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An Abf1p C-terminal region lacking transcriptional activation potential stimulates a yeast origin of replication

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ABSTRACT

Although it has been demonstrated that eukaryotic cellular origins of DNA replication may harbor stimulatory elements that bind transcription factors, how these factors stimulate origin function is unknown. In *Saccharomyces cerevisiae*, the transcription factor Abf1p stimulates origin function of ARS*121* and ARS*1*. In the results presented here, an analysis of Abf1p function has been carried out utilizing LexA(BD)–Abf1p fusion proteins and an ARS*121* derivative harboring LexA DNA-binding sites. A minimal region which stimulates origin function mapped to 50 amino acids within the C-terminus of Abf1p. When tested for transcriptional activation of a LacZ reporter gene, the same LexA(BD)–Abf1p fusion protein had negligible transcriptional activation potential. Therefore, stimulation of ARS*121* may occur independently of a transcriptional activation domain. It has been previously observed that the Gal4p, Rap1p DNA-binding sites and the LexA–Gal4p fusion protein can replace the role of Abf1p in stimulating ARS*1*. Here we show that the stimulatory function of Abf1p at ARS*121* cannot be replaced by these alternative DNA-binding sites and the potent chimeric transcriptional activator LexA(BD)–Gal4(AD)p. Hence, these results strongly suggest that the Abf1p stimulation of replication may differ for ARS*121* and ARS*1*, and imply specificity in the Abf1p/ARS*121* relationship.

INTRODUCTION

Analyses of eukaryotic nuclear origins of DNA replication have been greatly facilitated by the biochemically and genetically tractable yeast, *Saccharomyces cerevisiae*. DNA sequences of *S.cerevisiae* were identified which enabled the extrachromosomal replication of plasmids (1). These sequences, known as ARSs (autonomously replicating sequences), act as genuine origins of replication (2,3). While a subset of ARSs have been demonstrated to function as origins in their native chromosomal location, all chromosomal origins identified to date correspond to ARSs (for a review, see ref. 4).

Detailed characterization of a number of ARSs has identified individual elements which together elicit full activity (4). The only highly conserved sequence that is essential for function in ARSs is the 11 bp [(A/T)TTTAT(A/G)TTT(A/T)] ARS consensus sequence (ACS) (5,6). The ACS is part of a larger essential element, which varies in size depending on the ARS. Auxiliary elements are found on both sides of the essential element. Elements are often systematically referred to as residing in domain A, B or C (7). Domain A currently refers to the entire essential ACS-containing region as defined by linker substitutions (4). Domains B and C currently refer to the regions outside of domain A, 3′ and 5′ to the T-rich strand of the ACS, respectively (4).

It has been shown that the ARS essential sequences are specifically recognized by ATP-dependent DNA-binding proteins (8,9). Genetic evidence has implicated one of these, the ORC, in the initiation of DNA replication (10,11) as well as in transcriptional silencing (12,13). Genetic and/or physical interactions with ORC have been identified for a number of factors which have also been implicated in the initiation of DNA replication (14–19).

An auxiliary element is located at several ARSs is the DNA-binding site for Abf1p. Abf1p is an essential, multifunctional protein, with roles in DNA replication and in the transcriptional activation and repression of a number of diverse genes (for a review, see ref. 20). The function of Abf1p in replication has been examined in greatest detail at ARS*121* and ARS*1*, each of which contain auxiliary Abf1p DNA-binding sites. At ARS*121*, a series of linker substitutions through the Abf1p DNA-binding sites were utilized to demonstrate a direct, strong correlation between *in vitro* binding of Abf1p to the ARS and *in vivo* ARS function (21). In addition, at the semi-permissive temperature in *abf1(ts)* strains, ARS*121* function was severely reduced (22). Linker substitution analyses at ARS*1* also demonstrated that elimination of Abf1p binding *in vitro* abolished stimulation of ARS*1* by the Abf1p DNA-binding site (23).

How Abf1p stimulates ARS function is unknown. It has been shown that transcriptional activators can substitute for Abf1p function at the ARS*1* origin (23). Transcriptional activators are known to stimulate replication for a number of eukaryotic viruses. Studies in viral systems have indicated that auxiliary factors

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which stimulate DNA replication can do so in a number of ways (for reviews, see refs 24,25). For example, stimulation of bovine papilloma virus (BPV) DNA replication *in vitro* was mediated by the interaction of a transcriptional activation domain with the replication protein A (RPA) (26). Another mode of action, the alleviation of chromatin repression, required that the DNA-binding site of the auxiliary factor be located adjacent to essential core sequences (24,25,27). However, Abf1p DNA-binding sites can function in an orientation and distance independent manner as replication enhancers (28). Therefore, a role for Abf1p in overcoming chromatin repression may be less likely.

In this paper we report the results of experiments carried out to address Abf1p's role in replication. We have assessed the ability of LexA(BD)–Abf1p fusion proteins to stimulate ARS*121*. We show that a 50 amino acid region within the C-terminus of Abf1p is capable of stimulating ARS*121*. This LexA(BD)–Abf1p fusion protein lacks significant transcriptional activation potential. In addition, alternative factors, including known transcriptional activators, are unable to substitute for Abf1p at ARS*121*. These results indicate that ARS stimulation may occur independently of a transcriptional activation domain, and that ARS stimulation exhibits specificity at the ARS*121* replication origin.

MATERIALS AND METHODS

Strains and media

Saccharomyces cerevisiae strain FY251 *(MAT*a, *his3*Δ*200*, *leu2*Δ*1*, *trp1*Δ*63*, *ura3-52*; a gift of F. Winston, Harvard) was used. Chromosomal integrations at the BAR1 and ura3 loci of FY251 were carried out as described below. Selective media was 0.17% yeast nitrogen base/0.5% ammonium sulfate, supplemented with uracil, histidine, leucine and tryptophan as required (29). Non-selective media was 1% yeast extract/2% bactopeptone containing galactose or glucose at 2% final concentration (29). *Escherichia coli* HB101 was used for plasmid preparations.

LexA(BD) (binding domain) fusion constructs

Plasmid pHLO (constructed in this lab) is a derivative of pOBF1 (30) and encodes a 6His–LexA(BD)–Abf1p fusion. The 6His is present to facilitate purification of certain LexA(BD)–Abf1p fusion proteins for studies not described in this manuscript. LexA(BD) refers to LexA residues 1–87 (31). To facilitate construction of deletions, an *Nde*I site is present at the LexA(BD)–Abf1 junction. In addition, an *Xba*I site and stop codons in all three reading frames immediately follow the Abf1 gene stop codon. LexA(BD)–Abf1 fusions were generated with convenient restriction enzyme sites or by cloning a PCR product generated with appropriate oligonucleotides. The Abf1p residues present in each fusion are indicated. The construct encoding LexA(BD)–Gal4(AD)p (activation domain) was made by first cloning the *Kpn*I–*Sau*3AI fragment from pGAD424 (Clonetech) into the *Kpn*I–*Bam*HI sites of pUC19, and then moving an *Eco*RI(filled)–*Xba*I fragment from the resulting plasmid into the *Nde*I(filled) plus *Xba*I sites of pHLO. For the LexA(BD) construct, pHLO was digested with *Nde*I plus *Xba*I, end filled and re-circularized. Constructs were diagnosed by restriction analysis and verified by DNA sequencing.

Expression of fusion proteins

All fusion proteins were expressed from a disrupted non-essential chromosomal locus. For chromosomal integration we utilized the plasmid pbar1::GAL1P (constructed in this lab), which is a derivative of pRS405 (32). This plasmid harbors (i) LEU2 for selection, (ii) a *Sal*I cassette for directing gamma integration/disruption at the BAR1 locus, and (iii) an *Eco*RI–*Bam*HI GAL1 promoter from pMH101 (30). When the LexA(BD)–Abf1 *Bgl*II fragment from pHLO is inserted in the correct orientation into the *Bam*HI site of pbar1::GAL1P, the encoded protein is placed under the control of the GAL1 promoter, as previously described for ABF1 (30). For each pHLO derivative, the appropriate *Bgl*II fragment was moved into the *Bam*HI site of pbar1::GAL1P. Plasmid with a monomer insert in the correct orientation was then prepared, and gamma integrations were carried out as previously described (32). Plasmid was linearized with *Hin*dIII, and the linear DNA was used to transform the yeast FY251. This transformation process targets integrative transformation at the BAR1 locus of FY251 with high efficiency. The BAR1 gene is non-essential and encodes a protease that degrades α factor (33). Purified transformants were screened by Southern analyses for correct integration. Westerns, with an anti-LexA serum (kindly provided by Erica Golemis, Fox Chase Cancer Center), indicated that each fusion was appropriately produced at comparable levels. The size of the fusion proteins produced was within the range known to diffuse into the nucleus (34,35).

Yeast centromeric plasmids

The yeast centromeric plasmid YCpA121 harbors a truncated version of ARS*121* known as A121 (21). The truncation removes the Abf1p DNA-binding sites present at ARS*121*, but leaves the essential core and the auxiliary A+T rich element (21,36). To test LexA(BD)–Abf1p fusions for their ability to stimulate origin function, we utilized YCpLEX/A121 (constructed in this lab) in which eight tandem LexA DNA-binding sites (37) are inserted into the *Kpn*I site of YCpA121. YCpABF1/A121 [previously called YCpA121c(–), (28)] harbors a synthetic Abf1p DNA-binding site inserted into the *Kpn*I site of YCpA121. For substitution experiments, appropriate oligonucleotides were kinased, annealed and inserted into the *Kpn*I site of YCpA121. YCpGAL4/A121, YCpRAP1/A121 and YCpREN1501/A121 harbor a Gal4p DNA-binding site (31), Rap1p DNA-binding site (38) and the REN1501 element (Raychaudhuri and Eisenberg, unpublished), respectively. Since these elements stimulate replication in an orientation independent manner (28,23; Raychaudhuri and Eisenberg, unpublished), each was analysed in a single orientation. The inserts, 5′ to 3′ along the same strand as the T-rich strand of the ARS*121* essential core element, are as follows: GAL4 GGTACCTACGTACGGAAGACTCTCC-TCCGAGATCTGGT-ACC, RAP1 GGTACCTTTTTTCAAGGTTGATGGGTTTT-TGCAATATCGTACC, and REN1501 GGTACCTAGTATATTGC-TATGTATGGTTTGCGTACC. The structure of ARS*121* and the ARS*121* derivatives described above are shown in Figure 1.

Transcriptional activation reporters

For transcriptional activation assays of LexA fusions, we utilized the LacZ reporter borne on a pSH18-34 plasmid (39), or a derivative thereof. For qualitative assays we utilized pSH18-34, which harbors sequences from the 2μ plasmid for maintenance in

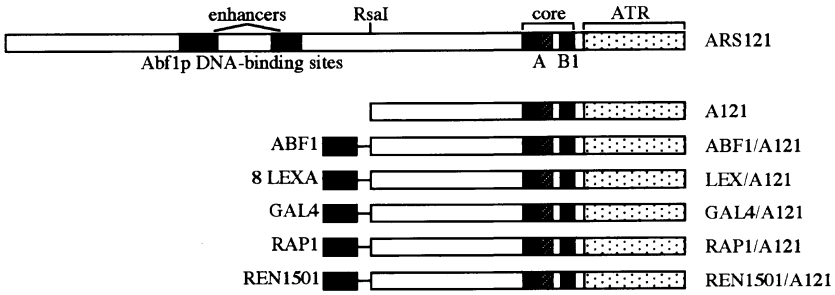

Figure 1. Structure of ARS*121* and ARS*121* derivatives. (**Top**) The 489 bp *Msp*I–*Hin*dIII ARS*121* fragment. ARS*121* is composed of an essential core plus two auxiliary elements, an A+T rich element (ATR) and a replication enhancer (Abf1p DNA-binding sites) (21,28,36). These elements and a *Rsa*I site present in ARS*121* are indicated in the diagram. The ARS*121* core is ~35 bp long and is composed of two regions (36). One region (A), from position 1 to ~23, harbors the ACS plus adjacent sequences and corresponds to domain A. This region is intolerant to linker substitutions (36). The filled-in box depicts the ACS and the hatched box designates ~12 bp 3′ to the T-rich strand of the ACS. The second region of the core spans positions from ~30 to ~35, as indicated by a combination of deletion, linker substitution and linker insertion experiments (36). This region is depicted in the figure by a filled-in box, and is labeled as B1. The designation of B1 is utilized as suggested by consideration of (i) subsequent analyses of ARS*1*, ARS*307* and ARS*305* (23,53–55), and (ii) identification and characterization of ORC (8,50,51). The two regions, (from 1 to ~23) and (from ~30 to ~35), are part of a single entity, the core, as demonstrated by linker insertion experiments (36). (**Bottom**) The derivatives of ARS*121* utilized in this paper are shown. The name of each ARS is indicated on the right. Each ARS is present as an *Eco*RI–*Hin*dIII fragment inserted into the *Eco*RI–*Hin*dIII sites of the yeast centromeric plasmid YCp5-3 (21). A*121* is the *Rsa*I–*Hin*dIII fragment of ARS*121*. The filled box adjacent to A*121* designates the various protein DNA-binding sites. The name of each site is indicated in capital letters to the left. ABF1, GAL4 and RAP1 refer to single DNA-binding sites for the yeast proteins Abf1p, Gal4p and Rap1p. In LEX/A121, eight tandem DNA-binding sites for the *E.coli* protein LexA are present adjacent to A*121*. REN1501 refers to a single copy of the REN1501 element, an enhancer of replication at ARS*1501* (Raychaudhuri and Eisenberg, unpublished). The DNA sequences of these sites are described in Materials and Methods.

yeast. For quantitative assays, we used a chromosomally integrated derivative of pSH18-34. The regulatory LexA DNA-binding sites present in this case are those of YCpLEX/A121. To allow for chromosomal integration, we removed the 2μ sequences, replacing the pSH18-34 *Spe*I fragment with an *Xba*I HIS3 cassette. Plasmid DNA was linearized with *Apa*I, and the linear DNA was used to transform the appropriate strains to Ura^+, His^+ autotrophy (40). Purified transformants were screened by Southern analyses to identify those with correct integration.

Mitotic stability and transcriptional activator assays

Mitotic stability assays (four to five independent transformants each) were performed as described elsewhere (21). With all fusion proteins, or when testing Gal4p, galactose was the carbon source instead of glucose. Mitotic stabilities are reported as the percentage of cells harboring the plasmid following ~14 generations of non-selective growth. Quantitative β-galactosidase assays were carried out as described (39,41) utilizing extracts prepared from cultures grown in galactose-containing media. Extracts were prepared by breakage with glass beads. Time-course experiments verified linear β-galactosidase activity beyond the time-point utilized for the assays. Activities are reported in Miller units (41) standardized to micrograms of protein and represent the average of three or four assays. Protein concentrations were determined by the Lowry method.

RESULTS

A region of Abf1p which stimulates the ARS*121* replication origin

To address Abf1p stimulation of origins of DNA replication, we tested the ability of LexA(BD)–Abf1p fusions to stimulate an origin harboring LexA DNA-binding sites. Origin activity was tested by plasmid mitotic stability measurements. Yeast centromeric plasmids (YCp) contain an ARS element for replication and a CEN element for segregation. Since the CEN element promotes efficient plasmid segregation, the mitotic stability of a YCp is a commonly used indicator of the efficiency of the ARS element, the yeast origin of replication borne on the plasmid.

All fusions were constructed, integrated and expressed from the BAR1 chromosomal locus as described in Materials and Methods. All fusion proteins were expressed to comparable levels as demonstrated by western analysis using anti LexA antibodies (unpublished). The effect of these fusion proteins on replication was measured by the mitotic stabilities of YCpLEX/A121 and YCpA121 plasmids. These plasmids harbor A121, a version of ARS*121* deleted from Abf1p DNA-binding sites (Fig. 1). In LEX/A121, eight tandem LexA DNA-binding sites were inserted adjacent to A121, as shown in Figure 1. The ability of LexA–Abf1p fusions to stimulate ARS function resulted in elevated mitotic stability of YCpLEX/A121 relative to YCpA121.

As shown in Figure 2, three LexA(BD)–Abf1p fusion proteins, bearing residues 1–323, 326–589 or 482–731, were used to survey the length of Abf1p for replication function. Of these three fusions, only LexA(BD)–Abf1(482–731)p, harboring the C-terminal 250 residues of Abf1p, stimulated the YCpLEX/A121 origin. The stimulatory effect was expressed as a ratio of the mean mitotic stabilities of the plasmids YCpLEX/A121 and YCpA121 (Fig. 2). Further analysis was then used to delimit the active region(s) more precisely (Fig. 2, bottom). Truncations from the N-terminus beyond amino acid residue 635 abolished activity, as did truncations from the C-terminus beyond residue 684. Analysis of additional fusions indicated that LexA(BD)–Abf1(635–684)p was sufficient to stimulate ARS*121*. All fusions harboring residues 635–684 provided origin stimulation, and all truncations which removed this region abolished the stimulation of replication. Furthermore, an internal deletion of residues 635–684 from the LexA(BD)–Abf1(482–731)p fusion also abolished stimulation of replication. While not excluding the possible

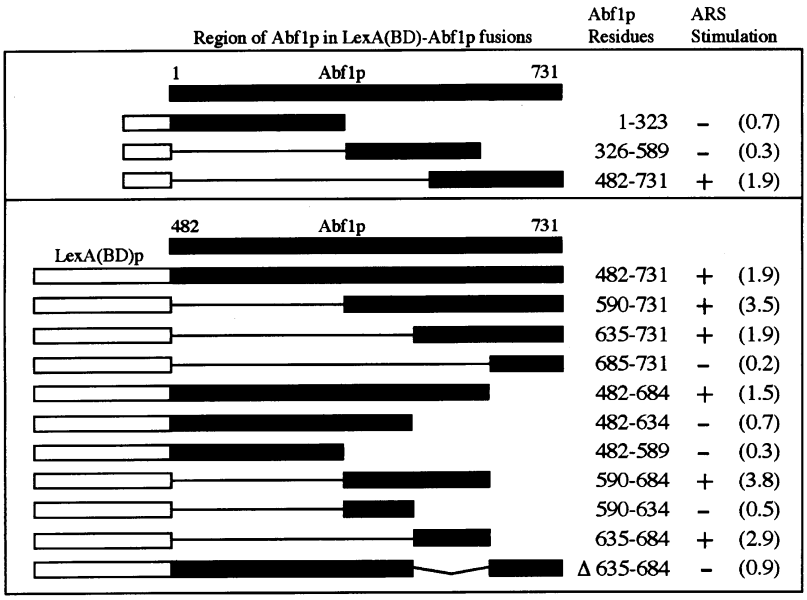

Figure 2. Identification and localization of a region of Abf1p which stimulates ARS*121*. LexA–Abf1p fusion proteins were tested for their ability to stimulate ARS*121* by measuring the mitotic stabilities of the yeast centromeric plasmids YCpA121 and YCpLEX/A121. These plasmids differ only in the version of ARS*121* that they harbor (Fig. 1). For each fusion protein tested, mitotic stability measurements of YCpA121 and YCpLEX/A121 were carried out in parallel as described in Materials and Methods. The '+' sign indicates that the mean mitotic stability of YCpLEX/A121 was greater than that of YCpA121. In each '+' case the mean – S.D. mitotic stability of YCpLEX/A121 was higher than the mean + S.D. mitotic stability of YCpA121. The '–' sign indicates that the mean mitotic stability of YCpLEX/A121 was less than or equal to that of YCpA121. In the absence of a fusion protein, the mitotic stabilities of YCpLEX/A121 and YCpA121 are equivalent (± S.D.). The numbers in parentheses express the ratio of the mean mitotic stabilities of the plasmids YCpLEX/A121 and YCpA121. The LexA DNA-binding domain [LexA(BD)] present at the N-terminus of each fusion protein is indicated by the unfilled box. Abf1p regions are indicated by the filled box, and the numbers in the 'Abf1p residues' column indicate the Abf1p residues present.

contribution of other regions of the protein, these results indicate that a 50 amino acid domain of Abf1p, residues 635–684, is capable of stimulating ARS function when tethered via a heterologous DNA-binding domain.

The Abf1p domain that stimulates replication has negligible transcriptional activation potential

Abf1p is also a transcription factor, functioning at promoters of a diverse number of genes (for a review, see ref. 42). Abf1p DNA-binding sites act as moderately potent transcriptional activators (38,43) and the same is true for LexA(BD)–Abf1p fusion proteins harboring C-terminal regions of Abf1p (44; Wiltshire and Eisenberg, unpublished). Substitution experiments of alternative transcription factors for Abf1p at ARS*1* suggested that origin stimulation by factors such as Abf1p required a transcriptional activation domain (23).

To determine if the LexA(BD)–Abf1(635–684)p fusion protein constituted a transcriptional activator, we carried out transcriptional activator assays with a LacZ reporter gene. Yeast harboring the reporter plasmid pSH18-34 (39) and expressing LexA(BD)–Abf1(635–684)p produced a white colony with only a hint of extremely pale blue color following extended growth on X-gal containing plates (unpublished observations). This analysis was followed up by quantitative assays performed with a chromosomally integrated derivative of pSH18-34, harboring the reporter LacZ gene, in which the regulatory LexA DNA-binding sites present are those of YCpLEX/A121 (Fig. 1). As shown in Figure 3, the quantitative analysis confirmed the qualitative observations, namely LexA(BD)–Abf1(635–684)p as well as LexA(BD)p had negligible transcriptional activation potential. Under the same conditions the LexA(BD)–Gal4(AD)p fusion protein resulted in approximately three orders of magnitude higher levels of β-galactosidase activity (Fig. 3).

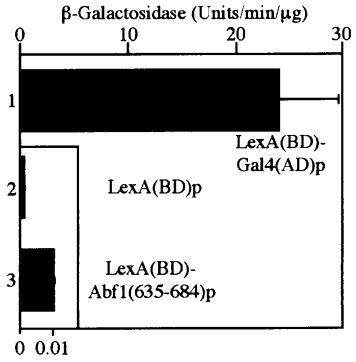

Figure 3. Transcriptional activator assays. Quantitative β-galactosidase assays were carried out as described (Materials and Methods), utilizing ONPG (*o*-nitrophenyl β-D-galactopyranoside) as the substrate. Cells expressed either (1) LexA(BD)–Gal4(AD)p, (2) LexA(BD)p or (3) LexA(BD)–Abf1(635–684)p. Extracts from cells expressing LexA(BD)p or LexA(BD)–Abf1(635–684)p provided extremely low, albeit detectable β-galactosidase activity. These rows are boxed off with an expanded scale.

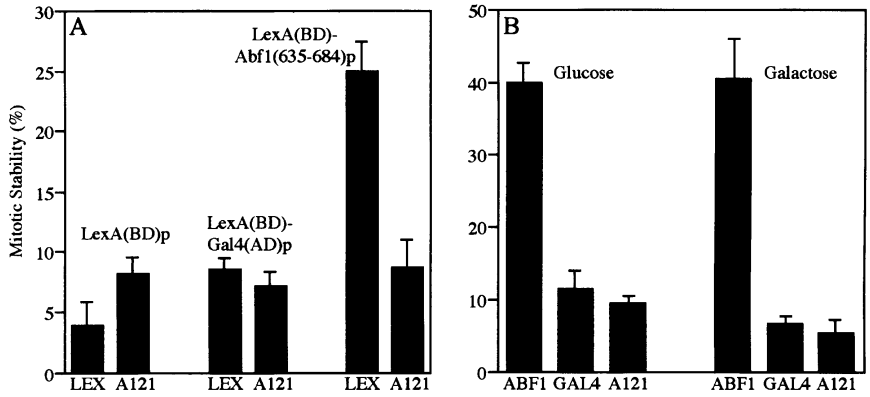

Figure 4. Substitution of transcriptional activators for Abf1p at ARS*121*. Mitotic stabilities were determined as described in Figure 2. (**A**) The mitotic stabilities of YCpLEX/A121 and YCpA121 were determined in cells expressing proteins LexA(BD)p, LexA(BD)–Gal4(AD)p, or LexA(BD)–Abf1(635–684)p. These are the same proteins assayed for transcriptional activation in Figure 3. The expressed protein is indicated above each pair of columns, and the plasmid assayed for mitotic stability is indicated at the bottom of each column. LEX and A121 refer to YCpLEX/A121 and YCpA121. (**B**) The mitotic stabilities of YCpABF1/A121, YCpGAL4/A121 and YCpA121 were determined in FY251 in glucose and in galactose. The carbon source is indicated above each set of columns, and the plasmid is indicated at the bottom of each column. ABF1, GAL4 and A121 refer to YCpABF1/A121, YCpGAL4/A121 and YCpA121, respectively.

Substitution of transcriptional activators for Abf1p

Although LexA(BD)–Abf1(635–684)p lacked transcriptional activation potential, we nonetheless considered it possible that a transcriptional activation domain may be capable of ARS*121* stimulation. We therefore tested LexA(BD)–Gal4(AD)p, harboring the Gal4p activation domain, for its ability to stimulate ARS*121*. The results of these mitotic stability assays are presented in Figure 4A, along with the results for LexA(BD)–Abf1(635–684)p and LexA(BD)p. While LexA(BD)–Gal4(AD)p served as a potent transcriptional activator (Fig. 3), it provided negligible ARS stimulation (Fig. 4A). In contrast, LexA(BD)–Abf1(635–684)p provided negligible transcriptional activation (Fig. 3), but stimulated the ARS (Fig. 4A). Hence, it appears that there is no direct correlation between the ability of a protein to activate transcription and its ability to stimulate the ARS*121* replication origin.

Although the chimeric LexA(BD)–Gal4(AD)p transcriptional activator failed to stimulate the origin, we considered it possible that native transcription factors might nonetheless be capable of stimulating ARS*121*. To test this possibility, we replaced the Abf1p DNA-binding site of YCpABF1/A121 with a Gal4p DNA-binding site, yielding YCpGAL4/A121. Mitotic stabilities were assayed in glucose and in galactose, and the results are shown in Figure 4B. The Abf1p DNA-binding site stimulated ARS*121*, but the Gal4p DNA-binding site did not.

Although Gal4p is capable of ectopically stimulating ARS*1* (23), Gal4p is not known to be naturally associated with any origin of replication. We therefore tested two elements which are naturally found to stimulate origin activity: a Rap1p DNA-binding site and the REN1501 element of ARS*1501*. Rap1p stimulates a synthetic HMRE origin (45) and could functionally replace Abf1p at ARS*1* (23). REN*1501* is a recently identified replication enhancer element which stimulates ARS*1501* (Raychaudhuri and Eisenberg, unpublished). We carried out mitotic stability analyses of YCpRAP1/A121 and YCpREN1501/A121, harboring a Rap1p DNA-binding site and the REN1501 element, respectively (Fig. 1). As shown in Figure 5, the Rap1p DNA-binding site and the REN1501 element were unable to functionally replace the Abf1p DNA-binding site at ARS*121*. Thus, the stimulation of ARS*121* appears to exhibit a specific requirement for Abf1p.

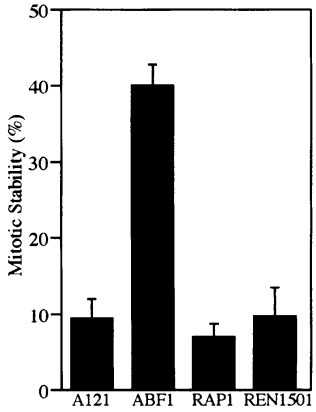

Figure 5. Specificity of ARS*121* stimulation. The Abf1p DNA-binding site of YCpABF1/A121 was replaced with two other elements naturally found to stimulate origins, a Rap1p DNA-binding site and REN1501. Rap1p is a multifunctional protein like Abf1p, and REN1501 is a recently identified enhancer of ARS*1501*. Mitotic stabilities were assessed in FY251 as described in Figure 2. The plasmid analyzed is indicated at the bottom of each column. A121, ABF1, RAP1 and REN1501 refer to YCpA121, YCpABF1/A121, YCpRAP1/A121 and YCpREN1501, respectively.

DISCUSSION

The results presented here indicate that a 50 amino acid region within the C-terminus of Abf1p is capable of stimulating the ARS*121* origin. Origin stimulation by LexA(BD)–Abf1(635–684)p and by Abf1p require tethering of the protein to the ARS. Obviously, a functionally important region of Abf1p is the DNA-binding domain, even if only to serve a tethering role. It has been shown before that the DNA-binding domain of Abf1p spans an extended region, from residue 1 to ~530 (46), including a possible zinc finger structure located at the N-terminal portion of the protein (46,47).

Three LexA(BD)–Abf1p fusions, harboring Abf1p residues 1–323, 329–589 and 482–731, were utilized to survey the length of Abf1p for DNA replication activity. Of these three fusion proteins, only LexA(BD)–Abf1(482–731)p stimulated ARS*121*. Subsequent analysis delimited the replication stimulatory region to residues 635–684 of Abf1p. While it is possible that Abf1p residues 635–684 serve as the sole ARS-stimulatory region of Abf1p, the experiments presented here do not rule out the existence of additional domains that may function in DNA replication. For example, (i) placement of the LexA(BD)p at the N-terminus of Abf1p(1–323) or Abf1p(329–589) might have obscured another stimulatory region; (ii) a stimulatory region might have been disrupted by the breakpoints at Abf1p residues 323 and 329; (iii) exposure of a stimulatory region may require the binding of Abf1p to its cognate DNA-binding site.

Functional redundancies have often been observed within a given factor which activates transcription (48). It is unknown if this is true for factors which stimulate replication. We have recently replaced the ABF1 gene with a version that encodes Abf1p deleted of residues 635–684. The resulting strain appears to have wild type growth rates and exhibits wild type stability levels for plasmids harboring ARS*121* (Wiltshire and Eisenberg, unpublished). Thus, if the 635–684 region of Abf1p stimulates ARS function as part of Abf1p, this region is likely to be one of at least two functionally redundant domains. It is interesting to note that Abf1p harbors two overlapping, functionally redundant domains for viability (44). The Abf1p (635–684) region, which stimulates ARS*121* function as a fusion protein, overlaps with one of the two redundant domains.

Previous studies indicated that Abf1p DNA-binding sites and LexA(BD)–Abf1p fusion proteins are capable of acting as transcriptional activators (38,43,44). In fact it has been shown that the C-terminal 132 amino acids (residues 600–731) exhibit transcriptional activation potential when fused to the LexA(BD) (44). Using our experimental system we have observed that the LexA(BD)–Abf1(635–684)p fusion protein had very little transcriptional activity. However, larger fusions, in agreement with other studies, exhibited more significant transcriptional activation potential (unpublished). Hence, the region encompassing amino acids 635–684 may be a part of a larger C-terminal Abf1p domain involved in transcription.

Clearly, the C-terminal region of the Abf1p protein harbors the ability to activate transcription and stimulate the function of a replication origin. In our functional analysis we observe a lack of a direct correlation between the ability of a fusion protein to stimulate replication and its ability to activate transcription (unpublished observations). Namely, fusion proteins that were more active in transcription were not necessarily more active in replication, suggesting that the function of the two activities may be separable. The resolution of this question, however, may require further functional analysis by point mutations within the C-terminal region of Abf1p. A separation of function has been noted for a viral DNA replication system. The BPV E2 protein stimulates both replication and transcription. Detailed analysis of the E2 protein indicated that a replication-stimulatory domain coincided with, but was genetically separable from, a transcriptional activation domain (49).

While a region of Abf1p which stimulates replication may genetically be separable from a region of Abf1p that can act as a transcriptional activator, it is nonetheless possible that other transcriptional activation domains might stimulate ARS*121*. However, transcriptional activators LexA(BD)–GAL4(AD)p and Gal4p appeared unable to substitute for Abf1p. Moreover, the Rap1p DNA-binding site and REN1501 element were also unable to substitute for the Abf1p DNA-binding site, again indicating specificity of the Abf1p stimulatory effect on ARS*121* activity. Therefore, even if Abf1p (635–684) is part of a larger domain involved in transcriptional activation, this domain harbors a capacity apparently lacking from other transcription factors.

Although it could be argued that the inability of alternative transcription factors to functionally replace Abf1p at the ARS*121* origin is a result of inaccessibility of these factors to their cognate DNA-binding sites, we view this possibility as unlikely. All heterologous binding elements were inserted into a position at which the Abf1p DNA-binding site is functional.

In contrast to ARS*121*, ARS*1* exhibits promiscuous stimulation by a variety of transcriptional activators, such as Gal4p, Rap1p and a LexA(BD)–Gal4(AD)p fusion (23). These results strongly suggest that the same functional element, an Abf1p DNA-binding site, may function differently at different ARSs. Specifically, it is possible that regions of Abf1p required for stimulation of ARS*121* may be genetically separable from those required for stimulation of ARS*1*.

The results obtained strongly suggest a specific requirement for Abf1p at ARS*121*. This apparent specificity of the Abf1p/ARS*121* interaction is not an exception. Recent studies from this laboratory have demonstrated the presence of a replication enhancer at ARS*1501* (Raychaudhuri and Eisenberg, unpublished). This apparently novel enhancer, termed REN1501, does not appear to bind Abf1p, Rap1p or other known transcription factors. Substitution experiments demonstrated that only the Abf1p DNA-binding site, but not the sites that bind Rap1p or Gal4p, could partially replace the REN1501 element in stimulating ARS*1501* (Raychaudhuri and Eisenberg, unpublished). Thus, among the three ARSs (ARS*1*, ARS*1501* and ARS*121*) at which substitution experiments have been carried out, a spectrum of specificity in the action of DNA replication enhancers has been observed. Such specificity may reflect the diversity of ARS composition. Domains A of ARSs are non-identical, and ARSs harbor a differing nature, number and arrangement of auxiliary elements.

It appears that ORC/ARS*1* interactions are Abf1p independent (50,51). However, this situation may be different at ARS*121*. We previously demonstrated that Abf1p is one of at least three factors required *in vitro* for the formation of a stable multiprotein complex at essential sequences of the ARS*121* origin of replication (9,52). The binding properties and certain physical properties of one of the activities, core binding factor (9,52), indicated that it could be ORC or ORC-related. The results presented in our current analyses indicate that Abf1p may stimulate ARS*121* and ARS*1* activity by different mechanisms. Taking into consideration all available data, a simple model emerges. Abf1p at ARS*121* may stimulate origin function by acting to stabilize a complex at the origin (9,52). In contrast, Abf1p at ARS*1* may stimulate the origin by interacting with other factors, perhaps RPA (23,51).

A differential requirement for Abf1p stabilization of an origin complex could arise from divergent ORC DNA-binding sites. For example, such divergence is apparent in a comparison of the essential sequences of ARS*121* and ARS*1*. The ARS*121* domain A is 18–23 bp in length and its ACS is a 9/11 match to the consensus ACS (36), whereas the ARS*1* domain A is only 15 bp

in length and its ACS is an 11/11 match (23). Studies on the strength of ORC/ARS interactions for different ARSs have not been reported.

How Abf1p and/or LexA(BD)–Abf1(635–684)p carry out their stimulation of ARS*121* is unknown. In simple terms, these factors could work by directly altering DNA structure or by protein–protein interactions. As Abf1p potently stimulates ARS function even at distances >1 kb (28), models invoking direct alterations of DNA structure seem less likely. The fact that LexA(BD)–Abf1(635–684)p harbors a region of Abf1p outside of the Abf1p DNA-binding domain, lends further support to the notion that Abf1p functions at ARS*121* through contact(s) with other proteins. Therefore, identification and characterization of factors which interact with Abf1p should provide further insight into the mechanism of origin stimulation by Abf1p.

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REFERENCES

1 Stinchcomb,D.T., Struhl,K. and Davis,R.W. (1979) *Nature* **282**, 39–43.
2 Brewer,B.J. and Fangman,W.L. (1987) *Cell* **51**, 463–471.
3 Huberman,J.A., Spotila,L.D., Nawotka,K.A., el-Assouli,S.M. and Davis,L.R. (1987) *Cell* **51**, 473–481.
4 Newlon,C. (1997) In DePamphilis,M.L. (Ed.), *DNA Replication in Eukaryotic Cells.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA. In press.
5 Broach,J.R., Li,Y.Y., Feldman,J., Jayaram,M., Abraham,J., Nasmyth,K.A. and Hicks,J.B. (1983) *Cold Spring Harb. Symp. Quant. Biol.* **2**, 1165–1173.
6 Van Houten,J.V. and Newlon, C.S. (1990) *Mol. Cell. Biol.* **10**, 3917–3925.
7 Celniker,S.E., Sweder,K., Srienc,F., Bailey,J.E. and Campbell,J.L. (1984) *Mol. Cell. Biol.* **4**, 2455–2466.
8 Bell,S.P. and Stillman,B. (1992) *Nature* **357**, 128–134.
9 Estes,H.G., Robinson,B.S. and Eisenberg,S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11156–11160.
10 Bell,S.P., Kobayashi,R. and Stillman,B. (1993) *Science* **262**, 1844–1849.
11 Bell,S.P., Marahrens,Y., Rao,H. and Stillman,B. (1993) *Cold Spring Harb. Symp. Quant. Biol.* **58**, 435–442.
12 Micklem,G., Rowley,A., Harwood,J., Nasmyth,K. and Diffley,J.F. (1993) *Nature* **366**, 87–89.
13 Foss,M., McNally,F.J., Laurenson,P. and Rine,J. (1993) *Science* **262**, 1838–1844.
14 Li,J.J. and Herskowitz,I. (1993) *Science* **262**, 1870–1874.
15 Liang,C., Weinreich,M. and Stillman,B. (1995) *Cell* **81**, 667–676.
16 Loo,S., Fox,C.A., Rine,J., Kobayashi,R., Stillman,B. and Bell,S. (1995) *Mol. Biol. Cell* **6**, 741–756.
17 Hardy,C.F. (1996) *Mol. Cell. Biol.* **16**, 1832–1841.
18 Hardy,C.F. and Pautz,A. (1996) *Mol. Cell. Biol.* **16**, 6775–6782.
19 Zou,L., Mitchell,J. and Stillman,B. (1997) *Mol. Cell. Biol.* **17**, 553–563.
20 Campbell,J.L. and Newlon,C.S. (1991) In Broach,J.R., Jones,E.W. and Pringle,J.R. (Eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces cerevisiae: Genome Dynamics, Protein Synthesis, and Energetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA. pp. 41–146.
21 Walker,S.S., Francesconi,S.C., Tye,B.K. and Eisenberg,S. (1989) *Mol. Cell. Biol.* **9**, 2914–2921.
22 Rhode,P.R., Elsasser,S. and Campbell,J.L. (1992) *Mol. Cell. Biol.* **12**, 1064–1077.
23 Marahrens,Y. and Stillman,B. (1992) *Science* **255**, 817–823.
24 DePamphilis,M.L. (1993) *Trends Cell Biol.* **3**, 161–167.
25 Pederson,D.S. and Heintz,N.H. (1994) *Transcription Factors and DNA Replication.* R. G. Landes Company, Austin, TX, USA.
26 Li,R. and Botchan,M.R. (1993) *Cell* **73**, 1207–1221.
27 Li,R. and Botchan,M.R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 7051–7055.
28 Walker,S.S., Francesconi,S.C. and Eisenberg,S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4665–4669.
29 Sherman,F. and Fink,G.R. (1986) *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
30 Francesconi,S.C. and Eisenberg,S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4089–4093.
31 Brent,R. and Ptashne,M. (1985) *Cell* **43**, 729–736.
32 Sikorski,R.S. and Hieter,P. (1989) *Genetics* **122**, 19–27.
33 MacKay,V.L., Welch,S.K., Insley,M.Y., Manney,T.R., Holly,J., Saari,G.C. and Parker,M.L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 55–59.
34 Brent,R. and Ptashne,M. (1984) *Nature* **312**, 612–615.
35 Osborne,M.A. and Silver,P.A. (1993) *Annu. Rev. Biochem.* **62**, 219–254.
36 Walker,S.S., Malik,A.K. and Eisenberg,S. (1991) *Nucleic Acids Res.* **19**, 6255–6262.
37 Ruden,D.M., Ma,J., Li,Y., Wood,K. and Ptashne,M. (1991) *Nature* **350**, 250–252.
38 Brand,A.H., Micklem,G. and Nasmyth,K. (1987) *Cell* **51**, 709–719.
39 Ausubel,F.M., Brent,R., Kingston,R., Moore,D., Seidman,J., Smith,J. and Struhl,K. (1987) *Current Protocols in Molecular Biology.* John Wiley and Sons, Inc., New York, NY, USA.
40 Orr,W.T., Szostak,J.W. and Rothstein,R.J. (1983) *Methods Enzymol.* **101**, 228–245.
41 Miller,J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.
42 Mager,W.H. and Planta,R.J. (1991) *Mol. Cell. Biochem.* **104**, 181–187.
43 Buchman,A.R. and Kornberg,R.D. (1990) *Mol. Cell. Biol.* **10**, 887–897.
44 Goncalves,P.M., Maurer,K., van Nieuw Amerongen,G., Bergkamp-Steffens,K., Mager,W.H. and Planta,R.J. (1996) *Mol. Microbiol.* **19**, 535–543.
45 Fox,C.A., Loo,S., Rivier,D.H., Foss,M.A. and Rine,J. (1993) *Cold Spring Harb. Symp. Quant. Biol.* **58**, 443–455.
46 Halfter,H., Kavety,B., Vandekerckhove,J., Kiefer,F. and Gallwitz,D. (1989) *EMBO J.* **8**, 4265–4272.
47 Diffley,J.F. and Stillman,B. (1989) *Science* **246**, 1034–1038.
48 Ptashne,M. and Gann,A. (1997) *Nature* **386**, 569–577.
49 Ferguson,M.K. and Botchan,M.R. (1996) *J. Virol.* **70**, 4193–4199.
50 Rao,H. and Stillman,B. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 2224–2228.
51 Rowley,A., Cocker,J.H., Harwood,J. and Diffley,J.F. (1995) *EMBO J.* **14**, 2631–2641.
52 Shakibai,N., Kumar,V. and Eisenberg,S. (1996) *Proc. Natl. Acad. Sci. USA* **93**,11569–11574.
53 Theis,J.F. and Newlon,C.S. (1994) *Mol. Cell. Biol.* **14**, 7652–7659.
54 Rao,H., Marahrens,Y. and Stillman,B. (1994) *Mol. Cell. Biol.* **14**, 7643–7651.
55 Huang,R.Y. and Kowalski,D. (1996) *Nucleic Acids Res.* **24**, 816–823.