as the actin probe.

Fig. 10. Detection of the SIVmac102 nef protein. COS-1 cells were transfected with pSV2gpt (lane 1), SIVmac102 (lane 2), SIVmacBA (lane 3), an expression clone utilizing the intact nef gene from SIVmac102 (lane 4), or an expression clone utilizing the truncated nef gene from SIVmacBA (lane 5). After 48 hrs, the cells were incubated with [35S]-methionine and cysteine, and labeled proteins immunoprecipitated with a polyclonal serum derived from an SIVmac infected rhesus macaque. The 31 kd nef protein is indicated by an * in lanes 2 and 4. Gag proteins (55 kd precursor, 26 kd capsid protein, 19 kd matrix protein, 16 kd nucleic acid binding protein) present in lanes 2 and 3 are indicated by a "g".

Fig. 3


X
dF/R/E·76
dF/R/E·19
dE·76
1.20
ρ
1.10
2000
1000
0
Untreated
Trypsin
Triton
250
125
0
dE·10
100
50
0
dE·19
250
125
0
X
5
10
15
20
Fraction Number
p24 Antigen (pg/ml)
1 2 3 4
200
97
68
43
29
1 Triton & Trypsin
2 Triton
3 Trypsin
4 Untreated

Manuscripts in preparation, submitted, or published from this work in the last year

1. Dedera D, Hu W, Vander Heyden N, and Ratner L. Viral protein R (VPR) of HIV-1 and HIV-2 is dispensable for replication and cytopathogenicity. J. Virol. 63:3205-3208 (1989). (Appendix 2)

2. Polmar S, Whittier R, and Ratner L. Immunological and virological studies of a cohort of premature infants that received blood from the same HIV infected donor. In preparation.

3. Lee SJ, Fisher AG, Hu W, Ratner L, Looney D, Gallo RC and Wong-Staal F. The role of the carboxyl terminus of the HIV-1 transmembrane protein in viral transmission and cytopathogenicity. AIDS Res. & Hum. Retrovir. 5:441-449 (1989). (Appendix 3)

4. Hu W, Vander Heyden N, and Ratner L. Analysis of the function of viral protein X (VPX) of HIV-2. Virol. 173: 624-630 (1989). (Appendix 4)

5. Bryant M and Ratner L. Myristoylation-dependent replication of HIV-1. Proc. Natl. Acad. Sci. U.S.A. 87:523-527 (1990). (Appendix 5)

6. Bryant M, Huekeroth R, Kimata J, Ratner L, and Gordon J. Replication of human immunodeficiency virus 1 and Moloney murine leukemia virus is inhibited by different heteroatom-containing analogs of myristic acid. Proc. Natl. Acad. Sci. U.S.A. 86:8655-8659 (1989). (Appendix 6).

7. McNearney T and Ratner L. Lack of human retrovirus involvement in Felty's syndrome. In preparation.

8. McNearney T, Westervelt P, Thielan BJ, Trowbridge B, Garcia J, Whittier R, and Ratner L. Limited sequence diversity in envelope among biologically distinct HIV-1 isolates from a clustered outbreak. Proc. Natl. Acad. Sci. U.S.A. 87:1917-1921 (1990). (Appendix 7)

9. Dedera D, Vander Heyden N, and Ratner L. Restriction of HIV-1 replication by an inhibitor of envelope oligosaccharide processing. AIDS Res. & Hum. Retrovir. 6:785-794 (1990). (Appendix 8)

10. Ratner L., Vander Heyden N, and Dedera D. Blockade of glucosidase I and HIV and SIV Replication. Virol. Submitted.

11. Ratner L, Vander Heyden N, Garcia J, Polinksy M, and Westervelt P. Non-infectious envelope-free HIV-1 virus particles. AIDS Res. & Hum. Retrovir. Submitted.

12. Westervelt P, Gendelman H, and Ratner L. Two genetic determinants required for HIV-1 infection and replication in monocytes: viral protein R and envelope. In preparation.

13. Bryant M, Ratner L, Adams S, and Gordon J. Mechanism of inhibition of HIV-1 with heteroatom substituted myristic acid analogs. In preparation.

14. Dedera D and Ratner L. HIV-1 Transmembrane envelope mutants which distinguish cell killing by fusion from single-cell mechanism. In preparation.

15. Dedera D and Ratner L. An open reading frame on the HIV-1 plus DNA strand is dispensable for virus replication and cytopathogenicity. In preparation.

16. Dedera D and Ratner L. Role of N-linked oligosaccharides in HIV-1 transmembrane envelope synthesis, processing, and function. In preparation.

17. Niederman T and Ratner L. SIV nef downregulates virus expression and mRNA accumulation. In preparation.

Invited reviews supported by this contract in the last year

1. Bryant M and Ratner L. The biology and molecular biology of HIV, in Pizzo PA and Wilfert C, eds. Pediatric AIDS: The challenge of HIV infection in infants, children, and adolescents. Williams & Wilkins, In press (1990). (Appendix 9)

2. Ratner L, Niederman TMJ, Lozeron H, and Bryant M. Structure and function of the negative factor (NEF) of HIV and SIV, in Kuma, A., ed., Advances in Molecular Biology and Targetted Treatment of AIDS. Plenum Press, In press (1990).

Abstracts supported by this contract in the last year

1. McNearney T, Thielan B, Westervelt P, Whittier R, Trowbridge D, Garcia J, and Ratner L. Biological significance HIV-1 envelope heterogeneity. Clin. Res. 1989.

2. Bryant ML and Ratner L. Myristoylation dependent assembly of infectious HIV-1. Clin. Res. 1989.

3. Haas DW, Tettelbach WH, Ratner L, Montefiori DC, Mitchell WM, and Hagedorn CH. Isolation and quantitation of eIF-4E in H9 lymphocytes. Clin. Res. 1989.

4. Ratner L and Niederman T. NEF responsive sequences (NRS) of the HIV-1 LTR mediate transcriptional suppression. Proceedings of the V International Conference on AIDS. 1989.

5. Ratner L, Hu W, and Vander Heyden N. Analysis of VPX function in HIV-2 infected cells. Proceedings of the V International Conference on AIDS. 1989.

6. Dedera D, Hu W, Vander Heyden N, and Ratner L. Viral protein R (VPR) of HIV-2 and HIV-2 is dispensable for replication and cytopathicity. Proceedings of the V International Conference on AIDS. 1989.

7. McNearney T, Westervelt P, Thielan BJ, Trowbridge D, Garcia J, and Ratner L. Biological significance of HIV-1 envelope heterogeneity. Proceedings of the V International Conference on AIDS. 1989.

8. Ratner L and Bryant ML. Mryistoylation dependent assembly of infectious HIV-1. Proceedings of the V International Conference on AIDS. 1989.

9. Westervelt P, McNearney T, Thielan BJ, Trowbridge D, Garcia J, and Ratner L. Limited sequence heterogeneity between lympocyte- and monocyte-tropic HIV-1 isolates. Cold Spring Harbor RNA Tumor Virus Meeting. 1989.

10. Ratner L, Vander Heyden N, and Bryant M. Mechanism of HIV-1 assembly - role of gag myristoylation and env synthesis. Proceedings of the VI International Conference on AIDS, 1990.

11. Ratner L, Bryant M, and Vander Heyden N. Role of envelope-gag interactions in HIV-1 virion assembly. Cold Spring Harbor RNA Tumor Virus Meeting, 1990.

12. Bryant M, Ratner L, Adams S, and Gordon J. Analogs of myristic acid block HIV-1 gag precursor processing nad prevent virus replication. Cold Spring Harbor RNA Tumor Virus Meeting, 1990.

13. Niederman T, Hu W, and Ratner L. SIV nef suppresses virus replication and mRNA accumulation. Cold Spring Harbor RNA Tumor Virus Meeting, 1990.

14. Niederman T and Ratner L. SIV nef suppresses virus replication and mRNA accumulation. Conference on Advances in Molecular Biology and Targetted Treatments for AIDS, Washington D.C., 1990.

15. Bryant ML, Ratner L, Adams S, and Gordon J. Analogs of myristic acid block replication and assembly of infectious HIV-1. 30th Icaac Conference, Atlanta, 1990.

16. Westervelt P and Ratner L. A determinants within the HIV-1 gp120 envelope protein critical for infection of primary monocytes. Cold Spring Harbor Vaccine/AIDS Meeting, 1990.

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4. Rosen C, et al. Nature 319: 555, 1986.
5. Muesing et al. Cell 48: 691, 1987.
6. Fisher A, et al. Science 237: 888, 1987.
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8. Luciw P, et al. Proc. Natl. Acad. Sci. 84: 1434, 1987.
9. Wong-Staal F, et al. AIDS Res. & Hum. Retrovir. 3: 33, 1987.
10. Miller. Science 239: 1420, 1988.
11. Kowalski M, et al. Science 237: 1351, 1987.
12. Fisher A, et al. Science 233: 655, 1986.
13. Ratner L, et al. AIDS Res. & Hum. Retrovir. 3: 147, 1987.
14. Ratner L, et al. Nature 313: 636, 1985.
15. Schultz AM and Oroszolan G. Vir. 133: 431, 1984.
16. Rhee SS and Hunter E. J. Virol. 61: 1045, 1987.
17. Kunkel L, et al. Proc. Natl. Acad. Sci.
18. Guyader et al. Nature 326: 662, 1987.
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