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Analysis of the interactions of functional domains of a nuclear origin of replication from *Saccharomyces cerevisiae*

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ABSTRACT

We have determined that *ARS121* is an efficient origin of replication on chromosome X of *Saccharomyces cerevisiae*. This origin is comprised of at least three distinct functional domains. One of these domains is the *ARS121* core sequence (~35 bp-long), which is essential for origin activity. This essential core contains an 11 bp sequence resembling (2 bp mismatch) the *ARS* consensus. Another important domain is an enhancer of DNA replication, which binds the OBF1 protein. The third domain, ATR (A/T-rich, ~72 bp), is auxiliary and works in either orientation, but only when located 3′ to the essential core. When fused to the *ARS121* core both the enhancer and the ATR domain act synergistically to enhance the activity of the origin. Furthermore, when fused to the essential core sequences of heterologous *ARS*s, *ARS1* and *ARS307*, the auxiliary domains also appeared to stimulate synergistically origin function. These results suggest that (i) in order to elicit maximal origin activity all three domains have to interact and (ii) activation of the essential core sequences at different origins of replication may share a common mechanism.

INTRODUCTION

Replication of eucaryotic nuclear DNA is initiated at the start of the S-phase and proceeds by multiple initiations along a chromosome, generating multiple replicons. Understanding the mechanism and regulation of eucaryotic DNA replication requires the elucidation of the structural organization of origins of replication. *Saccharomyces cerevisiae* provides an excellent model system for such studies since origins of replication amenable to biochemical and genetic manipulations have been isolated. In addition, these origins, called *ARS*s, were identified as DNA fragments that confer on plasmids bearing them the ability to replicate autonomously in yeast (1). Thus yeast provide a convenient system for *in vivo* analysis of origin activity.

Recently, compelling evidence has been obtained indicating that *ARS*s incorporated into plasmid DNA function as origins of replication *in vivo* (2,3). Furthermore, the presence of functional DNA replication origins was correlated with the location of *ARS* elements on chromosomes III, IV, V, and XII, strongly suggesting that *ARS*s are the origins of chromosomal replicons (4–7). Most *ARS*s are present as single-copy DNA in the genome with the exception of two families of repetitive *ARS*s. One family of repetitive *ARS*s is located on the tandemly repeated rDNA units on one arm of chromosome XII and the other is associated with the telomeres of yeast chromosomes (8,9). All *ARS*s share a common 11 bp, [A/T]TTTAT[A/G]TTT[A/T], sequence essential for origin function (10). *ARS121*, which is discussed in this paper, is an exception to this rule since it contains a sequence functionally homologous to the consensus that differs from the consensus sequence by a 2 bp. mismatch. Recently, Palzkill and Newlon (11), proposed an important role for the consensus sequences in origin function by demonstrating that synthetic copies of such a nucleotide sequence in tandem could sustain autonomous replication *in vivo*. Others have reported, however, that in naturally occurring *ARS*s the core consensus sequence alone is insufficient for optimal *ARS* function. Nucleotide sequences on either side of the core consensus are required, although the amount and the sequence of the flanking DNA varies depending on the *ARS* (1,12–14). In earlier studies we identified a DNA replication enhancer present in *ARS121* (15). This enhancer, which was also found in other *ARS*s, serves as a recognition site for binding the OBF1 protein (16,17). The recent isolation of the OBF1 gene has shown that OBF1, which is a phosphoprotein phosphorylated at serine and threonine residues (18), is identical to the ABF-I and BAF1 proteins isolated by others (19–21).

Here we have carried out a systematic analysis, by deletion and *in vitro* site-directed mutagenesis, to define, delimit and determine the structural organization of DNA elements (in particular the essential core sequence), in *ARS121*, that are important for origin function. This work provides evidence that a yeast nuclear origin is composed of at least three distinct

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functional units which must interact to attain maximal levels of origin activity. We also demonstrate that two of the *ARS121* domains can activate a heterologous origin core sequence, suggesting that yeast origins share a common mechanism for initiation of DNA replication.

MATERIALS AND METHODS

Strains and Growth Conditions

The *Escherichia coli* strain used for transformation and plasmid propagation was HB101(22). *Saccharomyces cerevisiae* strain 8534-8C (*MATα* ; leu2-3, −112; his4Δ34; ura3-52) was used for all plasmid transformation and maintenance studies (23,15). Bacteria were grown in LB broth or on LB plates at 37°C. Media were supplemented with ampicillin (100 μg/ml) where appropriate. Yeast were grown at 30°C in YPD (rich) and SD (minimal) media, prepared as described (24). SD was always supplemented with histidine (20 μg/ml) and leucine (30 μg/ml). Uracil was added at 50 μg/ml where needed.

Polymerase Chain Reactions (PCR)

The synthetic core of *ARS121* was prepared by PCR, using appropriate oligonucleotides as primers to produce a DNA fragment containing nucleotides 369−417 of the *ARS121* sequence (15) bounded by PstI sites. Similarly, the ATR was prepared as a DNA fragment containing the nucleotides 418−486 of *ARS121* sequence bounded by BglII sites. The functional core sequence of *ARS1* (13) was produced by PCR as a DNA fragment containing the nucleotides 823−872 (25). The core sequence of *ARS307* was produced as a DNA fragment containing the nucleotides 134−200, whereas the intact *ARS307* was prepared as a 200 bp fragment containing nucleotides 4 −200 of the *ARS307* sequence (11). The *ARS* core and ATR of *ARS121* were synthesized using purified plasmid DNA; the cores of *ARS1* and *ARS307*, as well as the 200 bp *ARS307* fragment, were synthesized using purified yeast genomic DNA, as described (26).

Plasmid Constructions and DNA Manipulations

Plasmids: YIp5, YCp5-3 (YIp5/CEN3), YCp5AB121, fs1, fs2, Δfs2, s3, Δs3, and s4, are as previously described (15,27). Sequence substitution and insertion mutagenesis of *ARS121* was carried out as before (15). Mutated *ARS121* DNA fragments: fs4, fs6, fs8, fs10, sp5, and sp10, were inserted into YIp5 cleaved with EcoRI and Hind III. These were tested for *ARS* function by their ability to transform yeast at a high frequency. Those containing an active origin were also put into YCp5-3 for determination of plasmid mitotic stability.

The deletions Δfs6 and Δfs10 were produced by cleaving the parent constructions (fs6 and fs10) with EcoRI and PstI and inserting the deletion fragments, using a single stranded oligonucleotide bridge (AGCTTGCA), into YIp5 cleaved by EcoRI and HindIII. Δfs10 was also inserted into YCp5-3 in a similar fashion. Δfs8 was constructed from a mutated *ARS121* containing an SphI site at the fs8 position by inserting the EcoRI/SphI fragment into pUC19 cleaved by EcoRI/SphI. This Δfs8 fragment was then moved into YIp5 as an EcoRI/HindIII fragment.

The synthetic *ARS121* reconstructions from individual elements were first performed in pUC19(Bgl) DNA, which is a modified pUC19 vector containing in the polylinker, in addition to a BamHI site, a BglII site in lieu of SphI (28). Then, the reconstructed origins were moved as EcoRI/HindIII fragments into YCp5-3 cleaved by HindIII. Prior to ligation the termini of the DNA fragments were filled in by T4 DNA polymerase. The p19CORE was prepared by inserting a DNA fragment, encompassing the sequence of *ARS121* between nucleotides 369 and 417, into the PstI site of pUC19(Bgl). This fragment, produced by PCR, was functional as an *ARS* (not shown). When inserted into YCp5-3 the plasmid obtained was YCpCORE . The ATR region of *ARS121* (nucleotides 418 to 489) was first inserted into pUC19 at the HindIII and PstI sites. This fragment was then excised as a HincII/HindIII fragment and inserted next to the core region, described above, at the HincII and KpnI sites, using a single-stranded oligonucleotide bridge containing sequences complementary to the KpnI and HindIII overhangs. Insertion of this origin into YCp5-3 yielded the plasmid YCpCATR. Plasmid constructions containing the *ARS* enhancer, in the form of two tandem synthetic OBF1 DNA-binding sites (15,28), were produced by inserting the dimer binding site as a BamHI/BglII fragment into the BglII site next to either the CORE or CATR constructs in pUC19(Bgl). Subsequent insertion of these origins into YCp5-3 yielded YCpCDBS and YCpRC121. YCpRCFL was first constructed by inserting a synthetic ATR, which was produced by PCR and terminated by BglII sites, into the BamHI site of p19CORE. Then, a dimer OBF1 DNA-binding site was inserted into the BglII site to yield the reconstructed origin. The plasmids YCpRCAU(+) and YCpRCAU(−) were constructed

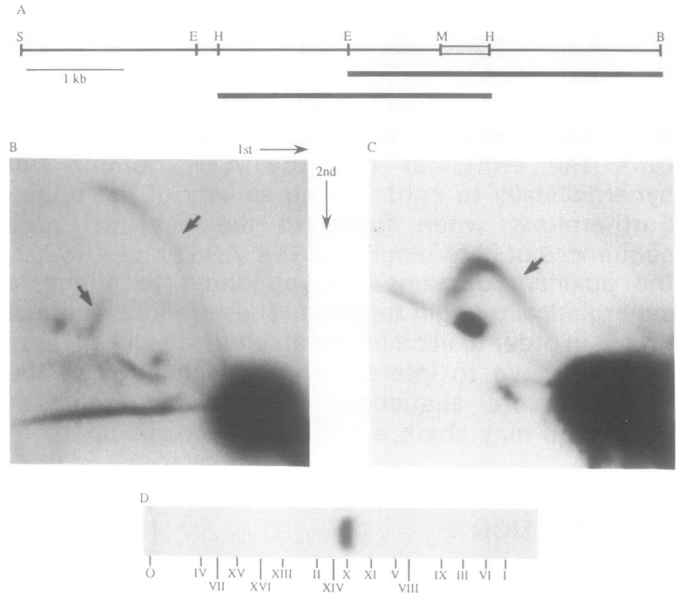

Figure 1. *ARS121* is associated with an origin of DNA replication on chromosome X. **(A)** Restriction map of a 6.6kb region of yeast chromosomal DNA containing *ARS121* (8). The shaded area indicates the 489 bp MspI/HindIII fragment containing *ARS121* activity and the two OBF1 DNA-binding sites. Thick lines identify the EcoRI/BamHI and HindIII fragments subjected to 2D electrophoresis in agarose gels as described in Materials and Methods. **(B)** and **(C)** Autoradiograms of blots of, respectively, EcoRI/BamHI and HindIII yeast genomic digests, which were subjected to 2D electrophoresis in agarose gels and probed with the MspI/HindIII fragment. In each panel, arrows identify major replication intermediates. Hybridization of the probe to non-replicating DNA is seen as a large spot in the lower righthand corner of each panel. **(D)** Identification of the chromosome containing *ARS121*. The MspI/HindIII probe was hybridized to yeast chromosomes separated by CHEF gel electrophoresis, obtained from Clontech Laboratories, Inc. (Palo Alto, CA). The gel was processed according to the manufacturers instructions. S; SalI, E; EcoRI, H; HindIII, M; MspI, B; BamHI, O; Origin of electrophoresis.

by inserting a synthetic ATR domain, bounded by BglII restriction sites, into the BglII site of p19CORE. This was followed by inserting an EcoRI/HindIII fragment, containing a dimer of the synthetic OBF1 DNA-binding site, into the EcoRI and KpnI sites of this plasmid, using a single stranded oligonucleotide bridge, as above. These constructs were then moved to YCp5-3 as above.

To produce the chimeric plasmids containing the essential cores of *ARS1* and *ARS307*, the cores synthesized by PCR were first cleaved with XbaI and PstI and then inserted into the multiple cloning site of pUC19. The ATR of *ARS121* was added as a PstI/HindIII fragment. Subsequently, a HindIII/EcoRI fragment containing two synthetic OBF1 binding sites was inserted into the EcoRI and KpnI sites, using a single stranded DNA bridge, as above. All chimeric constructs were then inserted as EcoRI/HindIII fragments into YCp5-3. Both *ARS1* and *ARS307* synthetic cores contain functional *ARS*s (data not shown). The 200 bp *ARS307* fragment, produced by PCR, which displayed wildtype levels *ARS307* activity (29),was inserted into the XbaI and HindIII sites of pUC19, and then moved as an EcoRI/HindIII fragment to YCp5-3. Plasmids were used to transform yeast to uracil prototrophy by the lithium acetate procedure (30).

Analysis of Plasmid Mitotic Stability

Plasmid mitotic stability analyses, under nonselective and selective conditions, were performed as previously described (27). Mitotic stability determinations were performed on at least four independent transformants for each plasmid construct.

Two-dimensional Agarose Gel Electrophoresis

Yeast chromosomal DNA was prepared for analysis of replication intermediates by glass bead disruption of logarithmically growing cells. Two-dimensional (2D) gel electrophoresis was carried out as described previously (2). About 50 to 100μg of yeast chromosomal DNA were digested with the appropriate restriction endonuclease(s), then subjected to electrophoresis in the first dimension on a 0.4% agarose gel (in 1× TBE) at 1VDC/cm. A slice of the first dimension gel was then embedded in a 1% agarose gel (in 1× TBE and 0.3 μg/ml ethidium bromide) and subjected to electrophoresis at 5VDC/cm at 4°C with buffer recirculation. The DNA was then transferred to Magna NT (Micron Separations, Inc.) and the blot was probed with the MspI/HindIII fragment of *ARS121* radiolabelled by the random hexanucleotide method (31). Filters were exposed to X-OMAT (Kodak) film for 3 to 12 days. Additional technical advise for both yeast chromosomal DNA preparation and two-dimensional gel electrophoresis was kindly provided by B.Brewer and W.Fangman (University of Washington, Seattle) and S.Greenfeder and C. Newlon (UMDNJ, Newark, NJ).

RESULTS

ARS121 is an origin of DNA replication on chromosome X

ARS121 was first isolated as a 6.6 kb DNA fragment (Fig. 1A) able to confer on plasmids the ability to replicate autonomously *in vivo* (8), presumably by providing the plasmid with a functional origin of replication. In earlier studies we delimited the functional origin in *ARS121* to a 489 bp MspI/HindIII restriction fragment (27), indicated by the shaded area in Fig.1A. Since we decided to systematically dissect the *ARS121* origin, it was important to determine whether this *ARS* functions as an origin of replication in its natural chromosomal setting. First, we mapped the location of *ARS121* by probing a gel containing separated yeast chromosomes with ^{32}P-labeled MspI/HindIII DNA fragment described in Fig.1A. The results of this experiment, shown in Fig. 1D, indicate that *ARS121* is a single copy *ARS* located on chromosome X.

To determine if this *ARS* is an active origin of DNA replication on chromosome X, we employed the two-dimensional (2D) agarose gel electrophoresis method for the analysis of replicating intermediates developed by Brewer and Fangman (2). In this experiment, yeast genomic DNA was digested with either BamHI/EcoRI or HindIII restriction endonucleases. The

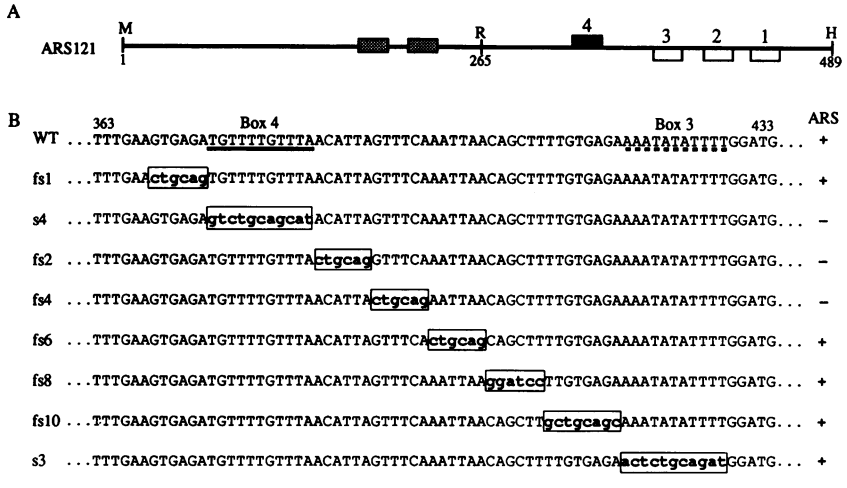

Figure 2. Substitution mutagenesis of the core and adjacent regions of *ARS121*. (A) Schematic presentation of the MspI/HindIII DNA fragment containing *ARS121* activity and the two OBF1 DNA-binding sites. Filled box indicates the essential near match to the *ARS* consensus sequence, while the open boxes designate the other consensus partial match sequences. Shaded boxes denote the two OBF1 DNA-binding sites. **(B)** Substitution mutations in *ARS121* and the resulting effect on *ARS* function and plasmid stability. All substitution mutations are in the entire 489 bp MspI/HindIII fragment; for clarity, only nucleotides 363 to 433 are shown. Thick underline indicates the essential near match to the core consensus sequence (box 4 in A) and dashed underline identifies a nearby consensus partial match sequence (box 3 in A). The sequence alteration made in each mutation is boxed and appears in lowercase type. Stability data (nonselective and selective) represent the percentage of the cell population that contains the plasmid, as described in Materials and Methods. Stability data for fs1 and s3 are from Walker, et al., (15). Nonsel.; Nonselective, Sel.; Selective, R;RsaI, others as in Figure 1. +; functional as a plasmid replication origin, −; does not contain a functional origin.

BamHI/EcoRI digest generates a 3.2 kb fragment containing the *ARS121* near the center of the linear fragment, while the HindIII digest releases a 2.9 kb fragment in which *ARS121* is situated at one end (Fig. 1A). Each of these digests was then subjected to 2D electrophoresis in agarose gels. Following electrophoresis, the DNA was probed with a radioactively labeled *ARS121* DNA fragment (the shaded MspI/HindIII region Fig.1A). The autoradiograms of the Southern blots are shown in Fig. 1B and C. The major arc seen in the autoradiogram of the BamHI/EcoRI genomic digest (upper arrow in Fig. 1B) is characteristic of replication bubble containing DNA fragments, while the small arc is characteristic of nearly completely replicated Y-shaped molecules (lower arrow in Fig. 1B). This pattern of arcs suggests that an origin of chromosomal replication is located within the EcoRI/BamHI fragment. If replication proceeds in a bidirectional manner with both forks moving at a similar rate, then the origin appears to be somewhat asymmetrically placed, in agreement with the location of *ARS121* in the restriction map shown in Fig. 1A. The intensity of the replication bubble-derived arc in Fig. 1B (upper arrow) compared with the very faint Y-shaped DNA-derived arc (lower arrow) suggests that *ARS121* is an active and efficient origin of replication. If this is true, then replication initiating near a restriction site should produce predominantly Y-shaped molecules when cleaved by the restriction endonuclease. This is indeed the case, as shown in Panel C, where probing a genomic HindIII digest with radioactively labeled *ARS121* revealed Y-shaped DNA molecules exclusively. Thus the predicted result has been confirmed, supporting the notion that *ARS121* serves as a site for initiation of replication on chromosome X. In addition, we have recently constructed a yeast strain in which the essential core sequence of *ARS121* on chromosome X was deleted. Only Y-shaped molecules were detected in a Southern blot of a BamHI/EcoRI digest probed by the *ARS121* DNA (data not shown), indicating that *ARS121* is the replication origin on chromosome X and that only a single copy of *ARS121* is present in the BamHI/EcoRI fragment.

The minimal origin of replication, the essential core sequence of *ARS121*, can be delimited to an approximately 35 bp-long DNA fragment

The property of a DNA fragment to confer on a plasmid the ability to transform yeast at a high frequency provides a means to analyze origin function *in vivo*. In addition, it is possible to assess the efficiency of an origin by measuring mitotic stability of a plasmid, provided the plasmid also contains a centromeric sequence to minimize the effect of nondisjunction.

We have shown that *ARS121* origin activity was located on a 489 bp MspI/HindIII DNA fragment (27, Fig. 1A). This DNA fragment contains several partial matches to the canonical *ARS* core consensus sequence and two binding sites for the *ARS* enhancer binding protein, OBF1 (Fig. 2A). One of those sequences, TGTTTTGTTTA (Fig.2A, box 4), was shown, by linker substitution mutagenesis (s4, Fig. 2B) to be essential for origin activity. In addition, a 6 bp sequence extending 3′ to the T-rich strand of box4 was also found to be essential (Fig. 2B). In order to determine the size of the essential core.we extended this analysis to encompass additional sequences around box 4. As shown in Fig.2B, we first constructed a series of 6 or 8 bp linker substitution mutations extending 3′ to the T-rich strand of box 4; we then tested these for *ARS* function and, when applicable, for plasmid mitotic stability. In addition to the previously described mutations, s4 and fs2, the linker substitution mutation fs4 also completely inactivated *ARS121* origin function. Mitotic stability analyses (Fig. 2B) of the flanking mutants, fs6, fs8, and fs10, indicated that, while this region could tolerate 6–8 bp sequence substitutions, the resulting *ARS*s did not function normally, suggesting that this region may also be important for origin activity. Interestingly, the flanking substitution mutation, fs8, decreased *ARS* function by approximately 15-fold, whereas the two mutations in flanking sequences, fs6 and fs10, caused a 2-fold decrease in origin efficiency. A substitution mutation, s3, that removes the core-proximal consensus partial match sequence (Fig. 2B), showed wild type *ARS* function. Wild type levels of origin activity were also observed with the fs1 mutation flanking box4 on the 5′ end (Fig. 2B). Therefore the minimal region required for *ARS121* function spans the sequence from the consensus-like box4 to the area defined by the fs10 mutation.

To further explore the importance of nucleotide sequences located 3′ to the T-rich strand of box4 and to estimate the minimum functional size of *ARS121*, we constructed, using the appropriate linker substitutions, a series of progressive external deletions into this region. Each deletion was then tested for *ARS* function (Fig. 3A). As previously described, the deletion Δs3 caused a significant reduction in origin function compared to the wild type *ARS* (Fig. 3A; 42). The next deletion towards the core

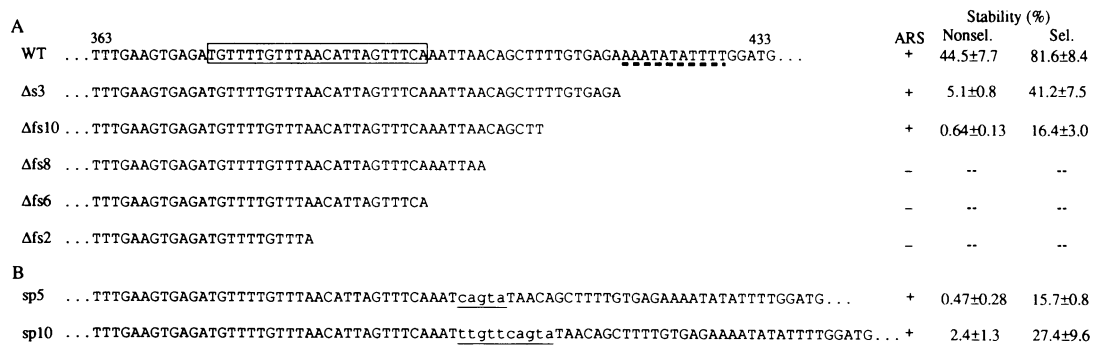

Figure 3. Deletion and insertion mutagenesis of the core and adjacent region of *ARS121*. Although the entire 5′ region of *ARS121* (to the MspI site, Fig.2) is present in each deletion mutation, only a portion is shown. **(A)** Progressive external deletions into the essential region of *ARS121*. Boxed sequence is the essential core of *ARS121* as determined by substitution mutagenesis. Other features as in Fig. 2. **(B)** Insertion mutagenesis within the core region of *ARS121* performed as described in Material and Methods. Lowercase underlined letters identify the sequence insertion made in the 489 bp *ARS121* MspI/HindIII fragment.

region, Δfs10, retained origin function yet was severely inefficient. Additional deletions, Δfs2, Δfs6, and Δfs8, into the region 3′ to box4 completely inactivated the origin (Fig. 3A).

Thus, the substitution and deletion analyses of the *ARS121* core indicate that the minimum sequence required for origin function is approximately 35 bp-long. This core can be divided into two regions based on the response to linker substitution and deletion mutagenesis. One region contains the *ARS* consensus-like sequence and other sequences intolerant to linker substitutions. In the second region, contiguous with the first, deletions inactivate the origin but sequence substitutions only decrease *ARS* efficiency. To determine if the two regions must be closely juxtaposed we placed 5 bp (one half helical turn) and 10 bp (one helical turn) insertions into a site situated between the two core regions (Fig. 3B). Both of these insertion mutations, sp5 and sp10, drastically reduced *ARS* function, suggesting that in order to retain wild type levels of origin activity the continuity of the sequences in the minimal origin must be maintained.

Reconstruction of *ARS121* from isolated, functional domains

In earlier studies we have shown that the region spanning box 3 and the HindIII site, which delimits the 3′ terminus of *ARS121* (Fig. 2A), was important but not essential for *ARS* function. We have also shown that single linker substitution mutations within this region have no apparent effect on origin activity (15). However, progressive deletions from the 3′ end caused a gradual increase in the level of plasmid instability. Deletion of this entire region, Δs3, caused a substantial reduction in origin activity (Fig.3A). We call this 72 bp region ATR (A/T rich) since it contains a higher A/T content (75%) than total yeast genomic DNA (62%) and we refer to it as a distinct functional domain of *ARS121*. Another distinct functional unit is the essential core sequence delineated in Figures 2 and 3. A third functional domain is the replication enhancer, which functions in a distance and orientation independent manner and binds the OBF1 protein (15). The nucleotide sequences of these elements are shown in Fig. 4.

In order to assess the contribution of each of these domains to origin function we have reconstructed origin activity from the individual functional elements. A diagram depicting these reconstructed origins and the relative plasmid stability data is shown in Figure 5A and B. The core sequence, although active, was extremely inefficient (~1% efficiency of wild type *ARS121*) in sustaining origin activity. Fusing the ATR to the core (YCpCATR) caused an approximately 6-fold increase in plasmid stability over the core itself (YCpCORE). Similar enhancement of origin function was achieved by fusing the enhancer (YCpCDBS), which consisted of two tandem synthetic OBF1 DNA-binding sites. In contrast, when both the enhancer and ATR domains were fused to the core, (YCpRC121) origin activity increased at least 100-fold over the core alone. These results show clearly that the *ARS* enhancers and the ATR work synergistically to stimulate the essential core sequence in origin function. The stability of the YCpRC121 plasmid, bearing the reconstructed origin, was approximately the same as the stability of the plasmid YCpAB121, which contains the entire 489 bp MspI/HindIII fragment (Fig.2A). Thus, this experiment shows that full *ARS121* activity can be recovered by fusing together the three isolated domains. Furthermore, the results demonstrate that the exact spacing between the elements is not crucial since 18 and 36 bp of a polylinker nucleotide sequence separated the core from the ATR and the enhancer domains, respectively. The fact that a polylinker bridge wedged between the domains is tolerated is also an indication that the three elements are functionally distinct.

The ATR domain works only when situated 3′ to the T-rich strand of box 4 of the essential core sequence

In earlier studies we have demonstrated that the OBF1 DNA-binding sites enhance *ARS* function in an orientation independent manner at varying distances from the essential core (15). In order to gain a better understanding of the potential role of ATR in origin function, we have examined the orientation requirement of the ATR in relation to the core sequence. First, as described in Fig. 6A, the ATR was fused to the core at the 3′ end in an inverted orientation relative to the natural situation. The two origins, containing the ATR in either orientation, were equally efficient (Fig. 6B). Therefore the orientation of the ATR, when positioned 3′ to the essential core, does not affect origin activity. In contrast, when fused upstream (5′ to) of the core region, in either orientation (YCpRCAU(+) or YCpRCAU(−)), the ATR

Core:
TGTTTTGTTT AACATTAGTT TCAAATTAAC AGCTT

ATR:
AAATATATTT TGGATGAATA GCAGAAAATA GAAATCTGGA
AATAAACAAA ACAGAGCCAA AATTCTAAGC TT

ARS Enhancer:
TCGTATTTAG TGATTATAAT AC

Figure 4. Sequences of the functional domains of *ARS121*. The sequences of the *ARS121* core and ATR are as defined in Figs. 2 and 3 and in the text. The *ARS* enhancer sequence represented is the region of *ARS121* protected from limited DNase I digestion as previously described (15,27).

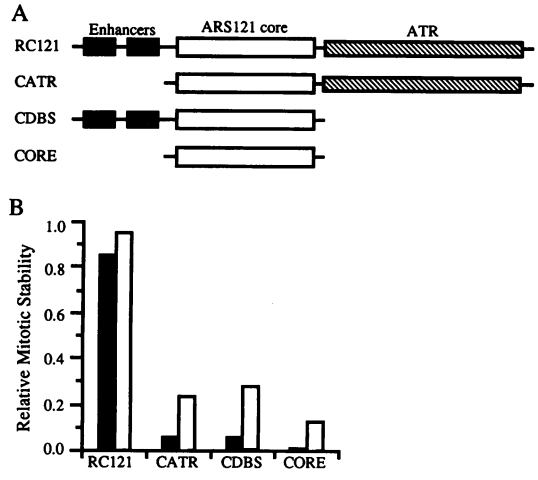

Figure 5. Reconstruction of *ARS121* from isolated elements. **(A)** Schematic presentation of *ARS121* reconstructed from isolated, functional domains as described in Materials and Methods. The *ARS* enhancers (Two, tandem, 22 bp synthetic OBF1 DNA-binding sites, 15) and the A/T-rich region (ATR, 72 bp) are denoted as stippled and cross-hatched boxes, respectively. For convenience of cloning the ARS121 essential core, denoted by the open box, we included an additional 8 bp of DNA comprising the region defined by the fs10 substitution mutation (shown in Figs. 2 and 3). Lines between elements indicate pUC19 polylinker DNA. The size of the polylinker separating the core from ATR is 18 bp, while 36 bp of polylinker separate the core from the enhancers. The essential core and ATR of *ARS121* occur in their natural relative orientation with respect to each other. **(B)** Histogram of stability data expressed as a fraction of the stability of the wildtype 489 bp *ARS121* fragment (Fig. 2). Stability data based on plasmid maintenance in nonselective and selective media are represented by filled and open boxes, respectively. The stability of the wildtype *ARS121* is shown in Fig. 2B.

was not functional. In this situation no stimulation of origin activity above the levels achieved by the core alone was observed (Fig. 5B). We ascribe this position effect on origin function to the ATR domain and not the enhancer since the latter has been shown to work equally well in either orientation irrespective of its relative position to the core (15).

An important relationship between the *ARS* consensus sequence and the 3′ flanking DNA has also been suggested in the studies with the *H4 ARS* (32). In this *ARS*, inversion of the consensus sequence in relation to the 3′ region appeared to inactivate the origin. However, since in this case the inversion mutation completely inactivated the *H4 ARS*, it is also possible that the inversion itself may have disrupted some other component of the essential core in addition to the relative orientation to an ATR-like domain. That this may be the case is supported by the fact that deletion analysis of *H4 ARS* indicates that the minimum essential region is larger than the 11 bp consensus sequence alone (32), as is the case for *ARS121*. Nevertheless, the findings with *ARS121* suggest that the essential core and ATR have to interact in a position specific manner to elicit maximal levels of origin activity.

The *ARS* enhancer and ATR of *ARS121* can enhance the origin activity of heterologous essential core sequences

Since the *ARS* enhancer and ATR play an important role in *ARS121* function, we reasoned that if a similar mechanism of initiation operates at other yeast origins, then these domains may stimulate the functional cores of other *ARS*s. To test this hypothesis, we fused the *ARS* enhancer and ATR to the core of *ARS1* and *ARS307*. It was especially interesting to examine how these two *ARS*s are affected by the auxiliary domains since the *ARS* enhancer (OBF1/ABF-I DNA-binding site) was found to be present in the wildtype *ARS1* and not in *ARS307* (33–37).

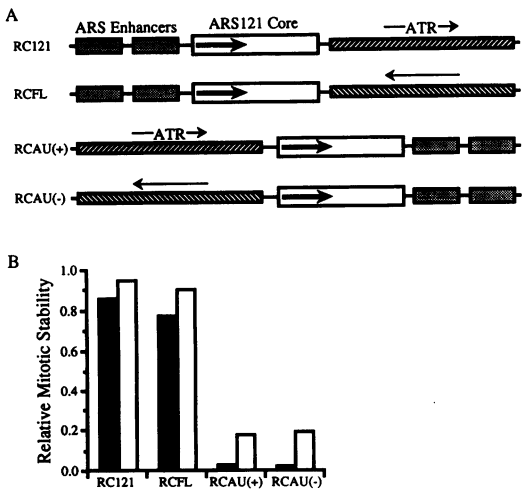

Figure 6. Determination of the orientation and position dependence for ATR function. **(A)** Schematic presentation of plasmid constructions used to test the orientation and location requirements of the *ARS121* ATR domain. Isolated components of *ARS121* were described in Fig. 4 and 5. The wide arrows within the *ARS121* core boxes indicate the 5′ to 3′ orientation of the T-rich strand of the essential consensus-like sequence. The thin arrows denote the 5′ to 3′ orientation of the A-rich strand of the partial matches to the *ARS* core consensus sequence present in the ATR (Fig. 2). **(B)** Relative stability data for the plasmids described in A. Histogram features are as described in the legend to Fig. 5

The core sequence of *ARS1* was prepared based on the deletion studies of Celniker, et al. (13). This 50 bp-long nucleotide stretch (Fig.7D) includes the 11 bp consensus (domain A) and about 34 bp of a sequence located 3′ to the T-rich strand of the *ARS* consensus. As shown in Fig.7B, the *ARS1* core alone (YCpC1) functioned as an extremely inefficient origin of replication. Fusion of the ATR and the enhancer elements of *ARS121* to the *ARS1* core, which formed the chimeric origin RC1, has augmented origin activity over the core alone (Fig. 6B). The activity regained was about equal to that of the wild type *ARS1*. This stimulation was dependent on both the ATR and the *ARS* enhancers, as shown in Fig.7B. In similar experiments with the 67 bp core sequence of *ARS307* (formerly *ARS C2G1*), as defined by Palzkill and Newlon (11), we observed an increase in *ARS307* function by the ATR and enhancers of *ARS121* over the *ARS307* core itself (Fig.7C). In this case, however, the chimeric *ARS* (YCpRC307) appeared to have only 35–40% efficiency of the wild type *ARS307*, suggesting that in addition to ATR and the enhancers another unidentified element may be necessary to recover full *ARS307* function. Nonetheless, the enhancer and the ATR domains of *ARS121* appear to act synergistically to activate core

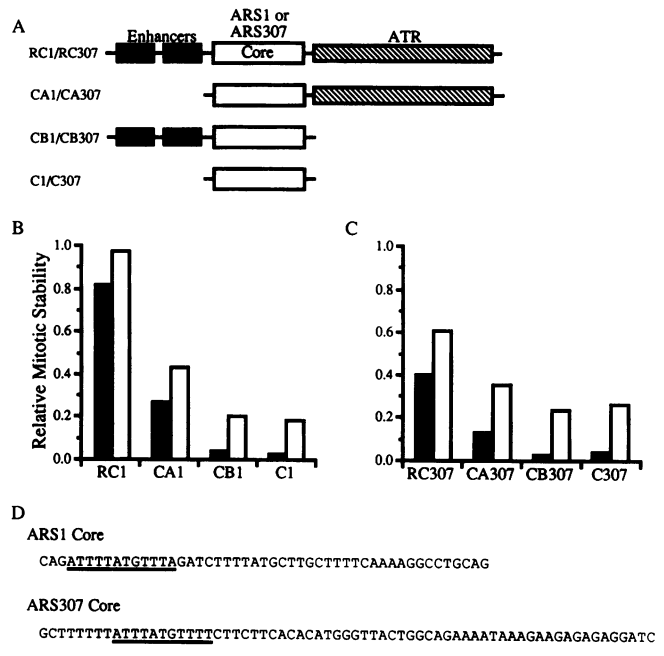

Figure 7. The OBF1 DNA-binding sites and the ATR of *ARS121* can stimulate the function of the minimal *ARS1* and *ARS307* core regions. **(A)** The open boxes depict the core sequences of either *ARS1* or *ARS307*, obtained as described in Material and Methods. The *ARS* enhancers (stippled boxes), the ATR (cross hatched boxes) and the T-rich strand of the *ARS* consensus sequence, which is embedded in the *ARS1* and *ARS307* cores, occur in the same relative orientation as that for the reconstruction of *ARS121* (Fig. 5). **(B)** and **(C)** Histograms showing the data for the stabilities of the *ARS1* and *ARS307* chimeric plasmids expressed as a fraction of the stability exhibited by the 340 bp HincII/HindIII fragment of *ARS1* (25) (B) and the 200 bp *ARS307* fragment obtained by PCR as described in Material and Methods (C), in YCp5-3 . Histogram features are as described in the legend to Fig. 5. The HincII/HindIII *ARS1* fragment contains domains A, B (including the OBF1-binding site), and a portion of C, as defined by Celniker, et al. (13). Its stability was 19.9 ± 6.0 and 66.5 ± 7.1% in nonselective and selective medium, respectively. The stability of the 200 bp wildtype *ARS307* cloned into YCp5-3 was 8.3 ± 1.9% in nonselective medium and 48.8 ± 7.9% under selective growth conditions. **(D)** The nucleotide sequences of the *ARS1* and *ARS307* cores used in the plasmid constructions described in panel A.

sequences of heterologous origins of replication, suggesting that the activation of different origin cores may occur by a common mechanism. This notion does not exclude the possibility that the action of different *ARS*s may be modulated by different regulatory proteins as previously suggested (23,38,39).

DISCUSSION

The analysis of *ARS121* in its native chromosomal location indicates that it is an active origin of replication on chromosome X. We have systematically dissected this nuclear origin by site-directed *in vitro* linker substitution mutagenesis and deletion analysis. These studies have delineated the boundaries of three distinct functional units, whose nucleotide sequences are described in Fig.4. One of these is the essential core comprised of a near match to the 11 bp *ARS* consensus sequence, adjacent nucleotides that are intolerant to linker substitution and sequences that are intolerant to external deletion. This 35 bp region is essential and sufficient for *ARS121* function, yet the essential core itself is a very weak plasmid replicator, suggesting that other components are necessary for optimal origin activity.

Extending 3′ to the core sequence is a region of high A/T content (75%) (Fig. 4), called the ATR, that by deletion analysis has been determined to have a role in the origin function of *ARS121*. Because of its relatively high A/T content, the ATR resembles the region found 3′ to the T-rich strand of the consensus sequence in other *ARS*s (11). The A/T enriched stretches of nucleotides in this region are concentrated in several clusters that partially match the core consensus sequence. Although the function of these sequences found 3′ to the core of many *ARS*s is unknown, Palzkill and Newlon (11) have suggested that they play an important role in origin function, perhaps by serving as binding sites for an initiation protein. We have found three consensus-like sequences in the ATR domain of *ARS121*. In earlier studies we demonstrated that a triple linker mutation,which eliminated all three consensus-like sequences, caused only a two-fold reduction in origin efficiency (15). A deletion of the ATR, however, resulted in a more drastic ten-fold reduction of origin function (Fig.3A). It appears, therefore, that proper arrangement of DNA sequences in the entire ATR is necessary for this domain to exert maximal stimulatory activity, not unlike the observations made for other *ARS*s (32,40).

A third domain we found to be crucial for full *ARS121* function is the *ARS* enhancer. This enhancer binds the OBF1 protein and stimulates *ARS* function in a distance and orientation independent manner (15). The origin reconstruction experiments (Figs. 5 and 7) have shown that the enhancer can exert its function only when the ATR is present. The enhancer and the ATR appeared to act synergistically to enhance the activity of the essential core sequence. These results suggest that the enhancer may have to interact with the ATR directly or indirectly through interactions with the origin core. Another type of interaction has been revealed by the experiment demonstrating that the ATR domain works in an orientation independent manner but only when located 3′ to the essential core, suggesting that the ATR may have to interact directly with the core to allow its function as a replication stimulator. It would appear, therefore, that in order to elicit maximal origin activity all three domains of *ARS121* must interact in a coordinated manner.

The precise role of these domains in origin function is not known. However, it is reasonable to assume that the interactions discussed above are mediated by proteins. It is possible that an initiation protein binds to the essential core sequence and forms a complex with the ATR domain directly or via interaction with another protein. Others have suggested that the region 3′ to the core consensus sequence comprises an element that is easily unwound to allow entry of the replication elongation machinery (41–43). The protein complex assembled at the core/ATR region may facilitate such an unwinding. Formation or stability of the protein complex assembled at the core/ATR region may also be promoted by the OBF1 protein, which binds specifically to the enhancer. We have already demonstrated that the enhancer works in an orientation independent manner even when situated at long distances from the core (15). Furthermore, we have suggested that this domain and its cognate protein, OBF1, interact with the origin core/ATR region by DNA looping, enabling a direct interaction of OBF1 with a component of the replication initiation apparatus. A prediction of such a model is the assembly of a replication initiation protein-DNA complex at the core/ATR region whose formation or stability is enhanced by the OBF1 protein.

One of the problems we have addressed is whether the mechanism of initiation of replication at different yeast origins shares something in common or is fundamentally different. To this end we have reconstructed origins by fusing the two auxiliary domains, the enhancer and ATR, to the core sequences of heterologous *ARS1* and *ARS307*. In both cases dramatic stimulation of origin activity was observed, suggesting that the events occurring during the initiation of replication at the different core sequences are fundamentally similar, in spite of the fact that little sequence homology exist among core sequences. A deeper understanding of the mechanism of initiation of DNA replication at the *ARS*, however, will require the isolation and characterization of the initiation proteins that, we presume, assemble as a protein-DNA complex at yeast origins of replication.

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