Forced binding of the origin of replication complex to chromosomal sites in *Drosophila* S2 cells creates an origin of replication

Gilles Crevel and Sue Cotterill*
Department Basic Medical Sciences, St Georges University London, Cranmer Terrace, London, SW17 0RE, UK

*Author for correspondence (s.cotterill@sgul.ac.uk)

Accepted 5 September 2011
Journal of Cell Science 125, 965–972
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doi: 10.1242/jcs.094409

Summary
Origins of replication in higher eukaryotes appear to lack specific sequence characteristics and those mapped often appear to be spread over several kilobases. This has complicated the study of site-specific events at origins of replication in vivo. Here we show that fusion of a Gal4-binding domain to proteins of the origin of replication complex (Orc) is sufficient to direct initiation to Gal4-binding sites inserted in the *Drosophila* S2 cell chromosome. The activation appears to go via an authentic route, taking place only in the S phase of the cell cycle and involving the formation of a prereplication complex. We have also shown that the origin-associated acetylation of histone H4 at K12 can be directed to the region of Orc binding by the presence of Orc. We expect that this system can provide a useful tool for the study of site-specific events at origins of replication in higher eukaryotes and a means to dissect Orc-dependent and Orc-independent events at origins.

Key words: Orc, DNA replication, Origin of replication, Gal4, *Drosophila* S2 cells, Histone acetylation

Introduction
In *Saccharomyces cerevisiae*, origin of replication complexes (Orcs) are formed by discrete specific DNA sequences (autonomously replicating sequences, ARSs), the core element of which is the 11 bp ARS consensus sequence (ACS) that is present in all origins (collated at http://www.oridb.org/index.php) (Nieduszynski et al., 2007). In other eukaryotes, however, the situation appears to be more complex. Origins seem to be spread over a much larger region of DNA and although some have reported that origins have some broad sequence characteristics in common, e.g. AT richness and proximity to transcriptional start sites (Segurado et al., 2003; Karnani et al., 2010; Stanojcic et al., 2008; Eaton et al., 2011), they do not have the same sequence specificity that occurs in *S. cerevisiae* (Cayrou et al., 2010). This has complicated the analysis of site-specific events in eukaryotic initiation.

Despite the differences in the nature of origins there is a high degree of conservation in the proteins that are needed to carry out the initiation of replication between *S. cerevisiae* and other eukaryotes. It is now quite well established that the primary event in initiation is the formation of the prereplication complex (preRC) consisting of the Orc complex (Orc1–Orc6), Cdc6, cdt1 and the Mcm protein complex (Mcm2–Mcm7). Subsequent to preRC formation, the binding of an array of other proteins, controlled by multiple protein phosphorylation events, leads to the formation of the pre-initiation complex (pre-IC) and ultimately the initiation of DNA replication (collated at http://DNAreplication.net) (Cotterill and Kearsey, 2009; Labib, 2010). Although many of the proteins involved in this process have been identified, less information is available concerning the characterisation of their precise biochemical functions and interactions.

One area that is unclear is whether the Orc complex alone can specify a site of DNA replication in a chromosome or whether the origin DNA sequence has functions in addition to Orc recruitment. A previous analysis in human cells suggested that Orc was capable of directing a site on a plasmid to act as an origin (Takeda et al., 2005). However, the structure of extrachromosomal plasmids might not be identical to that of the chromosome. Some functional distinctions between chromosomal and extrachromosomal locations have been observed (Willers et al., 2001) and, in addition, in *S. cerevisiae* the ARS sequences isolated via their ability to allow plasmid replication do not always function as efficient origins at chromosomal sites. It is therefore possible that additional determinants are necessary in the chromosome.

In this paper we have addressed the issue of whether the Orc complex is sufficient to specify the site of an origin of replication in the *Drosophila* S2 cell chromosome. To achieve this, we generated S2 cell lines containing Gal4-binding sites (UAS) located in the chromosome and also expressing Orc proteins fused to the Gal4-binding domain. The ability of the UAS region to act as an origin of replication can then be determined using quantitative PCR. A similar method was used to direct plasmid replication in human cells (Takeda et al., 2005) and has also been used to study the acetylation of origins in *Drosophila* ovaries (Aggarwal and Calvi, 2004).

Using this approach, we show that a Gal4-tagged Orc subunit is capable of directing preRC formation, origin activity and
acetylation of histone H4 at K12 (H4K12) to a UAS site in the chromosome of Drosophila S2 cells.

Results

Generation and characterisation of cell lines

These experiments required the production of stable cell lines with a Gal4-binding site (UAS) in the chromosome and that were expressing Gal4-binding domain tagged versions of Orc. The stable UAS line was manufactured first. We chose to insert a region containing five Gal4-binding sites, similar to the region already shown to act as an origin on plasmids in human cells (Takeda et al., 2005). Quantitative PCR suggested that seven copies of the UAS cassette were inserted into the chromosome (data not shown).

Cell lines established by this method were then stably transfomed with Orc subunits fused to a Gal4-binding domain. Takeda et al. observed activity with Gal4–Orc2 but not Gal4–Orc5 or Gal4–Orc6 al (Takeda et al., 2005), raising the possibility that fusion to Gal4 might inactivate some Orc subunits. We therefore made fusion proteins containing Orc2, Orc4 and Orc5. The Gal4 protein was cloned in frame at the C-terminus of each subunit with a V5 tag to allow detection and immunoprecipitation. In addition, a cell line expressing Gal4 in the absence of Orc was established as a negative control. As shown in Fig. 1, all cell lines appeared to express significant levels of the fusion proteins. Quantification suggested that the levels of the Gal4-tagged proteins were about five times those of the corresponding endogenous protein (supplementary material S1).

Gal4 and Gal4–Orc fusion proteins can bind to the chromosomal UAS

Chromatin immunoprecipitation (ChIP) analysis was performed to confirm that the Gal4-binding domain could act to direct the fusion proteins to chromosomal locations. Crosslinked DNA fragments were prepared as described and precipitated utilising the V5 tag. The DNA recovered was subject to PCR using primers in the UAS region as shown (Fig. 2A). Fig. 2B shows that each of the fusion proteins was bound to the UAS site in those lines where they were expressed. Binding of the Gal4 fusions to an unrelated region of the chromosome (the α-region of the Drosophila origin of replication, oriDα) was not observed using this methodology (data not shown), suggesting that their binding is confined to the specific region of UAS insertion.

Gal4–Orc fusion proteins can load other components of the preRC

To determine whether the Orc proteins were able to load other components of the preRC, the ChIP experiment was repeated but in this case the immunoprecipitation was carried out using antibodies against Orc1, Orc5, Cdc6, Mcm2 and Mcm5. An antibody against an unrelated human protein (TTC4) was used as a negative control. As can be seen from Fig. 3, Gal4–Orc5 appeared to be able to load all components of the preRC but not TTC4. By contrast, Gal4 alone could not load any preRC component.

The other Orc subunits each appeared to be capable of loading some of the components but not all (Table 1). Although this is likely to be a technical artefact caused by the spatial arrangements of components in the complex and the way that they are crosslinked (Toth and Biggin, 2000), it was decided that the primary construct used for further testing should the Orc5 fusion because this could be demonstrated to load all necessary components.

Gal4–Orc5 fusion protein can direct initiation to a chromosomal UAS site

The origin activity of the UAS was measured by quantitative PCR (qPCR) using the primers amplifying qUAS4 or qUAS8 for the UAS region (Fig. 2A). Positive and negative controls were taken directly from the previously characterised oriDα region (Ina et al., 2001). The positive control was the D fragment located in the origin. The negative control was the α-region located upstream of the D region and shown to have no origin activity. Prior to use, all primers were calibrated for qPCR (not shown). The DNA template was prepared by the λ-exonuclease method (Bielinsky and Gerbi, 1998). This removed all DNA fragments that did not have a 5′-RNA cap and therefore only bone fide replication products were included in the analysis.

Fig. 1. Gal4-tagged fusion proteins are efficiently expressed in S2 cells. Equivalent amounts of each of the cell lines shown were analysed by western blot using V5 antibodies. Predicted sizes are Gal4–Orc2, 86 kDa; Gal4–Orc4, 69 kDa; Gal4–Orc5, 69 kDa; and Gal4–Cdc6, 92 kDa.
Our initial experiments involved the use of unsynchronised cells and under these circumstances no enrichment of newly synthesised sequences at either the positive control or the UAS was observed (data not shown). However, in an asynchronous S2 cell population only 6–8% of the cells are in S phase. In addition, in order to detect origin activity in oriD, it is necessary to synchronise the cells in S phase (Takayo Sasaki, Florida State University, FL; personal communication). We therefore sought efficient ways of synchronising the cells. The final protocol used is described in the Materials and Methods (Ina et al., 2001) and Takayo Sasaki (personal communication). As can be seen in Fig. 4, this treatment efficiently synchronised S2 cells expressing either Gal4 alone or Gal4 fused to Orc5.

Time points were taken at 1, 2, 4 and 6 hours after release from a hydroxyurea (HU) block and analysed to determine whether initiation products could be detected by qPCR. This allowed determination of whether initiation had occurred and also whether it occurred at a particular point in the S phase. The results from this analysis are shown in Fig. 5. Using this technique, we were able to show that the D region of the oriD is enriched in nascent DNA, confirming that it is also used as an origin of replication in S2 cells.

For the UAS region, as expected, we could not detect any enrichment in nascent DNA when Gal4 alone was expressed, in

### Table 1. Loading of preRC components by Gal4–Orc2, Gal4–Orc4 and Gal4–Orc5

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<th>V5 tag protein</th>
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</tr>
<tr>
<td>Gal4–Orc4</td>
<td>+ (Orc2)</td>
<td>–</td>
<td>+</td>
<td>–</td>
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</tbody>
</table>

*Capable of loading the component, – not capable of loading the component.

*Orc proteins detected are given in brackets.

**Forced Orc binding drives initiation**

Fig. 2. (A) Schematic showing the location of the regions amplified (qUAS4 and qUAS8) using the primer pairs as described in Materials and Methods. 5XUAS indicates five tandem repeats of the UAS. (B) Gal4 fusion proteins bind to the UAS region when expressed in S2 cells. ChIP analysis was carried out on cell lines expressing Gal4, Gal4–Orc2, Gal4–Orc5 and Gal4–Cdc6 fusion proteins and also on cells not expressing a fusion protein (UAS) using the anti-V5 antibody for immunoprecipitation and the qUAS4 region for the amplification. The left-hand panel shows a titration of the input DNA for each cell line. The right-hand panel compares the amplification in the presence and absence of V5 antibody. In all cases, amplification of the UAS can only be seen when the V5 antibody is used for the immunoprecipitation.

**Forced Orc binding drives initiation**

Fig. 3. Gal4–Orc5 can load multiple components of the preRC onto the UAS site. ChIP analysis was carried in the Gal4 and Gal4–Orc5 cell lines using antibodies against Orc2, Orc5, Cdc6, Mcm2, Mcm5 and an unrelated human protein TTC4. Primers amplifying the qUAS4 region were for the DNA amplification. Amplification of the region was assumed to indicate that that protein was present at that region.

**Forced Orc binding drives initiation**

Fig. 4. Cell synchronisation. S2, Gal4, Gal4–Orc5 and Gal4–Cdc6 cell lines were treated as described in Materials and Methods to generate synchronous progression across the S phase. Flow cytometry was performed at the times indicated to determine the cell cycle state of the cells. Gal4, Gal4–Orc5 and Gal4–Cdc6 were also monitored for longer times but for the sake of clarity this data has not been included in the figures.
line with ChIP results, which showed that there was no assembly of a preRC on the UAS. However, with the Gal4–Orc5 fusion we were able to detect an enrichment of the UAS in the nascent DNA after the cells were released in fresh medium. In agreement with what was observed for the oriDx origin, the nascent DNA peaked in the middle of the S phase and the decreased again as the cells approached G2. This experiment was repeated three times using different time points. In all cases comparable timing and efficiency of nascent DNA amplification were observed. Comparable results were also obtained with the Gal4–Orc2 fusion (data not shown) suggesting that this activity is not confined to Orc5. This also adds support to the suggestion that the lack of complete preRC loading observed in the ChIP experiments was probably due to technical issues.

As further evidence that the enrichment was due to bone fide replication activity in the S phase of the cell cycle and not due to repair or products generated by the preparation method of the DNA, we were unable to detect enrichment if the samples were treated with RNase to remove the 5’-RNA cap prior to the λ-exonuclease treatment. In addition no products were detected if the cells were allowed to proceed into the G2 phase before DNA isolation (data not shown). These results show that the UAS can be used as an origin of replication when Gal4 is fused to Orc but not when only Gal4 is expressed.

**When Orc is directed to the UAS, acetylation of H4K12 is observed**

It has been shown that H4 acetylation at origins by the HBO1 histone acetylase is important for DNA replication licensing. (Miotto and Struhl, 2010). HBO1 has been shown to preferentially acetylate histone H4 on lysines K5, K8 and K12. In addition, tethering of the *Drosophila* HBO1 homolog (chameau) to the chorion locus has been shown to increase its origin activity (Aggarwal and Calvi, 2004). We therefore tested whether tethering Orc to a UAS could trigger the acetylation of histone H4. To do this, we performed ChIP using antibodies directed against acetylated H4K12. The x-region of oriDx, which does not have origin activity, was used as a negative control.

Fig. 6 shows that, consistent with previous observations (Fig. 2), the UAS region was efficiently precipitated using an anti-V5 antibody when S2 cells express either Gal4 alone or Gal4 fused to Orc5. However, when using an antibody against acetylated H4 the UAS was only precipitated when Gal4 fused to Orc5 is expressed. As expected, the x-region of oriDx was not precipitated with antibodies against either V5 or acetylated H4. Similar results were also seen with Gal4–Orc2 (data not shown), indicating that this effect is not specific to a particular Orc subunit. This suggests that when Orc is directed to the UAS, nucleosomes in the UAS region are acetylated on histone H4 at position 12.

**Gal4–Cdc6 is not able to direct replication to a chromosomal UAS**

Takeda et al. has shown that, in addition to the Orc fusions, a Gal4–Cdc6 fusion is capable of directing initiation to a site on a plasmid (Takeda et al., 2005). We therefore tested whether the same was true for a chromosomal location. We performed similar experiments to those described for Gal4–Orc5, but this time using a cell line expressing Gal4–Cdc6.

As seen in Fig. 1, the Gal4–Cdc6 fusion was well expressed in the stable cell line. Fig. 2 shows that it was also capable of binding to the UAS sites in the chromosome. However, we could not detect binding of any of the other preRC components to this site (data not shown). In addition, when we looked for initiation products at the UAS in Gal4–Cdc6 fusions we were not able to detect any at 2 hours (Fig. 5) or at earlier time points (data not shown). This therefore suggests that in our system a Gal4–Cdc6 fusion is not competent to load other components of the preRC or to initiate DNA replication.

One interesting fact, however, was that S2 cells expressing Gal4–Cdc6 did not show a sustained stop at G1 and proceeded through S in the presence of HU. Despite their different kinetics, these Gal4–Cdc6-containing cells did show a significant enrichment in the S phase (>75% at the 2 hour time point
shown), which should have allowed us to detect origin activation if it was present.

**Gal4–Orc and Gal4–Cdc6 are not able to sustain extrachromosomal replication of a plasmid containing a UAS**

Because we were not able to detect chromosomal replication promoted by Gal4–Cdc6, we tested whether it was able to promote replication of an extrachromosomal plasmid in S2 cells as previously reported for a Gal4–Cdc6 fusion in human cells. Therefore, S2 cells expressing either Gal4 or Gal4–Cdc6 were transiently transfected with a plasmid (pUAST) containing five UAS sites in tandem. Replication of the plasmid was monitored as described (Takeda et al., 2005) using an assay that utilises the fact that DNA replicated in mammalian cells is resistant to Dpn1 digestion (described in Materials and Methods). As shown in Fig. 7A, we were not able to detect plasmid resistant to Dpn1. This was true even using very long exposures of these gels, which should have allowed us to detect replication of 1% of the DNA. Similar results were obtained with a different plasmid pMTV/UAS (data not shown). This suggests that in our system the plasmids are not replicated or are replicated very inefficiently. This was consistent with the failure of Gal4–Cdc6 to allow chromosomal replication. Unexpectedly, however, we could not detect plasmid replication stimulated by Gal4 fusions to Orc2, Orc4 or Orc5 (Fig. 7A).

To further investigate the cause of the lack of plasmid replication, we used ChIP to analyse the level of binding of preRC components in cell lines expressing Gal4–Orc5. As shown in Fig. 7B although binding of Gal4–Orc5 could be detected at the UAS, little Orc2 was detected and, more importantly, no binding of Mcm5 could be observed. This leads us to suggest that in the *Drosophila* S2 cell system Orc binding is not sufficient to allow preRC binding or plasmid replication.

**Discussion**

**Gal4–Orc5 alone is sufficient to define a chromosomal site of initiation of DNA replication in *Drosophila* S2 cells**

Our results suggest that Orc binding to a chromosomal site is sufficient to allow the synthesis of nascent DNA at that site in S2 cells. They further show that Orc binding directs preRC complex
formation, suggesting that at these ‘artificial origins’ DNA replication initiates by a route analogous to that occurring at natural origins of replication in eukaryotic cells.

The initiation directed by Gal4–Orc5 starts at a similar time in the cell cycle to that seen for oriD\(\alpha\), which has been suggested to be an early firing origin (Ina et al., 2001; MacAlpine et al., 2004). This suggests that some of the Gal4–Orc5-directed initiation also occurs early in S phase. However, in comparison with oriD\(\alpha\), Gal4–Orc5-directed initiation is spread over a longer period of the S phase. This could reflect UAS sites in different genomic regions firing at different times. This extended period of initiation complicates the analysis of the efficiency of the UAS origin compared with oriD\(\alpha\) and is further complicated by the fact that it is not known whether all seven copies of UAS integrated into the genome are active. However, taking the absolute level of nascent strands detected over the course of the cell cycle, the initiation at the UAS appears to be of the same order of magnitude as that seen at oriD\(\alpha\), even allowing for the assumption that all of the seven UAS sites are equally active. Further analysis, mapping the initiation from each of the seven UAS sites individually, would need to be carried out to determine the absolute efficiency of replication stimulated by Gal4–Orc5.

We do not think that the observed initiation is an artefact caused by our synchronisation procedure because we only detected nascent UAS fragments in cell lines carrying a Gal4–Orc fusion. In addition, detection of initiation at the previously characterised oriD\(\alpha\) origin also requires that the cells be synchronised. It is likely that our inability to detect nascent strands in unsynchronised cells was caused by the low percentage of cell in S phase at any one time (6–8%).

**Gal4–Orc5 binding can direct H4K12 acetylation to the UAS site in S2 cells**

Results from several laboratories have suggested that histone acetylation at the site of an origin of replication has a positive effect on its efficiency of firing (Miotto and Struhl, 2010; Goren et al., 2008; Vogelauer et al., 2002; Aggarwal and Calvi, 2004). We observed that UAS sites in cells expressing Gal4–Orc5 show clear acetylation of H4K12. Because the acetylation is not seen if the Gal4 protein binding to the UAS is not fused to an Orc subunit, it must be due to Orc binding or to the process of initiation itself. This dependency on Orc binding suggests that the histone acetylation per se cannot be a fully independent determinant of the efficiency of origin firing. This does not exclude the possibility that the level of histone acetylation is important in determining the efficiency of the origin, but suggests that control of the acetylation levels takes place via an interaction of the responsible factors with Orc.

**Gal4–Cdc6 appears unable to define a chromosomal site of initiation of DNA replication in Drosophila S2 cells**

Although Orc is capable of defining an origin of replication, we were unable to see DmCdc6 acting in the same capacity. This was surprising because Takeda et al. reported that human Cdc6 drives replication of a plasmid in human cells (Takeda et al., 2005). Although the cell cycle characteristics of the synchronised Gal4–Cdc6 cells were altered, at the time points analysed the cells were predominantly in S phase, and therefore if nascent DNA was present we should have been able to detect it. We cannot rule out the possibility that the fusion of the Gal4-binding domain to Cdc6 inactivates the protein, although it clearly does not do so for the human protein. In addition, it is also worth noting that tagged DmCdc6 cannot be fully inactive as it was able to affect the characteristics of the synchronisation of cells expressing the fusion protein. The cells did not show a clean stop in HU and at 2 hours after the removal of HU a significant percentage of the cells were already the G2 phase of the cell cycle. There are a number of reports in the literature describing how mammalian cells adapt and allow replication with reduced nucleotide pools (e.g. Hanada et al., 2007). In addition, if wild-type S2 cells and cells expressing Gal4 or Gal4-tagged Orcs are left for extended periods of time (>24 hours) they too are able to continue through S phase in the presence of HU. How Gal4–Cdc6 speeds up progression through the HU block is not clear. Cdc6 has been implicated in S phase checkpoints and the control of S–M transition, although the available data (reviewed by Borrado and Mendez, 2008) suggest that it might have been expected to activate S phase checkpoints. In addition, overexpression of DmCdc6 in S2 cells (Crevel al., 2005) showed minimal effects for levels of DmCdc6 higher than those observed here. It therefore remains a possibility that the Gal4 fusion altered the activity of the protein, either preventing it from directing initiation or allowing it to promote a cellular state in which nascent fragments are processed so as to be undetectable.
Gal4–Orc5 cannot drive replication of a plasmid in Drosophila S2 cells

Another surprising observation from our data was that, although Gal4–Orc5 could promote initiation in the chromosome, it was not able to direct plasmid replication. We do not think this was a technical problem because we were clearly able to retrieve residual unreplicated DNA from the cells. In addition, at least 50% of the transfected cells contained the plasmid. This suggests that the requirements for initiation at a chromosomal site are different from those on a plasmid. Alternatively, it could represent a difference between Drosophila and human cells, or between non-transformed S2 cells and transformed HEK293 and C33a cells (Takeda et al., 2005). Differences in replication initiation between transformed and non-transformed cells have already been documented (e.g. Dorn et al., 2009; Di Paola et al., 2010).

Conclusion

We have demonstrated that it is possible to direct initiation of DNA replication to a specific chromosomal site only by the forced binding of an Orc subunit. The replication appears to occur through the formation of a preRC and also shows at least one origin-associated histone modification, suggesting it goes by a route similar to that occurring at natural origins. We therefore expect that this will prove a useful model for the future investigation of replication initiation at chromosomal sites in vivo.

Materials and Methods

Antibodies

Rabbit polyclonal antibodies against Drosophila Mcm2, Mcm5, Orc2, Orc5 and Cdc6 have been previously described (Crevel et al., 2001; Crevel et al., 2005). A rabbit polyclonal antibody against histone H4K12 was purchased from Abcam (Ab1761). Mouse monoclonal antibody against V5 was purchased from Abcam (clone SV5-PK1; Ab27261).

Manufacture of stable cell lines

A 2077 bp fragment containing five tandem UAS sites was excised from pUAST (Brand and Perrimon, 1993) with Stu1 and Nde1 and inserted into the EcoRI site of pMT/V5HisA (Invitrogen). It was stably transfected with pCoBlast using the calcium phosphate method, and selected using blasticidin. Of pMT/V5HisA (Invitrogen). It was stably transfected with pCoBlast using the calcium phosphate method, and selected using blasticidin.

S2 cell synchronisation

In order to synchronise S2 cells just after the G1–S boundary, cells were cultured in medium with no HU. Following this treatment, S2 cells go synchronously through S phase in 4–6 hours. For the preparation of nascent DNA, time points were taken between 0 and 6 hours.

ChIP analysis

ChIP was performed according to a published method (Breitling and Orlando, 2005) with the following modifications: the immunoprecipitates were recovered using protein A Dynalbeads (Invitrogen); the beads were subsequently washed with successive low salt buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 150 mM NaCl) and high salt buffer (20 mM Tris-HCl pH 8, 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 500 mM NaCl) prior to analysis.

Preparation of nascent DNA

Nascent strand preparation was carried out by the λ-exonuclease method (Bielinski and Gerbi, 1998). In short, DNA purified from replicating S2 cells was heat-denatured and loaded onto a linear sucrose gradient. DNA fragments of 500–1200 bp were precipitated and phosphorylated on the free 5′-ends using polynucleotide kinase. The phosphorylated DNA fragments were treated with λ-exonuclease, which degraded all the DNA not protected on their 5′-ends by RNA primers.

Quantitative PCR

The DNA was amplified using the Promega GoTag qPCR Master Mix following the manufacturer’s instructions. Primers were designed using the Primer3 online tool (Rozen and Skaltsky, 2000). For the oriDr site, primers were designed in the D and S regions as defined (Ina et al., 2001). For the UAS amplification, primers were designed in the region qUAS4 and qUAS8 as shown in Fig. 2. The sequences for all primers used are listed in supplementary material Table S1.

Assay for extrachromosomal plasmid replication and protein binding

S2 cells expressing either Gal4 or Gal4 fusion proteins were transiently transfected with pUAST or pMTVUAS using the calcium phosphate method. After 3 days, extrachromosomal DNA was extracted using buffers A1, A2 and A3 from the NucleoSpin plasmid kit (Macherey-Nagel, Düren, Germany), precipitated from the cleared supernatant and resuspended in Tris-EDTA buffer. The DNA was either digested with ApalI (three fragments) or ApalI and DpnI for the detection of plasmid replicated in the eukaryote cell and transferred to nylon membranes by Southern blotting. The DNA was detected using the qUAS4 DNA (Fig. 2A and Table 1) labeled with 32P. Alternatively, DNA was crosslinked with formaldehyde and the cells lysed and processed for ChIP as described above.

Acknowledgements

We would like to thank Isabelle Crevel, Lynn Grignard, Aleks Ivetic, Stephen Kearsey and David Szuts for critical reading of the manuscript.

Funding

This work was funded by Cancer Research UK (CRUK) [grant number C980/A6615].

Supplementary material available online at

http://jcs.biologists.orglookup/suppldoi:10.1242/jcs.094409/DC1

References


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