Methods

Cell Culture

The HeLa cell line (Scherer et al., 1953) and its derivates, were cultured at 37°C with 5% C02 in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 µg/ml penicillin and 50 µg/ml streptomycin. Cultures at ~80% confluence were routinely split 1:5 in 10 cm culture dishes as follows. The cells were washed twice in prewarmed PBS. 1 ml PBS containing 0.25% (w/v) Trypsin was added to the dishes and placed at 37°C for 5-10 minutes. After the cells were detached from the dishes, 1 ml prewarmed culture medium was added and the cells transferred to a 50 ml falcon tube. Cells were spun down at 150 g and plated in new dishes with fresh culture medium.

Insects cells were cultured at 26°C in Grace's medium (for Sf9 cells) or TMN-FH medium (for Hi-5 cells) containing 10% heat inactivated FCS, 2 mM L- glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, 0.1% Pluronic F-68 and 1x Yeastolate. The Sf9 cells grew well as suspension culture and Hi-5 cells as loosely adhered cultures in Petri dishes. For Sf9 cells, 50 ml suspension cultures were maintained in 250 ml glass bottle, with shaking at 110 rpm in the 26°C incubator. For Hi-5 cells, 15 cm dishes are used to infect cells. Starting culture always had the cell density of 0.5 million cells per ml. Cells were split twice in a week and care was taken for the cell density to never exceeded 3 million cells per ml.

Human cell line transfection method

Roti Fect reagent from Roth was used to make all transfections. Cells were grown to 60 – 80 % confluence. 10ug of qiagen purified DNA was used for a typical 10cm dish transfection. In tube A, 500ul of optimem (serum free medium)

is added to DNA. In tube B, 500ul of optimem (serum free medium) is added to 30ul of Roti Fect reagent. Now contents of both tubes are mixed gently. The DNA – Lipid complex is allowed to form by incubating the transfection reaction for 30 minutes at room temperature. While complex is forming rinse the cells with PBS and refill the dish with fresh serum free medium. Add DNA – Lipid complex on the cells and mix gently. Incubate for 4-6 hours. Remove the transfection medium and add complete medium to cells.

Generating stable cell lines

The HeLa cells were used to generate stable cell lines with constitutive expression of recombinant protein. A new technique of sorting sub cloning

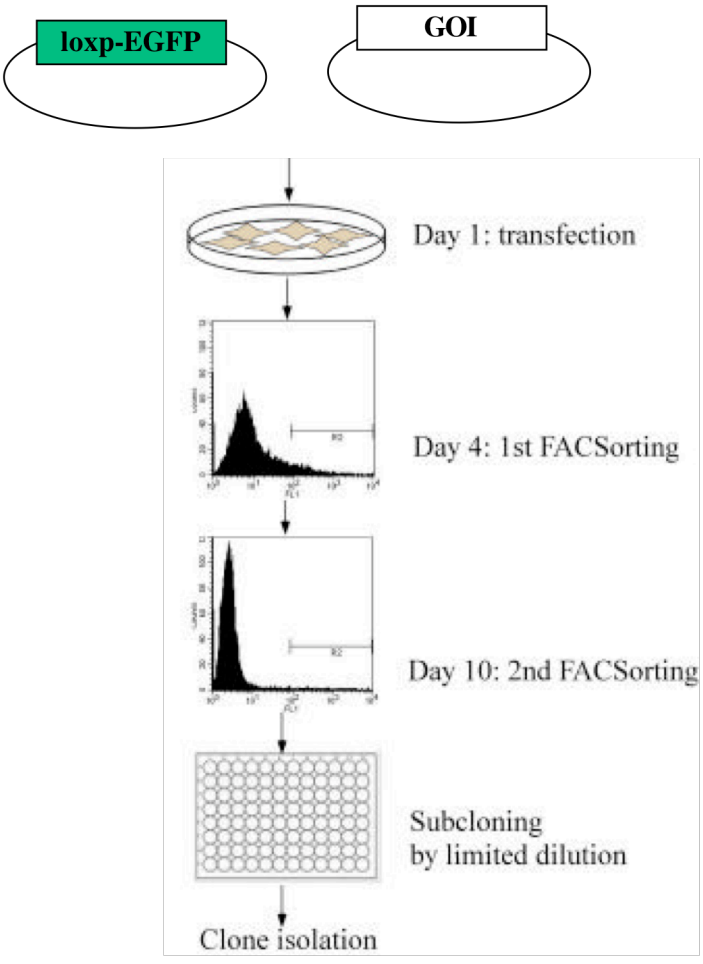

Fig.5 Algorithm to get stable clones using sorting subcloning method. Loxp-EGFP plasmid has EGFP flanked by loxp sites in same direction. After the stable clone has been isolated. Cre recombinase is used to excise the EGFP (Liu W., PhD thesis)

(Fig. 6) is used to generate stable cell lines. Plasmid carrying gene of interest is co--transfected with loxp-EGFP plasmid. Roti Fect transfection was done in 35 mm dishes exactly as described above. GFP positive cells are sorted on day 4 and day 10 after the transfection. Cells after second sorting were grown in limited dilution of approx. single cell per well. The cells were expanded and screened for positive clones using western blots. EGFP is kicked out from the positive clones by CRE recombinase expression.

Flow cytometry

Flow cytometry, or fluorescence-activated cell scanning or sorting (FACS analysis), is the measurement of characteristics of single cells suspended in a flowing saline stream when they flow past a series of detectors. The fundamental concept is the cells flow one at a time through a region of integration where multiple biophysical properties of each cell can be measured at rates of over 1000 cells per second. These biophysical properties are then correlated with biological and biochemical properties of interest.

Cells subjected for FACS analysis in this study were either expressing fluorescent protein EGFP or stained with propidiumiodide (PI) as described. Cells grown on 6-well plates were harvested by centrifuging at 1200 g for 3 min as described, and then suspended in 400 µl of PBS with 0.1% EDTA in Falcon tube 2054. The cells were scanned on the FACSCalibur station (Becton Dickinson Inc.) using proper laser settings according to the suggested manufacture protocols. In order to keep the resolution as accurate as possible, the acquisition flow rate was controlled around 200 events per second by choosing proper scanning speed on the FACScalibur or diluting the cells with PBS. Dead cells

and cell debris were excluded by gating the cells with a FSC threshold of 200. Doublet discrimination (DMM) to distinguish between clumped and mitotic cells was set for FL2. One negative cell line was included as autofluorescence control in each FACS analysis in order to adjust the instrumental setting properly for the positive fluorescent samples. The instrumental settings were kept consistent for all batches of samples during the time course. 10,000 cells were set as the defined scanning events for every sample. Data acquisition and control of the flow cytometer was performed with the CellQuest program (BD Biosciences, Heidelberg, Germany).

FACSorting in this study was set for collecting EGFP positive or negative cell populations. Cells grown in T25 flasks were harvested and counted. The cells were diluted with PBS at a concentration of 2X 10E6 cells per ml. The cell suspension was pipetted into 5 ml Falcon round bottom tubes (Falcon tube 35-2235) through cell-strainer cap to break up any clumped cells. The cells were sorted on FACSVantage (Becton Dickinson Inc.) and the subpopulations were collected into 15 ml Falcon tubes with 3 ml complete medium.

Transfection of Sf9 cells with bacmid for baculoviruses preparation

Two ml of Sf9 cells with a density of 0.5 million cells per ml were put in each well of a 6-well plate. The cells were let to stand for one hour at RT. In the mean time a mixture 200 µl Grace's media (without FCS), 15 µl DAC-30 (a kind gift from the Rezka lab, MDC, Berlin) and 10 µl Bacmid DNA (10 µg) were made and incubated at RT for 30 min. After 30 min 800 µl Grace's media (without FCS) was added to this mixture and the mix was added to Sf9 cells in the six well plates. The Sf9 cells were washed once with Grace's media (without FCS) before adding the transfection mix. The cells were incubated with 1 ml transfection mix at 26°C for 4 hours. The transfection mix was removed and 2 ml of fresh Grace's complete media was added to the culture. Cells were further grown for at 26°C for 72 hours.

After 72 hours the culture supernatant was collected and one ml of this supernatant (containing baculoviruses) was added to a 60 mm dish containing 5 ml culture volume (2.5 million cells), for further amplification of the baculovirus.

Recombinant DNA techniques

Conventional DNA preparation protocols

All standard methods like plasmid preparation, cloning methods, electrophoresis on agarose gels in TBE or TAE etc were done according to methods provided by Molecular cloning book (Sambrook and Russel, 2001) or according to the manufacturer's instructions. Modifications of any of these protocols are indicated where necessary.

Elution of DNA from agarose gel pieces

A homemade electrophoresis device ('salt trap') was used for elution of DNA fragments from agarose gel pieces. The apparatus was filled with 0.5 X TBE and agarose gel pieces with DNA was placed in the slots. Any air bubbles in the passages of the apparatus were carefully removed. 80 µl of salt trap solution (3 M Sodium Acetate, 0.025% w/v Bromophenol blue dye, pH 7) was loaded in the salt trap passage. The apparatus was connected to power pack and run at constant 150 Volts for 30 minutes. Then, the blue colored salt trap solution was collected in 1.5 ml tubes and equal volume of isopropanol was added to the tube. The precipitated DNA was immediately centrifuged at 4°C, 20000 g for 20 min. The pellet was washed once in 70% ethanol dried and re-dissolved in desired volume of double distilled water.

Biochemical methods of protein purification

Cell lysis and nuclear salt extraction method

HeLa cells and insect cells were both lysed using the same protocol. Cells were harvested and washed twice in ice cold PBS. Cells were then either lysed immediately or snap frozen in freezing buffer (PBS, 2 mM MgCl2 and 10% glycerol). For lysis, ice-cold lysis buffer (PBS, 2 mM MgCl2, 0.1% NP40, 10% glycerol and 1 mM PMSF) was added to the cells. All further steps were done at 4°C. Cell were uniformly mixed and incubated for 5 min. The nuclei were pelleted by spinning the lysate at 1500 g for 4 min. The nuclei pellet was washed once in lysis buffer and salt extracted in lysis buffer with 300 mM KCl for 1 hour. The soluble proteins were collected by spinning at 20000 g for 20 min. The nuclear salt extract was used for ORC immunoprecipitations and HA affinity purification experiments.
A typical experiment with Sf9 cultures, used cells from 50 ml baculovirus infected cells (pre infection cell count of 0.5 million cells per ml). The cell pellet was lysed in 8 ml lyisis buffer. Isolated nuclei were extracted in 2 ml of lysis buffer with 300 mM salt.

SDS-gel electrophoresis and Immunoblotting

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels used in this work were 1 mm thick, 8 cm wide and 9.2 cm long. The gels were poured in Hoefer mini VE Basic gel frames. The electrophoresis tank and upper chamber of the gel container were filled with 1x SDS running buffer (25 mM Tris-HCl, 192 mM glycin, 0.1% SDS). Proteins were separated through 10% or 12% polyacrylamide minigels, followed by coomassie staining, silver staining or immunoblotting for protein detection. The gels were

electrophoresed at 150 V for first half an hour and then at 200 V till the bromophenol dye reached the bottom. Power was supplied from a BioRad PowerPac 400 (Pharmacia).

Transfer of proteins to PVDF membranes

Before use PVDF membranes (Immobilon P, Millipore) were treated as follows: membranes were placed in 100% methanol for 1 minute, transferred into 60% methanol. The membranes were soaked in 1x transfer buffer (25 mM Tris, 192 mM Glycin) for at least 5 minutes. Polyacrylamide gels containing separated proteins were placed on PVDF membranes sandwiched between, 2 mm whatmann paper, 1mm whatmann paper presoaked in 1x transfer buffer and placed in a semi-dry blotter (Bio-Rad). The proteins were transferred at 15V for 60 minutes. Power was supplied from a BioRad Power Pac 200 (Pharmacia). The efficiency of the transfer was confirmed by staining the PVDF membrane with Poncue-S.

Immunoblotting

PVDF membranes containing bound proteins were blocked with super blotto solution (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20, 0.5% NP40, 0.5% BSA, Fraction V, 2.5% non-fat dried milk) for one hour at RT or overnight at 4°C. The membranes were incubated with primary antibodies diluted in super blotto for 1-2 hour at RT or overnight at 4°C. The membranes were washed twice in 1x TBST (100 mM Tris-Hcl, pH 8.0, 1.54 M NaCl, 1% V/V Tween 20) for 5 minutes and incubated for one hour at RT in super blotto containing a 1:5000 dilution of horseradish peroxidase (HRP) conjugated secondary antibody. The membranes were washed three times, for 10 minutes each in 1x TBST and the bound antibodies were visualized by chemiluminescence.

Chemiluminescence

Immunoreacted proteins were detected using the SuperSignal Pico West (Pierce). Equal volumes of the Luminol/Enhancer and Stable Peroxide solutions were mixed, poured onto the immunoblotted PVDF membranes and incubated for 2 minutes at RT. Excess solution was drained away, the blot placed in clear plastic folder and exposed to Biomax MR film (Kodak). The film was developed through an AGFA Curix 60 machine (AGFA, Germany).

Immunoprecipitation (IP)

All immunoprecipitations were done using nuclear extracts prepared by the method described earlier. When using anti-serum for IPs, the nuclear extract was pre cleared with pre immune serum from the same rabbit. 10 µl of preimmune serum and 7.5 µl protein A coupled sepharose beads (Pharmacia) was added to 400 µl of nuclear extract (from 1 million cells) and incubated at 4°C on shaker for 2 hours. The beads were then spun down (1000 rpm, 4°C, 4 min) and 10 µl of anit-serum was added to the supernatant. The anti-serum was incubated with cleared nuclear extract overnight at 4°C on shaker. Next day, 7.5 µl Protein A coupled beads were added to the tube and incubated further for 2 hours at 4°C. The IP beads were spun down (150 g, 4°C, 4 min) and washed 5 times with total of 5 ml lysis buffer (0.1% NP40, 1X PBS, 2mM MgCl2, 1mM DTT, 10% Glycerol).

When using a monoclonal antibody for immunoprecipitation, either antibody coupled to beads was purchased or covalently coupled. 2.5 µl of the antibody-coupled beads were added to 200 µl of nuclear extract (from 2-3 million cells) and incubated overnight at 4°C on shaker. The beads were washed with lysis buffer next day, 5 times with a total of 5 ml buffer. Then the beads were boiled in 1X SDS loading buffer and separated on SDS-PAGE. The proteins were transferred on PVDF membrane and detected by immunoblotting.

HA-affinity purification of recombinant protein complex

Sf9 cells were infected with baculoviruses coding for different HsOrc proteins and were harvested 60 hours post infection. Cells were lysed and nuclear extract was made following the method described before. 25 µl of 50% HA ab-agarose slurry was added to 1 ml of nuclear extract (corresponding to 1.25 X 107 infected Sf9 cells) and incubated overnight at 4°C on an overhead rotor. The antibody binding was done in siliconized 1.5 ml eppendorf tubes. The beads were washed 5 times with a total of 5 ml ice cold lysis buffer. The proteins bound to the antibody beads were eluted by cleaving off the tag on Orc, using TEV protease enzyme (Invitrogen). For TEV digestions, 1X TEV protease enzyme buffer and 1 µl of TEV protease enzyme (10 units) was added to the washed beads in a total volume of 100 µl. The beads were incubated at 16°C for 2 hours for TEV digestion. The eluate was collected by spinning the tubes and taking the supernatant without disturbing the beads pellet. ORC complex from 12.5 million cells were eluted in 140 µl 1X TEV buffer. Care was taken not to let the antibody coupled agarose beads to dry at any step of the experiment.

TCA precipitation of protein

100% Trichloro acetic acid (TCA) solution was mixed with 1/10 volume of 10% Sodium deoxycholate (DOC) solution before use. The mixing was done at room temperature, on shaker for half an hour. 1/4 volume of TCA/DOC mixture was added to the protein solution, and was incubated on ice for 40 min after a quick vortex. The samples were centrifuged at 20000 g for 20 min at 4°C. The supernatant was poured off and 100 µl ice cold Acetone was added to the pellet. The samples were centrifuged again at 20000 g for 20 min at 4°C. The pellet was air dried at RT for 30 min to 1 hour. The pellet was dissolved in minimal volume of SDS-sample loading buffer. The presence of trace amounts of acetone

sometimes gave yellow color to the sample and it was titrated with 1 M Tris HCl pH 8.5 to get the normal blue color (color is indicative of the sample pH).

In vivo DNA binding assay

All transfections were made in HeLa cells by Roti fect transfection method. The expression vector TO4/LacZ has 2xtetO sequences surrounding the TATA box of the CMV promoter (i.e. a “repression reporter”). Tet repressors (TR6) or Tet transactivators in the cell switch the expression of ß-galactosidase OFF and ON in absence and presence of Dox, respectively. In contrast, the pTRE/LacZ plasmid has a Tet responsive promoter (TRE, containing multimerized tet operators in front of minimal CMV promoter i.e. an “activation reporter”). Here, the Tet transactivator switches the expression of ß-galactosidase ON and OFF in absence and presence of Dox, respectively. Tet repressor should not induce expression of ß- galactosidase. After 3 days incubation, cells were harvested and ß-galactosidase activity is measure by Galacto-Light Kit (Applied biosystems) as per manufacturer’s protocol. As an internal control 10ng firefly luciferase expressing plasmid is co-transfected with all expression vectors. Relative intensity of luciferase is used to normalize the ß-galactosidase activity.

Electrophorectic mobility shift Assay

EMSAs were performed as described previously (Baron et al., 1997). Breifly, HEK 293 cells were grown in 10 cm dishes to 50-60% confluency and transfected via the Lipofectamine procedure with 10 ug of plasmid DNA encoding the various Orc fusions. 72 hours post-transfection total cell extracts were prepared as described before. Aliquots of the extracts (10 ul) were mixed with 10 ul of binding buffer (20 mM MgCl2, 20 mM Tris, pH 7.5, 10% glycerol, 2 mg/ml herring sperm DNA and 1 mg/ml bovine serum albumin, 2X protease inhibitor cocktail, 2mM ATP) and 2fmol 32P-end labeled tetO DNA (34 bp synthesized oligos). After 25 min, the reaction mixture was loaded onto a 5% polyacrylamide/0.13%

bisacrylamide gel containing 5% glycerol. Electrophoresis was carried out in 0.5x TBE (45 mM Tris base, 45 mM boric acid and 1 mM EDTA) at 10 V/cm.

Short term plasmid Replication assay

Plasmids are transfected into HEK 293 cells with Roti Fect transfection as described before. Cells are harvested 72 hours post transfection. Plasmid is extracted by method of Hirt (Hirt) as follows. Cells were washed twice with PBS and lysed with 1% SDS in 1X TE (Tris 8.0, EDTA 10mM) on the plate itself. 5M NaCl is added to the final concentration of 1M. Lysate is incubated in 4°C overnight and centrifuged at 15000 xg for 30 min at 4°C. Supernatant is incubated with 200ug/ml of RNAase for 1 hour at 37°C. Subsequently, 100ug/ml of Proteinase K is added at 37°C for 1 hour. After phenol:choloroform extraction DNA is ethanol precipitated in suspended in TE buffer or autoclaved water. To analyze the replication, extracted plasmids are linearized and digested with DpnI together a unique cutter for 3 hour to overnight. The DpnI resistant bands are analyzed by southern hybridization using appropriate radiolabeled DNA probes. Efficiency of DpnI digestion is tested by spiking 1ug of bacterial plasmid from DH5α or GM47 (Dam - strain) into Hirt extracts from untransfected cells.

Southern Hybridization

Digested DNA is resolved on 1% agarose gel. After analyzing the gel under UV, it's incubated with denaturation buffer for 30 minutes to get appropriate target for the radiolabeled probe. Overnight capillary transfer is used to transfer DNA to charged nylon membrane. After UV cross-linking, membrane is incubated with pre hybridization buffer at 65°C for 2 - 4 hours. In the mean time, probe is radiolabeled with [α-P32] CTP using NEblot kit. For SV40 replication experiments, AgeI/BsrGI digested fragment of pEGFP-C1 (Clontech) was used as probe. For tetO dependent experiments, XcmI/BglII fragment cut out from scTet plasmid was used as probe. Overnight incubation with radiolabeled probe

allows hybridization of probe with specific target DNA on nylon membrane. Membrane is exposed to phosphor plate after three washes and analyzed with phosphor imager.

Biotin-streptavidin coupling

For a standard coupling reaction, either 100pmol of 77bp biotinylated annealed tetO or 150pmol of 34bp biotinylated annealed tetO is used per mg of streptavidin coated paramagnetic beads. Beads are suspended in 100 ul of high salt binding buffer after two washes in the same buffer. Afterwards biotinylated DNA is incubated with the beads for 1-2 hours at 25°C with gentle shaking. The magnet is applied to collect the bound DNA. After three stringent washes with binding buffer. DNA coupled beads are resuspended in 100 ul of same buffer and stored in 4°C

Biotin DNA- Protein interaction

10pmol of streptavidin coupled DNA is used in a standard binding reaction. Final binding reaction contains 1x binding buffer, 60mM NaCl, 1mM ATP, 2x protease inhibitor cocktail, 1mg/ml ssDNA, 2mg/ml BSA and 10 fold excess of Poly (dA). Poly (dT). Purified ORC is incubated with the binding reaction for 10 minutes at 4°C. Then tetO is added to the reaction. After 30 minutes incubation at 4°C, magnet is applied to collect the bound protein. Beads are boiled with 10ul of 1x SDS buffer after three stringent washes with 1x binding buffer. Protein is loaded on 10% SDS gel and silver stained.

Purification of recombinant ORC using Talon beads

In parallel to HA antibody purification, ORC is also purified through His-tag using talon beads (Clontech). Protein was expressed in 15 cm dishes with Hi-5 cells at a density of one million cells/ ml. Cells were harvested and washed twice with PBS. lysis buffer. Nuclear extract is made as described before. Typical volume of

nuclear extracts obtained from a 15cm dish is 1ml. To this, 50 -60ul of talon beads is added. Beads have to be washed twice in the lysis buffer before adding to nuclear extracts. After incubation at 4°C for 2 h, bound protein is spun down at 700g for 2 minutes. 4-5 washes with Lysis buffer (without 300mM KCl), His-tagged protein is eluated batch-wise in lysis buffer with 400mM imidazole, pH 5.0. Two rounds of elution at 4°C incubation for 30 min each, generally gives more than 80% of bound protein. Eluted protein is snap frozen and stored in -80°C.