Immunoprecipitation of Nef from Patient Isolates using Rabbit anti-Nef Serum

Labeling of cells:

$3xl0^6$ CEMX174 cells were infected with HIV NL4-3, HIV pCAD (Δ nef), SIVsmmh4i or with various HIV Patient Isolates (PI), until the development of syncytia. The infected cultures were washed twice, pelleted, re-suspended in I ml cys(-), met(-) RPMI with 10% dialyzed FCS and incubated for 2 hours at 37°C. The cells were then pelleted, re-suspended in I ml cys(-), met(-) RPMI without serum, and 250 µCi of ^{35}S protein label (NEN-Dupont NEG-072) was added. The cells were incubated for 5 hours at 37°C with shaking then pelleted and re-suspended in 3000 RIPA buffer.

Immunoprecipitation:

The cellular debris was pelleted and 100 pl of the supernatant was pre-incubated with 50 µl of protein A sepharose beads for 30 minutes at 37°C. The beads were pelleted and 5 µl of anti-Nef serum was added to the supernatant. The sample was then incubated for 1 hour at 37°C. After the addition of 30 µl of fresh protein A beads, the sample was incubated at room temperature for an additional 30 minutes. The beads were then pelleted, washed four times with RIPA and incubated at 70°C with 50 µl loading dye for 10 minutes. The beads were pelleted and the supernatant loaded onto at 15% SDS-polyacrylamide gel. The gel was run for 1.5 hours at 250 volts, dried, exposed to film for 16 hours and developed.

Procedure for Detection of Nef by Western Blot Using Rabbit anti-Nef Serum

Reagents:

Stacking gel: 4.5% Acrylamide (0.4% wieght/volume bisacrylamide); 130 mM Tris pH 6.8; 0.1% SDS	Running gel: 15% Acrylamide (0.4% weight/volume bisacrylamide) 370 mM Tris pH 8.0; 0.1% SDS
Running buffer: 250 mM Tris; 1.92 M glycine; 0.1% SDS	Transfer buffer: 250 mM Tris; 1.92 M glycine; 20% methanol
TBST: 10 mM Tris pH 8.0; 150 mM NaCl; 0.05% Tween 20	Loading Buffer: 100 mM Tris pH 6.8; 16% Glycerol; 20% SDS; 0.1% Bromophenol blue; 1.1 M beta mercaptoethanol
RIPA: 0.15M NaCl; 20mM Tris pH 7.4; 2 mM EDTA; 1% Triton X-100; 1% deoxycholate; 2 µg/ml aprotinin 2 µg/ml leupeptin; 1 µg/ml pepstatin; 100 µg/ml AEBSF	Prestained High Molecular Weight Standards: Gibco/BRL 26041-020

Western Procedure:

$3xl0^6$ CEMX174 cells were infected with patient isolates and collected by centrifugation when CPE was maximal. The cell pellet was re-suspended in 100 gl RIPA buffer, cellular debris was pelleted and the supernatant was diluted 1:2 with loading dye. A 15 µl aliquot was loaded onto a 15% acrylamide gel. The gel was run until the bromophenol blue dye reached the bottom then transferred to nitrocellulose at 80 volts for 1 hour. The blot was blocked with 2% BSA in TBST for 1 hour at room temperature. The blot was then incubated for thirty minutes with gentle shaking at room temperature with the primary antibody (polyclonal rabbit anti-HIV-1 nef antibody produced by Rockland from Nef purified in Swanstrom lab or polyclonal rabbit anti-HIV-1 nef antibody produced by Lee Ratner as a positive control, each diluted 1:4000 in 5 ml 2% BSA in TBST). The blot was washed four times with 20 ml TBST then incubated for thirty minutes with gentle shaking at room temperature with the secondary antibody (anti-rabbit IgG AP conjugate, Promega S373B). The blot was developed with 10 ml BCIP/NBT in 0.1 M Tris pH 9.5, 0.1 M NaCl, 5 mM MgCl2 (Promega S381C/ S380C). The reaction was stopped with distilled water after five minutes.