Supplemental Materials and Methods

Plasmids

The plasmids pGEX-INI1, pGEX-IN, pSH_2INI1, pGADNotINI1 and deletion fragments, pGADNotINI1(183-294), pSH_2IN, pBABEpuro-INI1, pCGNINI1 and pQE32-INI1 have been described previously (17-18). pQE32-INI1(141-304) was generated by insering the BamH1 to SalI fragment of pGADNot-S7 clone into the pQE32 plasmid (Qiagen). The random mutagenesis library of pGADNotINI1(183-294) was generated as described elsewhere (8). All site directed mutagenesis were carried out using the Quick Change Site Directed Mutagenesis Kit from Stratagene.

Purification of proteins

Freshly plated *E. coli* BL21DE3 cells were transformed with pQE32-HisINI1. Cells were grown from a single colony upto 5 liters of 2YT culture, induced at ~ 0.8 A_{600} with 1 mM IPTG for 3 hours at 37 ^{0}C. Cells were harvested and suspended in 40 ml of lysis buffer (50 mM sodium phosphate (pH 8), 50 mM NaCl, 5 mM $MgCl_2$, 20 mM Imidazole, 0.1 mM EDTA, 20 mM β-mercaptoethanol (b-ME), 0.5% IGEPAL, 5% glycerol and protease inhibitors). 5 mg/ml lysozyme was added and incubated at 4 ^{0}C with rotation for 1 hour following which cells were sonicated 8X at 40% setting with cooling in between. 100 ml of DNase I (10U/ml) was added and incubated at 4 ^{0}C with rotation for 1 hour. Salt concentration was raised to 500 mM NaCl with 5 M NaCl and the suspension centrifuged at 48K rpm for 45 min in a 50.2 Ti rotor. The supernatant was added to 5 ml (10 ml of 50% slurry) of pre-equilibrated (in lysis buffer) Ni-NTA beads and incubated at 4 ^{0}C for 1 hour with rotation. Beads were washed 3X in 35 ml each of wash buffer [50 mM sodium phosphate (pH 6.2), 2 M NaCl, 1% IGEPAL, 0.1 mM EDTA, 20 mM β-ME, 40 mM Imidazole, 20% glycerol, 5 mM phenylmethanesulphonyl fluoride (PMSF)]. The beads were loaded onto a 10 ml syringe column and washed with 100 ml of wash buffer and then 100 ml of wash buffer + 20 mM ATP. Bound proteins

were eluted with 35 ml of elution buffer [50 mM sodium phosphate (pH 7.4), 500 mM NaCl, 0.5% IGEPAL, 0.1 mM EDTA, 10 mM b-ME, 800 mM Imidazole, 10% glycerol, 5 mM PMSF]. The eluted proteins were dialyzed overnight against 1 liter of dialysis buffer A [20 mM potassium phosphate (pH 6.8), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol]. The dialyzed proteins were loaded onto 1 ml of hydroxylapatite (HAP) column pre-equilibrated with 50 ml of equilibration buffer B [20 mM potassium phosphate (pH 6.8), 0.1% Triton-X, 0.1 mM EDTA, 1 mM DTT, 10% glycerol]. The column was washed with 50 ml of wash buffer C [100 mM potassium phosphate (pH 6.8), 0.1% Triton-X, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 5 mM PMSF]. Bound proteins were eluted with 7 ml of elution buffer [500 mM potassium phosphate (pH 6.8), 0.1% Triton-X, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 5 mM PMSF]. The pH of the HAP eluate was changed from 6.8 to 7.2 by adding 500 mM potassium phosphate (pH 8) and then diluted in a beaker with 5X ice-cold dilution buffer (0.1 mM EDTA, 1 mM DTT, 10% glycerol) with slow mixing to reduce salt concentration to around 100 mM. The protein solution was then loaded onto a Mono-Q sepharose column pre-equilibrated with buffer containing 20 mM potassium phosphate (pH 7.2), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol. Bound proteins were eluted with a gradient of 100 mM – 1 M KCl.

For integration assays, IGEPAL was replaced with 0.1% Triton-X in all buffers. HAP eluates were step dialyzed against 250 mM potassium phosphate (pH 6.8), 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 5 mM PMSF (2 hours) and finally against 20 mM HEPES-KOH (pH 6.8), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol (2 hours). INI1(183-294) was also purified through three chromatographic columns viz Ni-NTA agarose, hydroxylapatite and Mono-Q sepharose. The same procedure as used for His-INI1 was used for His-INI1(183-294) in the Ni-NTA step. Eluted proteins were dialyzed against 1 liter of dialysis buffer A and loaded onto hydroxylapatite column pre-equilibrated with buffer B. After wash with buffer C, bound proteins were eluted with

100 mM potassium phosphate (pH 8), 0.1% Triton-X, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 5 mM PMSF and loaded directly onto a Mono-Q sepharose column. Bound proteins were eluted with a gradient of 100 mM – 1 M potassium phosphate (pH 8). His-tagged integrase was purified through a Ni-NTA column as described previously (3). All recombinant protein quantitations were done by Coomassie blue staining and densitometric scanning using BSA (New England Biolabs) as standard.

Yeast two-hybrid analysis

All yeast two hybrid assays were performed in the CTY5d-10 strain. To isolate mutants of INI1(183-294) that were defective in multimerization, pSH2INI1 and a random mutagenesis library of pGADNotINI1(183-294) were cotransformed into the CTY5d-10 strain and ~50,000 colonies were screened by blue-white selection using the colony filter lift assay method and X-Gal as substrate. pGADNotINI1(183-294) DNA harboring mutations were isolated from interaction-negative colonies, retransformed into *E. coli* XL1Blue strain, selected against media lacking leucine and plasmid DNA was isolated and sequenced. Isolated plasmid DNA harboring mutations were retransformed with pSH2INI1 into CTY5d-10 strain and interaction-negative mutants verified by colony filter lift assay method using X-Gal as substrate. Mutants of INI1(183-294) defective for interaction with integrase were isolated using similar procedure as described elsewhere (8). All quantitative yeast two hybrid assays were performed using ONPG (o-nitrophenyl-β-D-galactopyranoside) as substrate using standard protocol (Clontech). Yeast extracts were prepared as described (Das and Maitra, 2000). Approximately 30 μg of yeast extract expressing wild-type or mutant INI1(183-294) proteins were separated by SDS-PAGE and subjected to Western blot analysis using anti-GAL4AD antibody (Clontech) as probe.

GST pull-down and co-immunoprecipitation assays

To test interaction between GST-INI1 and His-INI1, GST and GST-INI1 were prepared as described before (3). About 4 μg of GST and GST-INI1 immobilized onto Glutathione sepharose 4B beads (Amersham-Pharmacia) were incubated with 2 μg of hydroxylapatite eluate of His-INI1 in 200 μl binding buffer (20 mM HEPES-KOH (pH6.8), 100 mM KCl, 5 mM $MgCl_2$, 1% Triton-X, 1 mM DTT, 0.1 mM EDTA, 100 mg/ml ethidium bromide and protease inhibitors). 1 μl of DNase I (10U/μl) was added to the mixture and incubated at 4 ^{0}C for 1 hr with rotation. Following incubation the beads were washed 5 times with 1 ml binding buffer. The washed beads containing bound proteins were boiled with SDS loading buffer, separated by SDS-PAGE and subjected to Western blot analysis using anti-His monoclonal antibody as probe. In later GST-INI1 pull-down experiments conditions as below was used. To test interaction between GST-IN and His-INI1, GST and GST-IN were prepared as described before (3). About 5 μg of GST or GST-IN were incubated with 2 μg of His-INI1 (wild type or mutant) proteins in 200 μl binding buffer (20 mM HEPES-KOH (pH 6.8), 5 mM $MgCl_2$, 1 mg/ml BSA, 0.1% IGEPAL, 1 mM DTT, 0.1 mM EDTA, 100 μg/μl ethidium bromide and protease inhibitors). Salt concentration was kept at 100 mM/150 mM/200 mM NaCl. 1 μl of DNase I was added to the mixture and incubated at 4 ^{0}C for 1 hr with rotation. Following incubation the beads were washed 4 times with 1 ml of wash buffer (20 mM HEPES-KOH (pH6.8), 100 mM/150 mM/200 mM NaCl, 0.5% IGEPAL, 100 μg/μl ethidium bromide, 1 mM DTT, 0.1 mM EDTA and 0.1 mM PMSF). The washed beads containing bound proteins were boiled in SDS loading buffer, separated by SDS-PAGE and subjected to Western blot analysis using anti-His monoclonal antibody as probe.

To test INI1 self-association *in vivo* 293T cells were transfected with 10 mg of pCGNINI1 (HAINI1), pBABEpuroFLINI1 (FLAGINI1) or both. 48 hours post-transfection cells were harvested, lysed in lysis buffer (20 mM HEPES-KOH (pH7.9),

150 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM DTT, 0.1 mM EDTA, 1% Triton-X and protease inhibitors) for 1 hour at 4 ^{0}C with rotation. Cell debris was removed by centrifugation and the supernatant treated with 0.033 U/ml of micrococcal nuclease (10U/ml) for 15 min at 30 ^{0}C. The supernatant was precleared with 20 ml of Protein A agarose beads and 2 mg of mouse IgG for 1 hour at 4 ^{0}C. The beads were removed by brief centrifugation and the supernatant subjected to immunoprecipitation using 20 ml of anti-HA agarose (SIGMA) overnight. The beads were washed 3 times with wash buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl). The bound proteins were boiled in SDS loading buffer, separated by SDS-PAGE and subjected to Western blot analysis using anti-FLAG (SIGMA) and anti-HA (SIGMA) antibodies as probes. The same co-IP conditions were used to immunoprecipitate YFP-IN bound to INI1(183-294)/S6 except that 1% Triton-X was added to the wash buffer.

Glycerol gradient centrifugation and gel filtration chromatography

Mono-Q sepharose eluate of His-INI1 at concentration of <5 nM or >100 nM was loaded onto a 15-35% glycerol gradient prepared in 20 mM potassium phosphate (pH 7.2), 100 mM KCl, 1mM DTT, 0.1 mM EDTA, 0.1% Triton-X, 1 mM PMSF. The oligomeric forms were separated by centrifugation at 40K rpm (SW41 Ti rotor) for 24 hours, respectively. Fractions (400-450 ml) were collected and subjected to TCA (Trichloroacetic acid) precipitation. The precipitated proteins were resuspended by boiling in SDS loading buffer, separated by SDS-PAGE and subjected to Western blot analysis using anti-His monoclonal antibody as probe. To compare the oligomeric status of wild type and mutant INI1 proteins eluted from hydroxylapatite column, ~ 5 nM of protein was loaded onto a 15-35% glycerol gradient prepared in 20 mM HEPES-KOH (pH6.8), 100 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1% Triton-X, 1 mM PMSF. Prior to loading, the protein was incubated in 500 ml of the same buffer for 3 hrs on ice. The oligomeric forms of INI1 (wild type and mutant) were separated by centrifugation at 40K

rpm (SW41Ti rotor) for 24 hours. Fractions (400 ml) were collected and analyzed as described above. For IN:INI1 complex formation IN and INI1 wild-type and mutant I264T, I268T proteins were mixed at a ratio of 1:4 in integrase reaction buffer and incubated on ice for 1 hr. Following incubation proteins were loaded onto a 15-35% glycerol gradient and centrifugation and analysis was carried out as above. Gel filtration chromatography was performed in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, with 200 mg of His-INI1(183-294) eluate from Mono-Q sepharose column. Fractions (500 ml) were collected, subjected to TCA precipitation, the precipitated protein boiled in SDS loading buffer and separated by SDS-PAGE and subjected to Western blot analysis using anti-His antibody as probe.

Joining assay, DNA binding assay and EMSA

The joining assays were performed with P^{32} labeled 3' pre-processed duplex DNA (U5.5 and U5.4) as donor DNA and pcDNA as target DNA as described before ( ) using the indicated amounts of proteins except that ~ 7 ng of radiolabeled substrate and 100 ng of target DNA were used. Salt concentrations were varied between 10 mM – 150 mM NaCl.

For gel retardation assay about 8 pmole and 16 pmole of hydroxylapatite eluate of wild-type INI1 or mutant INI1 was incubated with 200 ng of pcDNA in 20 mM HEPES-KOH (pH 6.8), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and 3 mM $MnCl_2$ and incubated at 30 ^{0}C for 1 hour. Protein-DNA complexes were resolved by 1% agarose gel electrophoresis and stained with ethidium bromide.

For EMSA ~7 ng of radiolabeled 3' pre-processed duplex DNA (U5.5 and U5.4) was incubated with 5 pmole of hydroxylapatite eluate of wild-type INI1 in strand transfer reaction buffer, at 30 ^{0}C for 1 hr. 10 X cold viral LTR DNA was added to the reaction mixture. Minor groove inhibitors were added to the reaction mixture as indicated. About

10 ml of reaction mixture was run on a 6% native polyacrylamide gel made in TBE and the gel was run at 10 mA for 2 hours. The gel was dried and exposed to X-ray film.

p24 ELISA and confocal microscopy

To produce viral particles the transducing vector pHR'CMVGFP, the gag-pol vector CMVΔR8.2, and the VSVg envelope vector pMDG were transfected into 293T cells at a ratio of 2:1:1. 5μg of pGCN-INI1 or pCGN- S6 wild-type and mutants were cotransfected into 293T cells at 30% confluency in 6-well plates using the calcium phosphate method (Chemicon). Media was changed 16hrs post-transfection. The supernatant and cells were collected 48hrs later. The viral supernatant was passed through a 0.2-μM cellulose acetate filter (Nalgene) and treated with HEPES. The cells were lysed using 80ul of RIPA buffer plus protease inhibitors. P24 ELISA (Perkin Elmer) was performed using the cellular lysate to measure intracellular p24 and the viral supernatant to measure virion associated p24. Lystates were analyzed by Western blot after protein quantitation. Cells were transfected at 30% confluency in slide chambers with 0.8ug of GFP/CFP-INI1, GFP/CFP-S6 wild-type and mutants and YFP-IN as described above. After 36hrs post-transfection, cells were fixed with 2% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, and treated with 1μg/ml RNase A for 30 min at 37˚C. Cells were then stained with 40μg/ml propidium iodide for 5 min. and coverslips were mounted on the slides using mounting medium (Vector Laboratories, Burlingame CA). Images were captured using a Leica confocal microscope. Images were analyzed using the software ImageJ (http://rsb.info.nih.gov/ij/).