Discussion

The Origin recognition complex (ORC) is the first protein to be loaded on the DNA during the ordered protein assembly known as pre-RC (Pre replicative complex). ORC binding to DNA initiates the binding of other pre-RC proteins like Cdc6 and Cdt1 to the origin. With the recruitment of MCM complexes to the origins, pre-RC formation is completed (Fig. 3) and bidirectional, semi-conservative DNA replication is initiated in the S phase, in a CDK dependent manner.The discovery of ORC in budding yeast (Bell and Stillman, 1992) leads to the identification of homologs of ORC in higher eukaryotes including humans.
Much of what is known about the initiation of DNA replication in eukaryotes has been discovered through the work carried out *in S. cerevisiae* (see introduction). The presence of genetically defined origins of replication (ACS) and the ease of genetic manipulation provides extremely useful *in vivo* and *in vitro* assays for studying the initiation of DNA replication in budding yeast. However, no such advantages are present in mammalian system and absence of any genetically characterized origin has further eluded the dissection of replication initiation mechanism. While the ScORC binds the DNA in a sequence specific manner, which as a matter of fact was the basis of its discovery, HsORC has not even a single genetically characterized specific DNA sequence, to date.

In this study, we have conferred sequence specificity to human ORC by fusing it with TetR DNA binding moiety. Various empirically designed Orc fusions were expressed in HeLa cells. Based on the protein expression profile, best possible Orc fusions were chosen for further studies. The functionality of DNA binding domain and fused Orc subunit was analyzed. HeLa cells stably expressing Orc fusions were made to analyze interaction with endogenous Orc subunits. The characterized fusion proteins were then tested for their ability to replicate plasmids carrying target DNA sequences in HEK 293 cells. We have thus

generated an extremely useful system where an engineered human ORC has been shown to initiate plasmid replication from defined DNA sites. Recently, an independent study has shown similar results by fusing Gal4 DNA binding domain with different Orc subunits (Takeda et al., 2005). In an attempt to further investigate ORC DNA binding in our system, engineered human ORC was overexpressed in insect cells. Finally, the affinity purified ORC is used to investigate DNA binding in the presence of specific and unspecific competitors.

Part I – Engineering a sequence specific DNA binding human ORC

The main objective of this study was to construct a system where in an artificial ORC is recruited to specific DNA sequences. The recruitment ORC to DNA is known to initiate PreRC formation and then DNA replication (Bell and Dutta, 2002). So, we hypothesized that recruitment of human ORC to specific DNA sequences through a heterologous DNA binding protein could potentially lead to pre-RC formation, which in turn triggers a preferential origin activity at these sites. A monomerized version of tet trans regulator named as scTet (Krueger et al., 2003) was fused at the N terminus of various Orc subunits. scTet with only one regulatory domain will minimize the steric hindrances and potential influence on the activity of trans regulator as well as the fused Orc domain. Moreover, with wtTetR one would get two copies of ORC per pre-RC, in contrast to widely held opinion of one ORC per pre-RC. Out of six Orc subunits, we used Orc2, Orc4 and Orc1 in this study. Unlike ORCs from other species, human ORC is known to form sub-complexes, with Orc2 or Orc4 being the part of core-complexes (Ranjan and Gossen, 2006). So using these subunits would ensure the detection of ORC activity at sub-complex levels. Moreover, *S.pombe* Orc4 has an N terminal AT hook that has been shown to recruit ORC to AT-rich DNA sites that specify origins in *S.pombe* (Chuang and Kelly, 1999; Lee, J. K. et al., 2001). Orc 4 from other species lacks this AT hook domain. So fusing a sequence specific DNA binding domain at N terminal of human Orc4 seem analogous to naturally occurring AT hook domain at N terminal of *S.pombe* Orc4. If additional AT-hook

domain does not interfere in the complex formation ability of *S.pombe* ORC then tetR fused human Orc4 was expected to integrate into a stable ORC complex as well.

In order to further avoid steric hindrance, arising during a bulky ORC formation, a flexible linker was inserted between Orc subunit and TetR moiety. However, such fusion constructs can be susceptible to proteolysis, hence we begin with two empirically designed linkers (Fig. 6). The F-linker is identical to the linker used to monomerize TetR protein (Krueger et al., 2003) and hydrophilic G-linker, which was used originally to fuse PCNA and GFP (Leonhardt et al., 2000) has been also shown to construct a functional Orc4 and GFP fusion (McNairn et al., 2005). As a standard nomenclature Orc4 subunits fused through F and G linkers were named 4F and 4G respectively. Similarly, Orc2 fused through F and G linkers were named as 2F and 2G. Orc1 fusion, which we selected later in this study, was made without any flexible linker, in order to test the effect of a flexible interface on replication activity. Although no external linker was used, the flexibility at junction between Orc subunits and Gal4 DNA binding domain is regarded as the main feature for catalytic domains to come in direct contact with adjoining target DNA sites without any interference (Takeda et al., 2005).

Expression of Orc fusions in mammalian cells

Plasmids expressing various Orc fusions were individually transfected in HeLa cells. A non-functional ORC due to the overexpression of Orc fusions would have been detrimental to cells, as studies in various species have shown that ORC is absolutely required for DNA replication (Bell et al., 1993; Bell et al., 1995; Dhar et al., 2001; Pflumm and Botchan, 2001). Overexpressed subunit could destroy the stoichiometry by incomplete subcomplex formation. Even though overexpressed protein fails to integrate in the complex, it could titrate out essential pre-RC proteins proving toxic to the cells. No apparent cellular toxicity was observed during the transient expression of Orc fusions in HeLa cells. Nevertheless the

interference in the endogenous ORC's stoichiometry can't be ruled out due to overexpression of Orc fusions. This effect would have been more prominent in a long run, however we harvested the cells 3 days after the transfection to test the expression levels.

Expression profiles from various Orc fusions showed that F constructs (both 4F and 2F) gave a lower molecular weight band when probed with αTetR or αFlag antibodies (Fig. 8). However, no such bands were visible with antibodies against Orc subunits for the same protein extracts. Most probably, these bands represented the proteolytic fragment containing scTet and Flag tag part of the fusion protein. As no proteolytic bands were observed for G constructs (both 4G and 2G), we selected these constructs for further studies. We observed a substantial decrease in the endogenous Orc2 expression level when 2G fusions were transiently expressed (Fig. 8B). This interplay between endogenous and exogenous Orc2 subunit points toward a cellular mechanism that maintains a constant Orc2 protein levels. Most likely, the participation of exogenous Orc2 protein in complex formation would have lead to the rapid degradation of excess of endogenous Orc2 subunit. Expression dynamics between endogenous and recombinant Orc2 has been reported in two independent studies as well. In first, RNAi mediated knockdown of endogenous Orc2 lead to the increase in the expression level of recombinant Orc2, which was stably expressed in mammalian cells (Anand Ranjan, unpublished data). In second, recombinant Orc2 was shown to suppress the expression of endogenous Orc2 (Radichev et al., 2006) However recombinant Orc2 with mutations in nuclear localization domain and ORC assembly domain showed no suppression of endogenous Orc2 protein. Hence, mammalian cells limit intracellular levels of Orc2, thus limiting the amount of functional ORC in the cell.

Sequence specific DNA binding by Orc fusions

Fusing two proteins together often result in the loss of functionality of either one or both components involved. The failure of Gal4-Orc5 and Gal4-Orc6 to stimulate the replication activity was mainly attributed to the negative effects of fusion on the individual proteins (Takeda et al., 2005). Hence, it was critical to analyze the functionality of TetR DNA binding moiety in the fusion proteins. *In vivo* Dox dependent DNA binding by Orc fusions confirmed that no negative effect has been caused to the tetO binding due to the fusion. The DNA binding activity as observed by ß- galactosidase intensity was similar for Orc fusions and wild type scTet (Fig. 12). Different studies suggest that recruitment of transcription activators to chromatin alters ORC binding due to the modification of chromatin structure (Aggarwal and Calvi, 2004; Danis et al., 2004). The TetR moiety itself is transcriptionally inactive; therefore in our activator assay only TetR moiety fused with transcriptionally active, VP16 domain was able to activate the expression of LacZ (Fig. 13). Orc subunits fused to TetR were not able to activate the transcription of LacZ, indicating that Orc fusions are transcriptionally inert as well. Even with the Gal4 DNA binding domain, Orc subunits showed no transcriptional activation (Takeda et al., 2005). Hence, this property of the Orc subunits seems to be valid irrespective of the DNA binding moiety.

Electrophoretic mobility shift assays (EMSA) with end labeled target DNA sequence (34bp tetO) was used to assess the ability of Tet moiety in Orc fusions to interact with tetO sequences *in vitro*. As there are no data available on the gel shifts by scTet proteins, I first established the conditions for the EMSAs with scTet. Protein extracts containing tetracycline transactivator (tTA) and scTet were incubated with P-32 labeled tetO. The result showed a clear Dox dependent DNA binding by scTet (Fig.11A). For EMSAs with Orc fusions, it was expected that the part of transiently expressed Orc fusions would interact with the endogenous Orc proteins to form subcomplexes. Hence, the shifts obtained in these experiments (Fig.11A) gave a qualitative measure of where to expect the shifts from individual Orc fusions. However the shifts obtained from the HEK 293 protein extracts overexpressing Orc fusions indicated only the individual Orc

fusion interaction with tetO (Fig. 11B), we confirmed it by preparing protein extracts from Sf9 cells infected with only one baculovirus expressing individual Orc fusions (Fig. 11C). With HEK293 protein extracts, we expected to see at least a weak signal representing shifts from the holocomplex or sub complexes. However, we could not see any such signal, except some Dox dependent smear (data not shown), which could possibly due to the binding of recombinant ORC. Another important aspect is the stability of the complex during the protein extract preparation and in the gel matrix. We found that increasing the final concentration of protease inhibitor to 2 folds more prevents any proteolysis occurring during the experimental procedure. However, running EMSA gels at room temperature as compared to 4ºC did not affect the results. In any case, under our experimental conditions, this binding does not seem to be stable as we were never able to obtain clear shifts from the complex *in vitro*. To date, not a single study has shown a clear human ORC DNA binding through EMSAs. It could possibly due to the intrinsic DNA binding by human ORC and no preference for a particular DNA sequence (Baltin et al., 2006; Vashee et al., 2003). With our system we are in a position to address this problem. Nevertheless it would require optimization of variables like non-specific competitors, choice of gel matrix, length of probe DNA etc.

Stable expression of Orc fusions by Sorting-subcloning

The initial attempts to generate stable cells for Orc fusions through traditional antibiotic selection method proved to be futile. Stable transgene expression is known to occur at very low frequency under the antibiotic selection pressure (Felgner et al., 1987; Gubin et al., 1999). Moreover, antibiotic selection induces some deleterious effects such as growth inhibition (Gu et al., 1992; Kim et al., 1998). The concentration of the antibiotic in the culture medium even had demonstrable effects on transgene expression within those antibiotic resistant cell lines (Schott et al., 1996). Moreover, Orc itself is a cell cycle regulated protein and the formation of a functional ORC complex is an absolute necessity

for DNA replication. As we didn't observe any apparent cellular toxicity during the antibiotic selection, we adopted ' sorting-subcloning' (see materials and methods) to generate stable cells lines for 4G. 'Sorting -subcloning' has already been shown (Liu W, Unpublished data) to be a successful strategy to obtain clones with sustained transgene expression over a long period of time. In contrast to antibiotic selection, this approach provides cells a consisitent culture condition. FACS sorting allows efficient separation of EGFP positive cells after the transfection. After the first round of sorting, EGFP expression in the majority of cells is most likely transient due to non-integration of the plasmid construct. A large portion of cells will revert to a EGFP negative state due to loss of the transfected DNA. Thus, a second round of FACS sorting is necessary. After two consecutive rounds of sorting EGFP positive cells have most likely a stably integrated transgene construct. As screened by western blot (Fig. 15) high efficiency of positive clones were obtained through 'sorting subcloning'. EGFP is an inert protein and has never been reported to have interfered with the normal cellular mechanisms. Never the less, flanking loxP sites in our system provide us with the possibility to excise EGFP by Cre expression (Fig. 14). The stable 4G clones thus obtained show a normal cell cycle progression (Fig 16.C) as observed by propidium iodide staining. Before, randomly integrating into the host choromosome the transfected DNA forms concatomer. Hence we expected the reduction of transgene's copy number to one. Consequently, after the Cre recombinase expression, we observed a decrease in the intensity of 4G band on the western blot (Fig. 16B). As expected, the expression of Cre recombinase would most probably have excised the 4G-transgene positioned internally to the loxP sites along with the EGFP.

Co-immunoprecipitaiton experiments in the stable 4G cell lines with the antibody against Orc1 show that 4G interact with endogenous Orc subunits (Fig. 17). This means that 4G participate in cellular ORC, indicating that inside the cell there exit two sets of complex. One, where Orc1 forms complex with endogenous Orc subunits and the other where 4G integrates into the complex instead of Orc4. As

we know that ORC has single copy of each subunit, it's not possible that both 4G and Orc4 can interact together with Orc1 in the same complex.

Target DNA dependent replication of the plasmid

Having well-characterized origins in *S. cerevisiae* has immensely facilitated the study of replication initiation mechanism. The initial discovery and characterization of ORC were possible due to the knowledge of ARS sequences (Bell and Stillman). Plasmid transformation experiments that were successful in budding yeast have failed to identify autonomously replicating sequences (ARS) in higher eukaryotes. Under these conditions, it is difficult to observe changes occurring during replication initiation mechanisms due to the lack of sequence specificity of origins. One way to circumvent this difficulty is to use SV40 replication system (Waga and Stillman). Replication of SV40 genome begins at specific 64-bp region and requires only large T antigen (the viral initiator protein) in addition to cellular replication machinery. Large T antigen binds to the SV40 origin and facilitates DNA unwinding and recruitment of the host replication factors. Although the SV40 system mediates DNA replication initiation in mammalian cells, it does not require pre-RC proteins. Hence it is not suitable for studying the formation of pre-RC. As a result, we aimed at creating a system where ORC is recruited to specified DNA sequences and triggers replication preferentially at these DNA sites. During the investigation we found that high levels of ORC expression is a necessity to be able to get a positive read out with short term replication assay using our system. As plasmid carrying multimerized tetO (target DNA sequences) didn't show any DpnI resistant band in HeLa cells stably expressing 4G. HeLa cells are known to express relatively low levels of endogenous proteins as compared to HEK293 cells. At these levels only a small fraction of transfected plasmid could possibility undergo replication. Given, the low efficiency of these short-term plasmid replication assays, it is likely that no signal was detected with the Hirt supernatants. Nevertheless our Hirt supernatant preparations showed an efficient extraction of the transfected plasmid as

observed by the southern blots with only linearized DNA (data not shown). However, we don't know what fraction of the transfected plasmid was able to make its way in the replication competent compartment of the cell. It is reasonable to assume that most of the DpnI digested plasmid never got a chance to reach the right cellular compartment.

We made use of SV40 origins to establish the short-term replication system in transient environment. T antigen is a sole viral initiator required to initiate replication from SV40 origin of replication. Plasmid carrying SV40 origin replicates efficiently in HEK 293 cells expressing large T antigen as observed by the presence of DpnI resistant band in the southern blots (Fig. 18). However, the intensity of the same band remains at the background level when tested with 293 cells without any large T antigen.

We observed plasmid replication in tetO7-4G (4G plasmid inserted with heptamerized tetO) and tetO7-2G (2G plasmid inserted with heptamerized tetO) in constrast to 4G and 2G plasmids, without having target DNA sequences (Fig. 19A). The inability of 4G and 2G plasmids to replicate was not due to the failure of protein expression, as immunoblots made with extracts from the transfected cells show efficient expression by all the plasmid constructs (Fig. 19B). Thus, the replication of a plasmid in our system is dependent on the presence of target DNA sequences on it. In case of tetO7-4G or tetO7-2G, engineered ORC binding to specific DNA sites would have triggered the replication initiation by recruiting other pre-RC proteins, as shown for Gal4-Orc fusions by the CHIP experiments(Takeda et al., 2005). However, due to the absence of tetO in 4G or 2G, no specific DNA – protein interaction would take place. Hence replication is not observed with these plasmids, although proteins were expressed efficiently. We have also observed plasmid replication in 293 cells co-transfected with Orc fusions (either 4G or 2G) expressing plasmid and a response plasmid carrying tetO sequences. However, having target DNA sequences on the same plasmid provides higher replication efficiency as compared to co-transfection. It would be

interesting to analyze the plasmid replication in 293 cells stably expressing Orc fusions.

On the analysis of the protein lysates obtained from HEK293 cells transfected with various Orc fusion plasmids, we observed that after a period of one week the replication positive plasmids, tetO7-4G or tetO7-2G, showed higher protein levels when compared with protein lysates from the cells transfected with replication negative plasmids, 4G or 2G (Fig. 20). Higher protein levels indicate higher protein expression most probably due to more copies of the plasmids as a result of replication inside the mammalian cells. Over a period of 7 days, transient expression would have decreased; as a result, the increase in plasmids copy number due to replication in mammalian was visible in terms of higher protein levels.

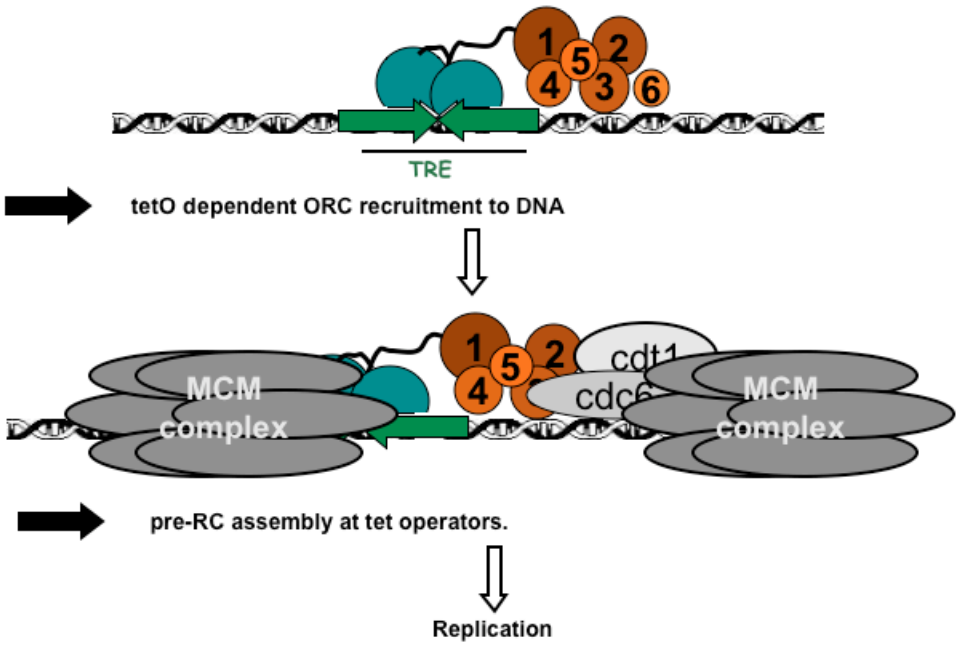

Fig. 26. Proposed model for tetO dependent plasmid replication by engineered human ORC. Based on the results that ORC fused with scTetR domain supported replication of plasmid containing tetO sequences, we proposed the above model. First, ORC is recruited to the target DNA sequences through TetR. After that, it recruits other proteins like Cdc6, Cdt1 and MCM complex in G1 to form a pre-RC at tetO. With the change in cdk activity the origin fires and starts the bi-directional, semi-conservative replication in S phase

One of the main advantages of using TetR is its Dox responsiveness. We had expected the system to be Dox controlled. Surprisingly no difference in the replication activity in presence of Dox was observed (data not shown). There could be various reasons for it. One of the most important is the mechanism of ORC formation and its recruitment to DNA. In a scenario where ORC is recruited to DNA as a holocomplex, adding Dox can control only TetR-tetO binding but not the intrinsic ORC-DNA binding. Hence as a result of its intrinsic DNA binding, once recruited to DNA ORC would remain bound to DNA even in the presence of Dox. To circumvent this problem, we treated cells with Dox before transfections so that expressed TetR-Orc fusions cannot bind to tetO. Even under these conditions, we observed similar replication activity in presence or absence of Dox (data not shown). So we decided to analyze Dox effect *in vitro* with purified ORC complex.

One of the ways to make this system inducible is the use of an inducible promoter for the expression of Orc fusions. However, choice of transcriptional activator and Orc fusion partner should be carefully made to avoid any competition for the target DNA by both of them. Nevertheless, we have created a system where recruitment of ORC to the target DNA sequences supports plasmid replication. Based on our results, we propose a model (Fig.26), which shows how replication would initiate at the target DNA sites through recruitment of artificial ORC at these sites. At first, artificial ORC is recruited to tetO as a holocomplex. This initiates the formation of pre-RC at these sites. With the change in cdk activity DNA replication begins in S phase

Part II DNA binding analysis of purified Engineered ORC

In vitro complex formation

Baculovirus based insect cell expression system has been used previously for the efficient purification of ORC (Giordano-Coltart et al., 2005; Ranjan and Gossen, 2006; Vashee et al., 2003). We utilized the same approach to investigate the *in vitro* complex forming ability by the Orc fusions. We observed an inverse co-relation between expression level and solubility for single subunit infections in insect cells (Fig.21). With soluble protein giving weaker bands in the whole cell lysates and vice versa. Affinity purification of nuclear extracts prepared from insect cells co-infected with different Orc subunits show the efficient integration of Orc fusions in the ORC complex (Fig. 22). With the increased molecular weight as a result of scTetR fusion, both 4G and 2G migrates at much higher molecular weight level as compared to their wild type counterparts. Orc6 does not form the part of stable complex as shown by glycerol gradient experiments (Ranjan and Gossen, 2006). So we did not include it in our affinity pull down experiments. Stoichiometry of various subunits in the complex is comparable to the wild type protein. Hence possibility of Orc fusions coming through a subcomplex and not from the holocomplex is quite unlikely. Orc1 fusions also integrate efficiently in a complex. However, these fusions were found replication incompetent during the short-term replication assay (data not shown). Hence, the failure to replication plasmid by Orc1 fusions was not due to the inability to form ORC.

Purified ORC binding with tetO

Human ORC does not effectively discriminate between so called origin and random DNA sequences (Vashee et al., 2003). However, it has several folds more affinity for poly(dA). poly(dT) sequences than naturally occurring DNA fragments. Preferential binding of HsORC to AT rich sequences is shared by

S.pombe ORC (Chuang and Kelly, 1999). Here ORC DNA binding is mediated by a unique N terminal AT hook domain in SpOrc4. Therefore it is interesting to speculate that HsORC may be recruited to DNA by its interaction with an AT hook protein. However, no evidence of such a protein has been reported to date.

His-tag at the C-terminus of the Orc1 subunit was utilized to purify the ORC in our experiments. The quality of purified ORC is analyzed on silver stained gel. Pull down experiments with biotinylated tetO showed efficient enrichment of purified ORC2G (Fig. 23). Hence, complex formation didn't mask the TetR tag, as it was able to recognize tetO sequence *in vitro*. Like plasmid replication assay, no Dox effect was observed for *in vitro* DNA binding by ORC2G (Fig.25A). This is highly unexpected because it is known that in the presence of Dox, the changed conformation of TetR can no longer bind to tetO. Moreover, we added 10 fold excess poly(dA). poly(dT) as competitor DNA to titrate out the intrinsic DNA binding by ORC. However due to some unknown mechanism ORC2G binds to tetO even in the presence of Dox. Now, it is also possible that ORC2G binding to tetO is unspecific and not tetO dependent. Our competition assay disproves this argument because, ORC2G – tetO binding can be titrated out by adding increasing amounts of untagged tetO in the reaction (Fig. 25). Where as 10 folds more of non-biotinylated tetO (specific competitor) substantially reduces ORC2G binding with biotinylated tetO, no effect on biotinylated DNA binding was observed by adding 10 folds more of poly(dA). poly(dT). To date, HsORC has been known to have preferential affinity for poly(dA). poly(dT). However, with our system we have conferred to HsORC, a more robust and higher affinity towards tetO. Although, we have shown that engineered human ORC binds preferentially to tetO, it would be interesting to probe further into non-responsiveness for Dox. It is widely accepted that DNA binding of ORC is through Orc1. So it is possible that intrinsic DNA binding activity of ORC due to Orc1 subunit is preventing to show any positive read outs for Dox effect, in our experiments. With the baculovirus expression system, one can express ORC (2-5) and test it for Dox responsiveness.

In conclusion, fusing N terminal of Orc subunits with a sequence –specific DNA binding protein doesn't interfere with the complex formation ability of ORC. *In vivo* such a fusion protein can replicate plasmid carrying the target DNA sequences. This could be an extremely useful assay for studying the functions of various initiator factors involved in pre-RC formation. Additionally, the utility of this system in long-term protein expression could be a very useful tool for gene delivery.

Future perspectives

Although replication initiator factors have been discovered for more than a decade now, their precise role in replication initiation is not known. With the extremely useful plasmid replication assay we could be able to dissect the functions of various initiator proteins involved in the pre-RC assembly. Orc fusions with point or deletion mutants can readily identify amino acids indispensable for DNA replication.

Owing to the unspecific DNA binding by HsORC, not even a single origin of replication has been genetically characterized to date. We can translate this system to the genomic level by using mammalian cell lines with tetO sequences stably integrated into their genome. These sites could function as an origin of replication where the mechanism of replication initiation can be efficiently analyzed.

Efficiency of the plasmid replication can be probed further by using different permutation and combination of target DNA sequences on the plasmid. One of the interesting possibilities is to insert the tandem arrays of heptamerized tetO and observe the increase in the DpnI resistant band intensity in southern blot. One of the major challenges would be to probe the cause of Dox ineffectiveness.

Self-replication of plasmid carrying the target DNA sequences shows the long-term protein expression. Such a system has practical implications in gene therapy. To date not even a single non-viral episomal vector for gene delivery has been reported. Viral episomal vectors require the introduction of viral based proteins, raising questions about their safe and long-term gene delivery. The efficiency of plasmid replication has to be optimized in our system before replication competent plasmid can be made useful for gene delivery. We have tried to improve the plasmid replication efficiency by inserting target DNA

sequences on the same plasmid expressing Orc fusions. Additionally, stable overproduction of the Orc fusion proteins could improve the plasmid replication efficiency. The incorporation of an element in the vector that promotes its attachment to chromosomes to ensure proper segregation could also be useful for its stable maintenance in the cell over many generations.

The inducible system would provide us an additional level of regulation for studying the replication initiation mechanism using at artificial origin sites. One indirect way to circumvent Dox ineffectiveness is to control the replication at the protein level by using an inducible promoter to derive the expression of Orc fusions. Under off state, promoter will not express the fusion protein and no replication of plasmid will take place. However as the promoter is switched on, expressed Orc fusion forms a complex with endogenous Orc subunits and initiate replication by binding the target DNA sites. In order to implement such a strategy, one must carefully choose the transcriptional activator and DNA binding moiety (for Orc fusions) to avoid competition between both of them for target DNA.

Summary

Origins of DNA replication are the cis elements, which are specifically recognized by trans factors called initiators. The Origin Recognition Complex (ORC), a six-subunit protein complex selects the sites of DNA replication in eukaryotes. A uniformly distributed origin sequences is a basic necessity for the precise duplication of eukaryotic genome. However in humans, no uniform pattern of origin selection is reported to date. This is mainly attributed to the unspecific DNA binding by human ORC.

The work presented in this thesis focused on the unspecific DNA binding activity of human ORC. We reconstituted a recombinant HsORC where sequence specific DNA binding is artificially imposed through a heterologous DNA binding domain. *In vitro* DNA binding analysis with purified recombinant protein showed the high degree of specificity for the target DNA sequences. The affinity of artificial protein for target DNA sequence was several folds more as compared to the affinity of the wild type protein for the AT rich sequences.

In a parallel approach using these engineered initiator genes, we tried to establish *in vivo* replication of the plasmid with specific sequences in mammalian cells. Recognition of initiator proteins to DNA sequences lead to the assembly of protein factors, which initiate DNA replication, preferentially at these sites. Plasmids without specific sequences failed to replicate in mammalian cells. This system provides an effective way to analyze the function of mammalian initiator proteins. Our study has practical implications for the development of episomal vectors in gene delivery.

Zusammenfassung

Origins für die DNA-Replikation sind cis-Elemente, die spezifisch durch trans-Faktoren, die man Initiatoren nennt, erkannt werden. Der ’Origin Recognition Complex’ (ORC) besteht aus sechs Untereinheiten und bindet die Origins bei der Initiation der DNA-Replikation von Eukaryoten. Die gleichmäßig auf der DNA verteilter Origin-Sequenzen sind Grundvoraussetzung für genaue Verdopplung des Genoms. Jedoch ist beim Menschen bisher keine einheitliche Origin-Sequenz bekannt. Der Grund dafür liegt in der unspezifischen DNA-Bindung der humanen ORC-Proteine.

Diese Arbeit beschäftigt sich mit der unspezifischen DNA-Bindung der humanen ORC-Proteine. Dafür wurden rekombinante ORC-Proteine hergestellt, die sich durch eine künstlich eingeführte DNA-Bindedomäne spezifisch an bestimmte DNA-Sequenzen binden können. Durch *in-vitro*-DNA-Bindungsanalysen konnte die Bindung dieser rekombinanten Proteine an spezifische DNA-Zielsequenzen gezeigt werden. Des Weiteren konnte eine deutlich höhere Affinität dieser Proteine zur Zielsequenz nachgewiesen werden, als das Wildtyp-Protein zu einer AT-reichen DNA-Sequenz.

In weiteren Versuchen wurden die rekombinanten Initiatorgene in vivo getestet, wobei die Replikation von Plasmiden mit den spezifischen Zielsequenzen in Säugetierzellen untersucht wurde. Aufgrund der Erkennung der Zielsequenzen durch die Initiatorproteine erfolgte die Rekrutierung von anderen Replikationsfaktoren, die daraufhin die Replikation der Plasmide verursachten. Dagegen zeigten Plasmide ohne diese Zielsequenzen keine Replikation in den Zellen. Diese Methode erlaubt eine effektive Analyse der Funktion der Replikationsinitiationsproteine von Säugetieren. Des Weiteren könnte dieses System Anwendungen bei der Entwicklung von episomalen Vektoren für die Gentherapie finden.

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Appendix

I. Abbrebiations

°C	degree celsius
aa	amino acid
AAA+	A superfamily also containing AAA proteins
ACS	ARS consensus sequence
Amp	ampere
ARS	autonomous replication sequence
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CDC6	cell division cycle 6
CMV	human cytomegalovirus
dATP	deoxy adenosine triphosphate
DNA	deoxyribonucleic acid
Dox	doxycycline
ds	double stranded
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
FCS	fetal calf serum
g	gram
HDAC	histone deacetlayase
Hs	*Homo sapiens*
IP	immunoprecipitation
K	kilo
Kb	kilo base pair

kDa	kilo dalton
l	liter
m	milli
M	molar
MCM	minichromosome maintenance
min	minute(s)
Orc	subunit of ORC
ORC	origin recogniton complex
ori	origin
PAGE	poly acrylamide gel
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylchloride
pre-RC	pre-replicative complex
PVDF	polyvinylidene fluoride
RFC	replication factor C
RPM	rotations per minute
RT	room temperature
sc	Single Chain
Sc	*Saccharomyces cerevisiae*
SDS	sodium dodecylsulfate
Sp	*Saccharomyces pombe*
ss	single stranded
SV40	simian virus 40
TEMED	tetramethylenediamine
TetR	tetracycline repressor
tetO	tetracycline operators
Xl	*Xenopus laevis*

II. Schematic representation of Orc fusion expression vectors

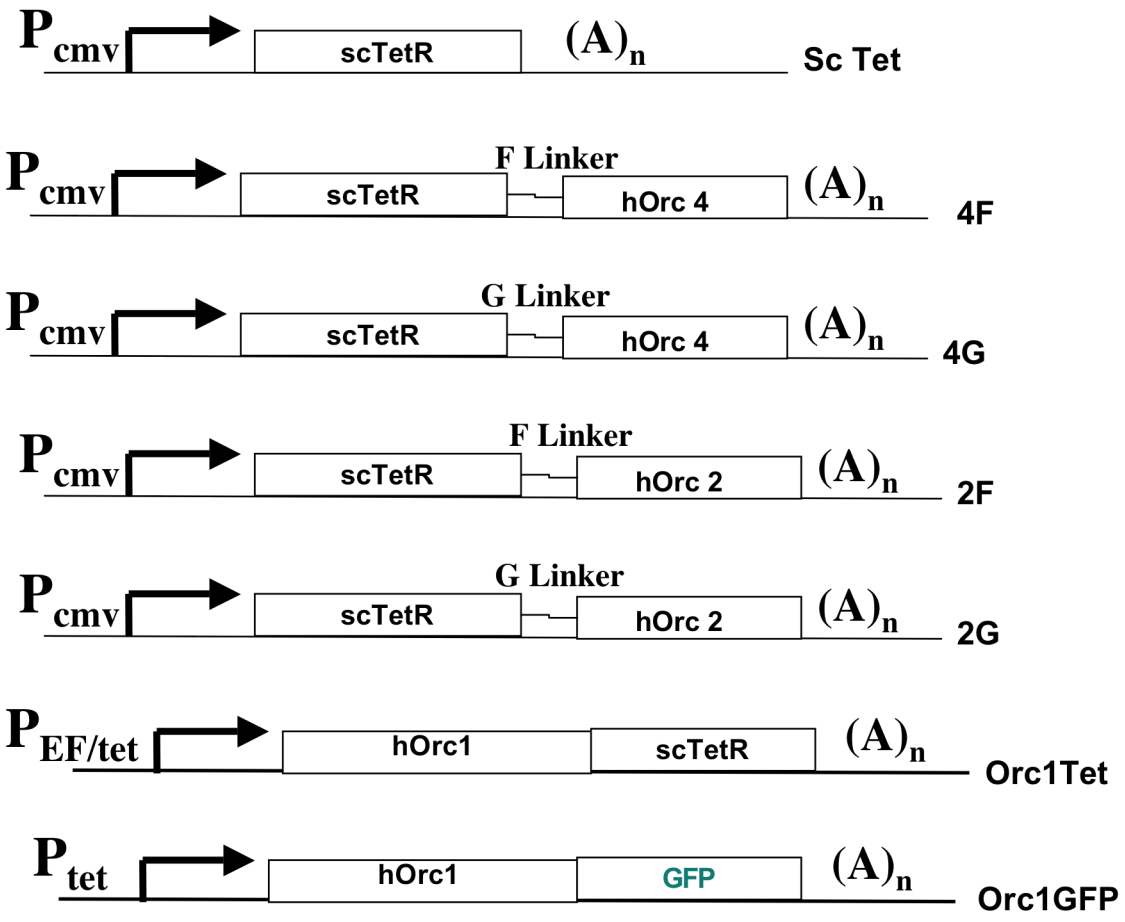

Fig. II Important elements present in the vectors expressing Orc fusions. Either constitutive CMV/EF promoters or inducible tetrycline controlled promoter.drives the expression of Orc fusions. DNA binding domain was fused at the N terminus of Orc2 or Orc4 and a flexible linker was inserted between TetR and Orc moeties . C terminus of Orc1 was used to generate Orc1 fusions and no flexible linker was used.

III. Schematic representation of replication positive Orc fusion vectors

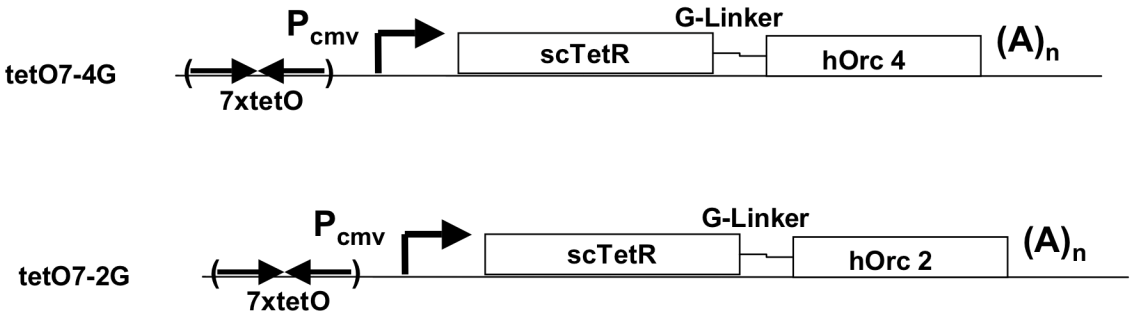

Fig. III Important elements present in the replication positive Orc fusions. A heptamerized tetO (7xtetO) insert cloned upstream of CMV promoter of previously described (Appendix Fig. II) 4G and 2G resulted in tetO7-4G and tetO7-2G, respectively.

IV. Schematic representation of response plasmids used for in vivo DNA binding experiments

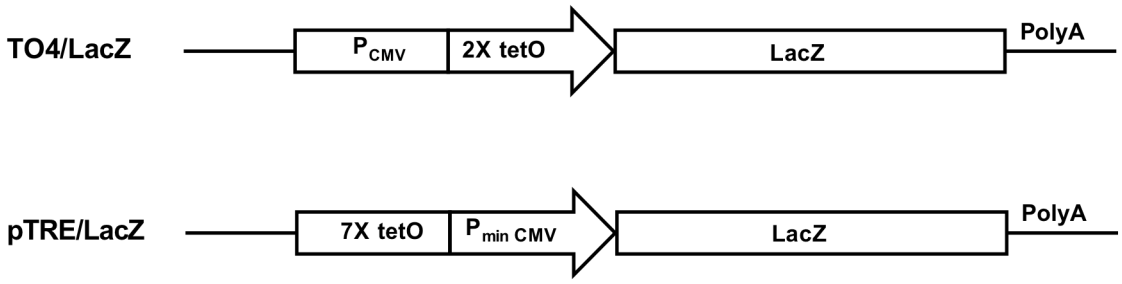

Fig. IV Important elements present in the repressor (TO4/LacZ) and activator (pTRE/LacZ) reponse plasmids. TO4/LacZ contains 2xtetO upstream of the TATA box in CMV promoter. Binding of any tet trans regulator to these sites will inhibit the expression of downstream lacZ gene. pTRE/LacZ contains a minimal CMV promoter. Binding of any transcriptional activator at heptamerized tetO (tet responsive element) sites upstream of minimal CMV promoter will trigger the expression of downstream lacZ gene. However, tet transregulators exhibiting no transcriptional activation would not result in lacZ expression.

V. Modified Sorting –subcloning strategy to generate stable cell lines for transgene of interest.

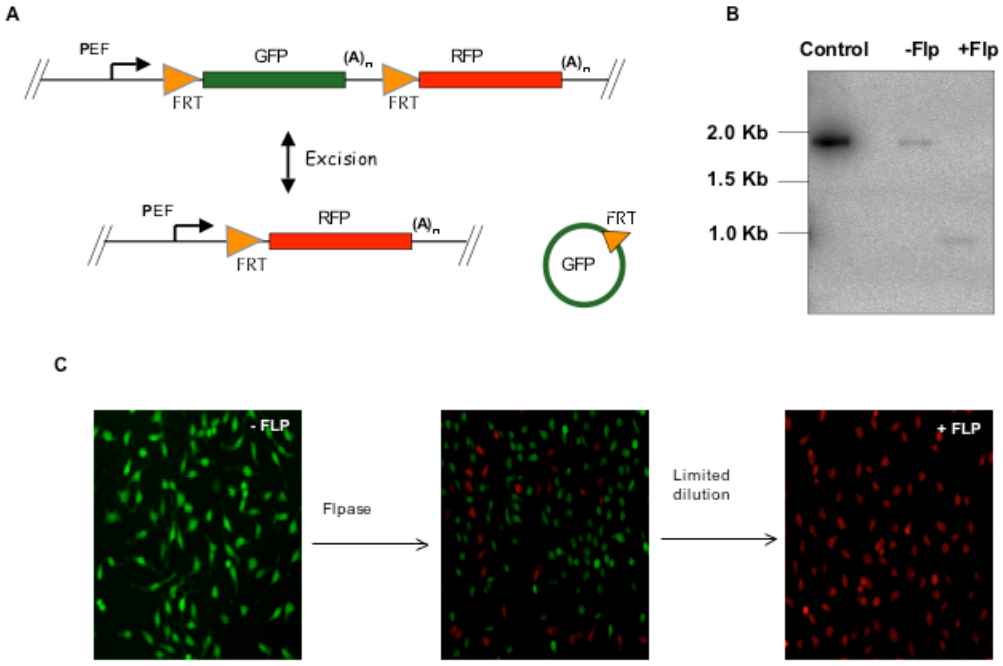

Fig. V Strategy to stably transfect any transgene of interest using a modified "Sorting-Subcloning"method.
HeLa cells were transfected with pFRT-EF-EGFP-mRFP. Stable clones were isolated by "Sorting-Subcloning" approach. A) Scheme to switch ON the expression of RFP (or any transgene) in stable GFP clones. B) The excision efficacy of Flp-recombination of FRT flanked EGFP was analyzed by southern blot. +FLP clones show a reduction of the band size (+Flp) whereas -FLP clones maintain the original band size, as observed in –Flp and control lanes. C) The change of fluorescent signal from green to red is observed under the fluorescent microscope after transfecting – FLP cells with recombinase expressing plasmid. Isogenic clones were picked by lmited dilution of this population. Instead of mRFP one can insert any transgene to obtain stable and homogebous expression using the above strategy.