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Research Article

A Yeast Chromosomal Origin of DNA Replication Defined by Multiple Functional Elements

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Although it has been demonstrated that discrete origins of DNA replication exist in eukaryotic cellular chromosomes, the detailed organization of a eukaryotic cellular origin remains to be determined. Linker substitution mutations were constructed across the entire *Saccharomyces cerevisiae* chromosomal origin, *ARS1*. Functional studies of these mutants revealed one essential element (A), which includes a match to the ARS consensus sequence, and three additional elements (B1, B2, and B3), which collectively are also essential for origin function. These four elements arranged exactly as in *ARS1*, but surrounded by completely unrelated sequence, functioned as an efficient origin. Element B3 is the binding site for the transcription factor–origin binding protein ABF1. Other transcription factor binding sites substitute for the B3 element and a trans-acting transcriptional activation domain is required. The multipartite nature of a chromosomal replication origin and the role of transcriptional activators in its function present a striking similarity to the organization of eukaryotic promoters.

ALTHOUGH CELLULAR ORIGINS OF DNA REPLICATION ARE poorly understood in most eukaryotes, short chromosomal sequences have been cloned in the yeast *Saccharomyces cerevisiae* that enable plasmids to replicate along with the cellular chromosomes in the S phase of each cell cycle (*1*). Such autonomously replicating sequences (ARS's) have been shown by two-dimensional agarose gel electrophoresis techniques to be authentic origins of replication in plasmids and in cellular chromosomes (*2, 3*). Interestingly, only a subset of *S. cerevisiae* ARS's are active origins at their native chromosomal positions (*3*).

All ARS's of both classes contain a match to a degenerate "ARS consensus sequence," 5′-$^{T}_{A}$TTTA$^{T}_{C}$$^{A}_{G}TTT^{T}_{A}$-3′ that is essential for ARS function (*4–7*). The sequence requirements of this element have been thoroughly characterized, and any one of numerous single point mutations in this sequence eliminates ARS function (*6*). Although the conservation of this element suggests that it is recognized by a sequence-specific DNA binding protein, no protein has yet been shown to function at this sequence. A single strand DNA binding protein that binds the T-rich strand of this sequence has been identified and purified (*8*), but is not known whether this protein functions in DNA replication.

A sequence (or sequences) flanking the consensus match is also essential for origin function. Such sequences have been poorly defined because deletion mutagenesis has often produced conflicting results. For example, deletion mutagenesis of an ARS revealed a strong dependence on the nature of the adjacent sequence (*9*). One effect of adjacent sequences that has been demonstrated is the suppression of ARS function when nucleosomes intrude on the origin (*10*). Despite the poor sequence definition, there have been several proposals for a role of the essential flanking region. Deletions that reduce origin efficiency have variously been correlated with the disruption of a DNA bend, a DNA unwinding element, weak homology to the ARS consensus sequence, and a nuclear scaffold attachment site (*11–15*). The binding of the protein ABF1 has also been suggested to be important for ARS function. Although ABF1 only binds to a subset of origins, deletions and substitutions that disrupt these ABF1 binding sites reduce the maintenance of plasmids (*16–19*).

For our study of the structure of a yeast origin of DNA replication, *ARS1* was chosen because it is a proven chromosomal origin of replication and the chromatin structure of this origin has been studied in plasmids and in the chromosome (*20, 21*). A deletion analysis has shown that *ARS1* can be divided into three domains, A, B, and C (*5*). Domain A contains an essential match to the ARS consensus sequence and domain B is a broad region to the 3′ side of the T-rich strand of the consensus match. Domains A and B are adequate for efficient ARS activity and all other ARS sequences examined displayed the same A-B organization (*7*). Domain C, however, may be present only in *ARS1*, and its weak effect was detected only in the absence of domain B. We have now undertaken a systematic search for functional elements in a 193-bp *ARS1* segment containing domains A and B and a part of domain C that shows strong ARS activity (*5, 18*).

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Four sequence elements in *ARS1*. Linker substitution mutations rather than deletion mutagenesis was used to avoid variation caused by the introduction of foreign DNA adjacent to the origin. This technique was first used to define sequence elements that constitute a eukaryotic promoter (*22*). We constructed 34 Xho I linker substitution mutations across the origin (Fig. 1). In 25 of the mutants, 8 bp of the *ARS1* sequence were replaced with an 8-bp Xho I linker sequence, and in the remaining 9 mutants, either a 7- or 9-bp sequence of ARS1 was replaced with the Xho I linker. Each *ARS1* derivative was in a vector that contains a centromere (*CEN4*) and the *URA3* selectable marker (*23*).

Origin function was measured by two assays. In the first assay, plasmids were transformed into *S. cerevisiae* strain SP1 (*24*), which contains the *ura3*-52 allele that reverts to *URA3*$^+$ at a low frequency. When this strain was transformed by plasmids with a functioning origin, *URA3*$^+$ transformants were obtained at high frequency (*1*). Only two plasmids, pARS/858–865 and pARS/865–872, failed to transform at high frequency (Fig. 1). These two linker mutations define a 15-bp sequence named element A, which is essential for *ARS1* function and includes the ARS consensus sequence.

Several linker mutants produced transformants that grew more slowly than the wild-type transformants on selective media. To measure the efficiency of these crippled origins, we used a plasmid stability test (*25, 26*). In this assay, purified transformants were grown in selective (uracil-free) media, diluted into nonselective media (containing uracil), and then grown for 30 hours or approximately 14 generations. The percentage of yeast cells that retained the plasmid was then determined. Since a cloned centromere provides circular plasmids with an efficient segregation function during mitosis, we assumed that changes in origin efficiency were responsible for altered loss rates of mutant plasmids. This assumption has been validated (*27*). We nevertheless expected all plasmid stabilities to be less than 100 percent because yeast cells lose small chromosomes more frequently than they lose large chromosomes (*27, 28*). With this assay, 43 percent of yeast cells, on average, retained the wild-type *ARS1* plasmid.

Three independent measurements of the plasmid stabilities for each of the linker scan mutants were determined (Fig. 2, top). Most of the mutants assayed resembled the plasmid stability of wild-type *ARS1*. Three clusters of mutations to the 3′ side of the T-rich strand of the A element were responsible for lower plasmid stabilities. These mutations defined the functional elements B1, B2, and B3, which were numbered in order of decreasing influence on plasmid stability (Fig. 2, top, and Fig. 1, top). For selected mutants, we also determined the rate of plasmid loss per generation (*26*). The plasmid loss per generation was determined as 5.8 ± 2.2 percent for the wild-type *ARS1* plasmid, 17.7 ± 4.2 percent for the B1 mutant pARS/835–842, 14.6 ± 2.7 percent for the B2 mutants pARS/802–808 and pARS/802–810, and 12.2 ± 3.3 percent for the B3 mutant pARS/757–764. Linker substitution mutations across *ARS1* therefore defined four sequence elements, one of which was essential and three of which were important for *ARS1* function.

The identification of four sequence elements necessary for efficient origin function raised the possibility that these four elements were sufficient to constitute an efficient origin. To test this possibility directly, we excised the *ARS1* segment from the plasmid and replaced it with a sequence constructed from synthetic oligonucleotides. This sequence contained elements A, B1, B2, and B3 spaced exactly as in the natural DNA. With the exception of a few restriction enzyme recognition sites, the remaining DNA consisted of an otherwise randomly generated sequence that was adjusted so that no base had the same identity as its counterpart in the natural sequence. We found that the four sequence elements alone were sufficient for efficient ARS function (Fig. 3).

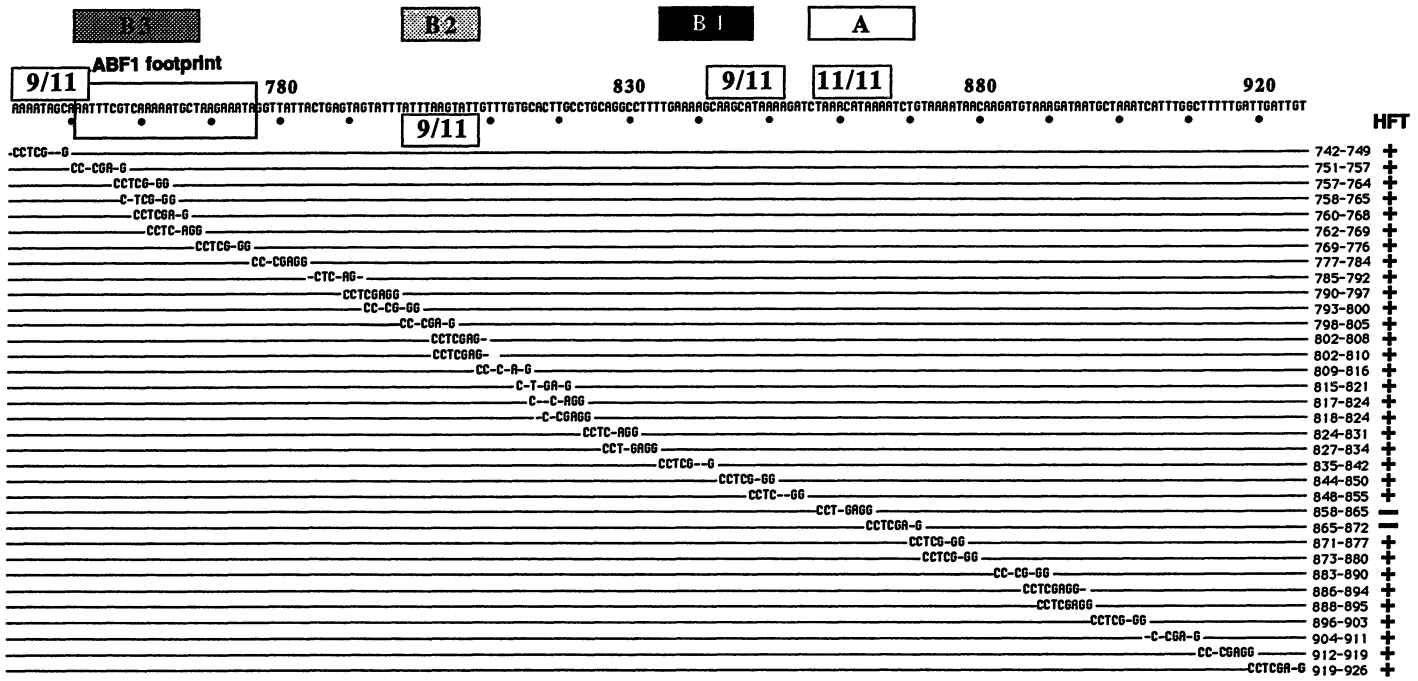


Fig. 1. Linker substitutions in ARS1. The wild-type *ARS1* sequence is shown near the top of the figure. Each line below the wild-type sequence represents an Xho I linker substitution mutant. For each mutant, sequence is shown only at positions that differ from the wild-type *ARS1* sequence. Toward the right side of each line are the linker coordinates of each mutant plasmid which represent the coordinates of the first and last position of the Xho I linker in the *ARS1* sequence. All plasmids contain the *URA3* selectable marker and a *CEN4* centromere. To the right of each mutant name is shown the result of the high frequency transformation (HFT) test, which was conducted in the *ura3*-52 strain SP1. A plus sign indicates that the plasmid produces transformants at high frequency. The functional sequences defined by the high frequency transformation test (A) and the functional elements defined in Fig. 2 (B1, B2, and B3) are marked by boxes at the top of the figure. Also marked are one perfect (11/11) and three partial (9/11) matches to the ARS consensus sequence and the region protected from DNase I digestion by ABF1 binding (*18*).

The 15-bp element A, defined by linker substitutions, contained an 11-bp match to the ARS consensus sequence. When element A was altered so that only the consensus match remained, the origin function was 20 times lower (Fig. 3). Therefore, sequences in addition to the ARS consensus sequence were important for the function of element A.

The importance of the elements B1, B2, and B3 was further studied by examining the properties of plasmids with singly or multiply inactivated elements. Mutations that disabled only one B element caused transformants to grow only slightly more slowly than transformants carrying the wild-type plasmid on selective medium. The simultaneous disruption of any combination of two elements, however, allowed high frequency transformation, but resulted in extremely slow growing colonies, particularly when B1 and B2 were simultaneously inactivated (Fig. 4). Plasmid stabilities of all the double mutants were below the lower reliable limit of our assay (0.2 percent). The transformation of yeast with a plasmid in which the elements B1, B2, and B3 were all mutated or with a plasmid in which the entire ARS was deleted in each case resulted in microcolonies that could not be passaged.

The above results demonstrate that the presence of any two of the three B elements is sufficient for reasonably effective origin function in the presence of element A. A question that therefore arises is whether multiple copies of one of these elements can substitute for two or three different B elements. To test this possibility, we constructed plasmids that have two or three copies of either element B1, B2, or B3 linked to the A element. The spacing between the elements resembled the spacing of the elements in the natural origin. Neither element B1 nor element B2, when present in multiple copies, increased the stability of plasmids above the lower reliable limit of the assay (0.2 percent) (Fig. 5). When multiple copies of the B3 element were present, transformants grew so slowly that a measurement of plasmid stability was not possible. Therefore, elements B1, B2, and B3 appear to be functionally distinct.

Function of element B3. Snyder *et al.* (*12*) reported the existence of bent DNA at ARS1 and showed that deletions that disrupt the bend decrease the stability of *ARS1* plasmids. A bend in a DNA molecule retards the migration of that molecule in a polyacrylamide gel. Linker substitutions that disrupt a DNA bend should therefore disrupt the retarded electrophoretic mobility caused by the bend. To map the sequences involved in DNA bending more precisely, the 193-bp *ARS1* derivative fragments were excised from the polylinker of each plasmid and subjected to polyacrylamide gel electrophoresis (Fig. 2, bottom). Linker substitutions that altered DNA bending overlapped with the B3 element, suggesting a role for DNA bending in element B3 function.

This interpretation, however, was complicated by the observation that the site-specific DNA binding protein ABF1 (ARS binding factor 1) binds to a sequence within the B3 element (*18*). ABF1 is a transcription factor–ARS binding protein that has been purified on the basis of its ability to effect a gel shift in *ARS1* DNA (*18, 29*). The region of DNA protected by ABF1 from deoxyribonuclease I (DNase I) digestion was determined earlier (Fig. 1) (*18*). This footprint covers a match to a consensus binding sequence that has been determined for the ABF1 protein (RTCRYNNNNNACG) (*17, 30*). The linker substitutions that define element B3 also disrupt this consensus match for ABF1 binding.

To determine whether ABF1 binding or DNA bending was responsible for B3 activity, we constructed mutations that disrupt only the DNA bend or only ABF1 binding. These were compared to wild type or the original B3 linker mutations (Fig. 6). The ABF1 binding sequence in *ARS1* was replaced with the stronger of the two ABF1 binding sequences between the *TUB2* and *YPT1* genes where ABF1 functions as a transcription factor (*31*). This change in sequence disrupted the DNA bend (Fig. 6B, lane 4), but maintained both ABF1 binding capability (Fig. 6C, lane 4) and full B3 activity (Fig. 6A, lane 4). Conversely, two point mutations in the ABF1 binding site at *ARS1* had no effect on DNA bending (Fig. 6B, lane 2), removed all traces of ABF1 binding (Fig. 6C, lane 3), and eliminated B3 activity (Fig. 6A, lane 2). Therefore, ABF1 binding, not DNA bending, is required for element B3 function.

The observation that the transcription factor–origin binding protein ABF1 functions at *ARS1* raised the question whether the binding site for the related protein RAP1 (*32*) would function in

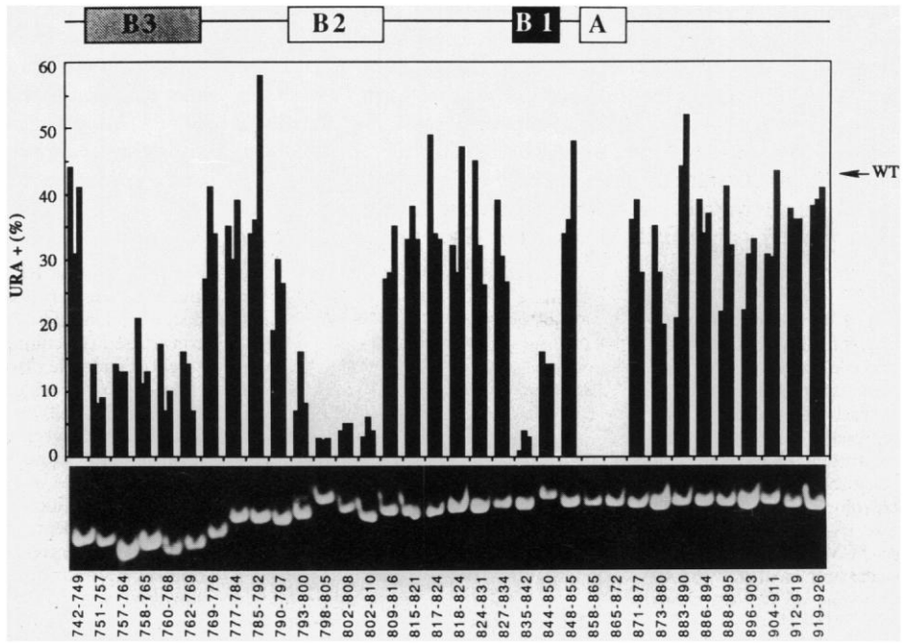


Fig. 2. Linker substitution mutations define functional elements at *ARS1*. (**Upper**) The bar graph of the plasmid stabilities of the linker substitution mutants (Fig. 1). The proportion of yeast cells that retained the wild-type *ARS1* plasmid or mutant derivatives after approximately 14 generations of growth in nonselective media (percent URA$^+$) was determined (*26*). The horizontal axis identifies the mutant plasmids from Fig. 1 by their linker coordinates. The vertical axis measures plasmid stabilities (percent URA$^+$) of the plasmids. For every mutant plasmid, the three bars represent the plasmid stabilities from three independently derived transformants. The average plasmid stability for nine transformants containing pARS/WTA from three independent transformations is indicated by the arrow marked "WT" on the right. Mutant plasmids pARS/858–865 and pARS/865–872 do not produce transformants that can be passaged, but are included with plasmid stabilities of zero to convey the topology of *ARS1*. Functional elements, defined either by clusters of mutations that display a reduced plasmid stability (elements B1, B2, and B3) or by mutants that fail to transform yeast at high frequency (element A), are indicated at the top. (**Lower**) Gel of plasmid fragments shows the altered DNA-bending properties of some of the *ARS1* derivatives. The *ARS1* fragment for each of the mutants was excised from their plasmid with Eco RI and Hind III, subjected to electrophoresis in an 8 percent polyacrylamide gel at 4°C (1.7 v/cm) for 60 hours, stained with ethidium bromide, and photographed.

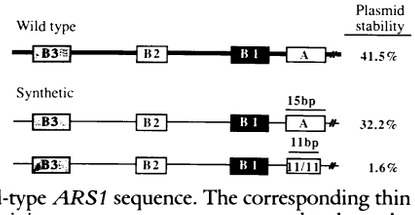


Fig. 3. Construction of an efficient synthetic origin of DNA replication. The region of the replication origin in plasmids pARS/WTA (**top**), pB3B2B1A (**middle**), and pB3B2B1ACS (**bottom**) is shown (*23*). The thick line represents wild-type *ARS1* sequence. The corresponding thin line in the two synthetic origins represents sequences unrelated to the wild-type sequence. The plasmid stabilities listed are average values of three independent determinations and were determined as described (Fig. 2). The box labeled 11/11 is the *ARS1* match to the ARS consensus sequence, whereas the 15-bp element A is the sequence defined by data in Fig. 2. The box labeled B3 is actually the ABF1 binding site and takes into account the data in Fig. 6, and therefore it represents less sequence than the B3 element defined in Fig. 2.

place of the ABF1 binding site. RAP1 binds at four ARS's associated with transcriptional silencers and displays properties similar to ABF1. To test whether the RAP1 binding site will function at *ARS1*, we inserted an oligonucleotide containing the RAP1 binding site derived from the ARS at the *HMR E* silent, mating-type locus in place of the ABF1 binding site (*33*). The spacing of the known protein recognition sequences with respect to the other ARS elements was not changed. The RAP1 binding site, in either orientation, fully substituted for the wild-type ABF1 binding sequence (Fig. 6A, lanes 5 and 6).

The observation that the ABF1 binding site can be successfully replaced with the binding sequence for the related RAP1 protein prompted an investigation into whether an unrelated transcription factor can also functionally substitute for ABF1. The transcription factor GAL4 was chosen for this analysis because it is an active transcription factor when cells are grown in media containing galactose, but inactive as a transcriptional activator in media containing glucose, thereby allowing the effects specific to GAL4 protein activity to be examined (*34*). The ABF1 binding site was replaced with a synthetic 17-bp GAL4 binding sequence that had, in an earlier study, proved to be effective in promoting transcription (*35*). The GAL4 binding site, in both orientations, successfully substituted for the ABF1 site when cells were grown in media containing galactose but failed to substitute when cells were grown in media containing glucose (Fig. 7). Therefore, the binding site for an unrelated transcription factor will functionally substitute for the ABF1 site, but only if the protein is in its active form.

To determine whether the transcriptional activation domain of GAL4 functioned at *ARS1*, the ABF1 binding site at *ARS1* was replaced with a synthetic LexA operator oligonucleotide (*36*). The stability of this plasmid was determined in cells that express either the LexA protein (which lacks a eukaryotic transcriptional activation domain) or a LexA-GAL4 fusion protein (which contains the GAL4 activation domain) from the ADH1 promoter. The presence of the GAL4 activation domain increased the stability of this plasmid by about 10 percent or one-and-a-half times (*37*). A more rigorous test was provided when the experiment was repeated in the absence of the B2 element. A plasmid was used that contains functional A and B1 elements, the B2 element disrupted by the linker substitution mutation 798–805, and a LexA operator in place of element B3. In this test, the transcriptional activation domain of GAL4 was the source of a sevenfold increase in plasmid stability (Fig. 8).

Organization of a cellular origin of DNA replication. Our findings reveal that a eukaryotic chromosomal origin of DNA replication is organized into a modular array of short functional sequences. One essential (A) and three important (B1, B2, and B3) elements are sufficient to constitute an effective origin of DNA replication. Together, the B elements were essential for origin function. Each of the B elements alone was not essential, even though they were functionally distinct. A similar modular arrangement exists at the transcriptional silencer at the *HMR E* locus where only two of the three elements A, E, and B, are necessary for silencing, even though each of the silencer elements is functionally distinct (*33*). The modular organization of the *ARS1* origin also resembles the arrangement of sequence elements seen in eukaryotic promoters. At most promoters, a single element, called the TATA box, is the only sequence absolutely required for the initiation of transcription. In this respect, the A element resembles the TATA box. Furthermore, other proximal elements and distal enhancer elements are also important for promoter function (*38*). The B elements identified in *ARS1* therefore resemble these transcription activation elements. In the initiation of transcription, the TATA element is bound by the central protein in transcription, TFIID, allowing a "basal transcription apparatus" to assemble around it. Transcriptional activator proteins bind to the proximal or enhancer elements to increase the frequency of transcriptional initiation. We propose that at an origin of DNA replication in yeast, element A provides an initiator function, which, like the TATA box of a eukaryotic promoter, is essentially inactive unless stimulated by other (B) elements we call activator elements.

The properties of element A make it an attractive candidate for initiator function. Of the four sequences shown to be competent for efficient *ARS1* function, element A is the only essential element. Contained within element A is a match to a degenerate sequence that has been found in every ARS, the "ARS consensus sequence" (*4, 6*). Although insufficient for full element A activity, the ARS consensus sequence is an essential part of the A element at *ARS1* and a consensus match is essential at every ARS tested. Two other

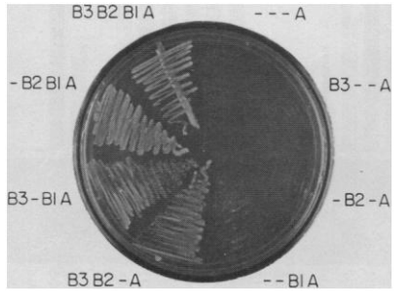


Fig. 4. Growth response to disabled B domains. Plasmid constructions in *S. cerevisiae* strain SP1 are shown. In each case a minus sign represents a mutation that inactivates the element. Primary transformants (or microcolonies in the case of - - - A) were streaked out on SCM plates (*26*) lacking uracil and incubated for 40 hours at 30°C. Mutations that disable elements B1, B2, or B3 are, respectively, linker substitutions 835 to 842, 802 to 808, and the double-point mutation described in Fig. 6. The only exception is the plasmid that has both elements B1 and B3 disabled. In this plasmid, the B3 mutation is the linker substitution 762 to 769.

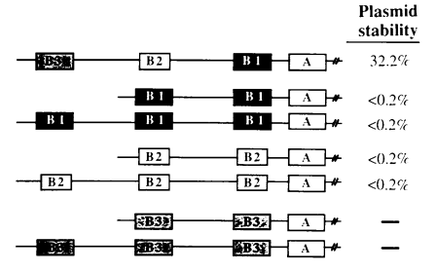
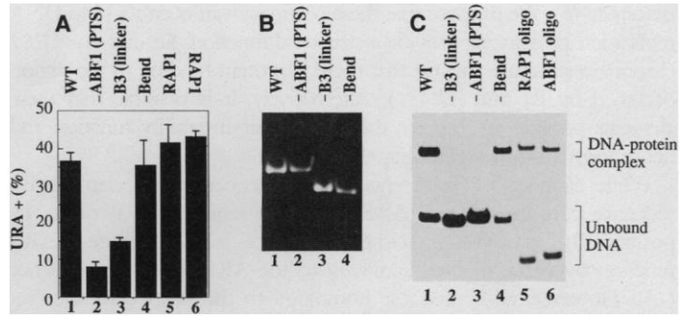


Fig. 5. Elements B1, B2, and B3 are functionally distinct. Plasmids were constructed containing element A and either two or three copies of each of the elements B1, B2, or B3. These plasmids were transformed into strain SP1 and the plasmid stability assay was conducted on three independent transformants for the top five plasmids. The plasmids with two or three copies of element B3 transformed at high frequency, but the transformants grew too slowly to perform the plasmid stability assay. The plasmids used were (from top to bottom) pB3B2B1A, pARS/B1B1, pARS/B1B1B1, pARS/B2B2, pARS/B2B2B2, pARS/B3B3, and pARS/B3B3B3 (*23*). The box labeled B3 takes into account the data in Fig. 6, and therefore represents a smaller sequence than the B3 element defined in Fig. 2.

Fig. 6. Function of ABF1 and RAP1 binding sites at B3. (**A**) Plasmid stabilities of the constructs described. The same assay was used as in Fig. 2. Averages and standard deviations of three independent determinations are shown. (**B**) DNA bending properties of *ARS1* derivatives. *ARS1* and derivatives were excised from the polylinker with Eco RI and Hind III and run on an 8 percent polyacrylamide gel at 4°C at 1.7 v/cm for 60 hours. (**C**) Gel-retardation analysis of wild-type and mutant protein binding sites. Whole-cell extracts were prepared and used as described (*48*). The probe used in each of the first four lanes was a 138-bp Sal I–Bgl II fragment that was excised from the plasmid indicated. In the last two lanes the probe consisted of synthetic double-stranded oligonucleotides. The 37-bp RAP1 oligonucleotide (lane 5) was used in the construction of the RAP1 plasmids. The 41-bp ABF1 oligonucleotide (lane 6) includes the ABF1 binding sequence contained in element B3. In the plasmids labeled RAP1, the ABF1 binding sequence at *ARS1* has been replaced with the RAP1 binding sequence at *HMRE* in either orientation. The spacing between the ABF1 or RAP1 binding sequences and element B2 is kept constant in all constructs. WT refers to the wild-type plasmid pARS/WTA; −ABF1(PTS) refers to the wild-type plasmid with two point mutations in the ABF1 binding site: a C to G transversion at position 756, and a T to C transition at position 758. −B3 (linker) is pARS/757–764 (see Fig. 1); −BEND is a plasmid that contains the stronger of the two ABF1 binding sequences that exist between the *TUB2* and *YPT1* genes (where ABF1 functions as a transcriptional activator) in place of the ABF1 binding sequence at *ARS1* (*31*).



lines of evidence support the initiator element hypothesis. First, very weak ARS activity has been reported within a 19-bp *ARS1* sequence that includes the 15-bp A element (*39*). Second, several copies of an ARS consensus element on a plasmid, in the absence of any other origin sequence, confer efficient ARS activity (*13*).

We suggest that there are at least two kinds of activators of the *ARS1* origin of DNA replication. The first group is defined by elements, such as the ABF1 binding site, that activate both DNA replication and transcription, whereas the second group of elements exclusively activates origins of DNA replication. De Villiers and colleagues (*40*) were the first to show that an origin of DNA replication of a eukaryotic DNA virus, polyomavirus, includes a transcriptional enhancer that can be replaced by other enhancers. The replication origins of numerous other eukaryotic viruses were subsequently shown to be stimulated by transcriptional enhancers (*41*). Our data establish that a cellular eukaryotic origin of replication is stimulated by a transcriptional activator that also can be replaced by other activators.

The demonstrated involvement of the same proteins in the activation of origin and promoter functions requires that these proteins are either endowed with two different biochemical activities or that they have a single activity that stimulates two distinct processes. Three lines of evidence suggest that transcription factors can stimulate eukaryotic viral origins and eukaryotic promoters by the same mechanism. First, the in vivo activation of a mammalian viral promotor—for example, the marine mammary tumor virus–long terminal repeat (MMTV-LTR)—and polyomavirus DNA replication by GAL4-VP16 are dependent on the VP16 activation region of this chimeric protein and are similarly influenced by mutations in the VP16 activating sequence (*42*). Second, some transcription elements function in both a distance- and orientation-independent manner to activate promoters and virus origins of DNA replication (*40, 41*). Third, transcription factors stimulate both a eukaryotic promoter and the SV40 replication origin in vitro in a process dependent on the use of chromatin as a template (*43, 44*). The relief of promoter repression by GAL4-VP16 in vitro has been shown to be dependent on the VP16-activating region (*44*).

We propose that some transcription factors activate cellular eukaryotic origins of DNA replication and transcriptional promoters by the same mechanism. Support for this proposal comes from our observation that the GAL4 transcription factor, which is thought to exist in the cell solely as a transcriptional regulator, can stimulate a cellular origin of DNA replication. In addition, our data and that of others indicate that transcription factor binding sites activate ARS function in a distance- and orientation-independent manner (*19*). Finally, we have demonstrated that the activation of a cellular origin by the protein chimera LexA-GAL4 is dependent on the transcriptional activation region of this protein. An attractive possibility is that the transcription activation region of some activators facilitates cellular origin function by influencing chromatin structure at the origin locus. Another possibility (*45*) is that the transcription activation function might directly interact with DNA replication proteins. These two possibilities are not necessarily mutually exclusive.

The evidence for a second group of origin activator elements is more tenuous. This group of activators exclusively stimulates origins of DNA replication. In contrast to the ABF1 binding site, the region encompassing elements B1 and B2 does not stimulate a truncated CYC1 promoter, suggesting that these elements do not bind transcription factors (*17*). The B1 and B2 elements, like the ABF1 binding site, qualify as replication-activating elements, since, even though they are individually not essential at *ARS1*, each element can combine with element A to transform yeast at high frequency [although with extremely low plasmid stabilities (*37*)]. Elements B1 and B2 do not appear to regulate the timing of origin initiation within the S phase as this regulation is due to sequences outside of *ARS1* (*20*). Another candidate for this second group of origin activators is the amplification control element, *ACE3*, a cis-regulatory element required for the amplification of an eggshell gene cluster in *Drosophila melanogaster*. No connection between the *ACE3*-mediated control of amplification and transcription regulation has been

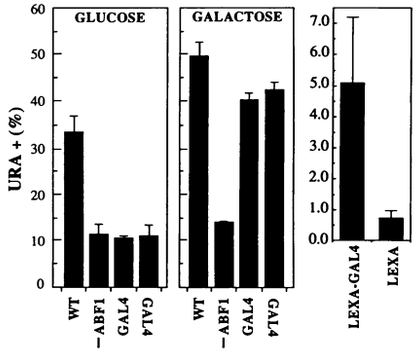


Fig. 7 (**left boxes**). A GAL4 binding site stimulates *ARS1* when cells are grown in galactose but not glucose media. Plasmid stabilities were determined in either glucose or galactose containing media as indicated in the figure. The plasmids labeled GAL4 contain the synthetic GAL4 binding sequence described (*35*) in place of the ABF1 binding sequence at *ARS1*. The spacing between the ABF1 or GAL4 protein binding sequences and element B2 was kept constant. WT is pARS/WTA and −ABF1 is the same plasmid as −ABF1(PTS) in Fig. 3. **Fig. 8** (**right box**). Activation of *ARS1* by LexA-GAL4. A plasmid was constructed that contained (i) functional A and B1 elements, (ii) the 798 to 805 linker substitution mutation in element B2, and (iii) the LexA operator sequence instead of element B3. Plasmid stabilities of the resulting plasmid, pARS/LexA,798–805, were determined in a *GAL80* strain in the presence of a second plasmid that produces either LexA or GAL4-LexA protein with the use of the *ADH1* promoter. A modification of the plasmid stability assay described in Fig. 2 was used to include the role of the second plasmid (*26*).

detected (*46*). We propose that these origin activators either bind DNA replication proteins or provide a structural function. Studies on *ARS1* chromatin structure suggest that there is protein binding in the region occupied by B1 and B2 (*21*). Alternatively, it is possible that these elements provide an intrinsic thermodynamic instability function and hence form a DNA unwinding element (*14*).

While element B1 represents a novel sequence, the element B2 sequence conforms to the ARS consensus sequence at 9 of its 11 positions. It has been suggested that sequences may contribute to ARS function by virtue of their similarity to the ARS consensus sequence (*13*). However, all appreciable homology to the ARS consensus sequence, apart from the essential initiator element, has been removed from the histone-4 *ARS* (H4 *ARS*) without reducing ARS function (*47*). In contrast to the larger element A, element B2 function appears to be entirely contained within an 11-bp element that is only partially related to the ARS consensus sequence and, unlike an ARS consensus sequence match (*13*), element B2 does not generate an efficient origin when reiterated. Furthermore, several point mutations that would either severely impair or eliminate the function of element A have no effect on the function of element B2 (*37*).

Finally, a paradox in yeast is that only a subset of the chromosomal sequences that allow plasmids to transform at high frequency are active origins of DNA replication at their native chromosomal positions (*3*). It is not unreasonable to assume that there are many more sequences in the yeast genome that meet the sequence requirements of the A element than there are active replication origins. Since, as reported above, element A only requires one -activating element to produce transformants at high frequency, we would expect more chromosomal segments to transform yeast at high frequency than there are origins of DNA replication in the chromosome. Perhaps an A element requires multiple replication-activating elements to be an active chromosomal origin like *ARS1*. If this is true, then the frequency of functioning origins in the chromosome may correspond with the frequency of A elements that are associated with multiple activating elements.

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23. Plasmid pARS/WTA is pUC119 with a 1.2-kb Hind III fragment carrying the *URA3* gene cloned into the Pvu II site in the lac *i* gene such that the Nde I site is near the polylinker; a 1.3-kb Bgl II–Nhe I fragment containing *CEN4* cloned into the other Pvu II site such that the Nhe I end is near the polylinker; and a 193-bp Hinf I–Rsa I *ARS1* fragment was inserted into the Sma I site in the polylinker. Plasmid pARS/WTB is pARS/WTA with the 1.3-kb *CEN4*-containing fragment inverted. Linker substitution constructs were made as follows. pARS/WTA was linearized with either Eco RI or Xba I and treated with exonuclease Bal 31 for various lengths of time. The DNA was then blunt-ended and recircularized with an Xho I linker (5′-CCTCGAGG-3′) inserted. Deletion endpoints of approximately 400 transformants were determined by sequencing. The endpoints of the two sets of deletions were compared, and pairs of deletions that overlap in *ARS1* by seven to nine bases were recombined at the Xho I linker site and a second appropriate site on the plasmid. Regions of *ARS1* left untouched by these linker substitutions were mutated by standard site-directed mutagenesis procedures. The synthetic origin plasmid pB3B2B1A is pARS/WTA with the ARS1 containing Sal I–Sac I segment replaced by the sequence

5′-TCGACTCTAGCTGTTCCTTCAACATTTGAGG GATCTAATAAGATA

TTCCCGGGTCGTCAAAAATGCTATCCG GATCTATTTATAATGATCAG

GGCAATTTAAGTATTAGACGG GATCCTCTAGAGTCGACAGATGAAA

AGCAAGCAAGCACTTCTCGACTAAACATAAAATCTATAAACGAGCT-3′

This sequence was constructed by sequential cloning of double-stranded oligonucleotides into a PUC119 polylinker. pB3B2B1ACS is identical to pB3B2B1A except that the element A sequence has been altered to 5′-GTAAA CATAA AAAGC-3′ by means of site-directed mutagenesis. The plasmids with multiple copies of a domain were constructed as follows. Plasmid pARS/817–824 was cut with Xho I, the ends were filled in, and the plasmid was recircularized with one copy of a Bam HI linker inserted. One or two tandem copies of the 41-bp Bam HI–element B1–Bgl II fragment obtained from this plasmid were inserted into the Bgl II site of pARS/756,758,802–808 such that an intact Bgl II site was restored next to element A. This generated plasmids pARS/B1B1 and pARS/B1B1B1. The plasmids pARS/B2B2, pARS/B2B2B2, pARS/B3B3, and pARS/B3B3B3 were constructed in the same manner, except that the starting vector was plasmid pARS/756,758,802–808,835–842, and synthetic oligonucleotides used in the construction of the synthetic ARSs were introduced. The sequence of one strand of these oligonucleotides is underlined above. Double and triple mutants were constructed with the use of site-directed mutagenesis of pARS/WTA or with recombining mutant plasmids at the Pst I site that naturally occurs between B1 and B2. Plasmid −ABF1(PTS) is pARS/756,758 and −BEND is pARS/−BEND. These two plasmids were constructed by site-directed mutagenesis of pARS/WTA. The pARS/RAP plasmids were constructed by annealing the oligonucleotides 5′-TCGAG ATATT GCAAA AACCC ATCAA CCTTG AAAAA AG-3′ and 5′-TCGAC TTTTT TCAAG GTTGA TGGGT TTTTG CAATA TC-3′ and inserting them, in both orientations, into pARS/777–784 in place of the short Sal I–Xho I fragment that contains the element B3. This oligonucleotide contains the RAP1 binding sequence found at *HMR E* (*33*) flanked by an Xho I and a Sal I site. The pARS/GAL4 and pARS/LEXA plasmids were constructed in the same way with the pARS/769–776 vector plasmid and either an oligonucleotide containing the synthetic 17-nt GAL4 binding sequence (*35*) flanked by 5′ overhangs complementary to the sticky ends produced by Xho I, or the synthetic LexA operator oligonucleotide of (*36*). Plasmid pARS/LexA,798–805 was derived from pARS/LexA by site-directed mutagenesis. The plasmids that produce the LexA and LexA-GAL4 proteins via the ADH promoter are pL50 and KL1027, respectively, and were provided by Roger Brent.
24. SP1 is *MATa, ura3*-52, *his3*, *trp1*-289, *leu2*-2,113, *ade8*, *can1*, and was provided by M. Wigler [T. Toda *et al.*, *Cell* **40**, 27 (1985)]. BJ926 is a/α *pep4*-3/*pep4*-3, *pre1*-126/*pre1*-126, *prb1*-1122/*prb1*-1122 [E. W. Jones, *Methods Enzymol.* **194**, 428 (1991)]. Synthetic complete medium (SCM) consists of glucose (2 percent, w/v), ammonium sulfate (4.8 g/liter), yeast nitrogen base without amino acids and without ammonium sulfate (1.6 g/liter); dibasic potassium phosphate (0.49 g/liter); leucine (30 mg/liter); adenine, uracil, histidine, methionine, and tryptophane (20 mg/liter); aspartic acid, threonine, and valine (16.3 mg/liter); phenylalanine (12 mg/liter); isoleucine, lysine, and tyrosine (7.3 mg/liter); and arginine, cysteine, proline, and serine at 5 mg/liter. YPD is 2 percent dextrose, 2 percent Bacto-peptone, and 1 percent Bacto-yeast extract.
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26. For the plasmid stability assay, *S. cerevisiae* strain SP1 was transformed with the plasmid of interest, by standard lithium acetate procedures [F. Sherman, G. R. Fink, J. B. Hicks, *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986)] and transformants were streaked on SCM-Ura plates. Single colonies were each inoculated in 5 ml of SCM-Ura broth and grown at 30°C. Twenty-two–hour cultures were diluted to an OD_{600} of 0.0003 in 3 ml of YPD broth and grown for 30 hours at 30°C. Cultures were then plated on SCM and SCM-Ura plates at dilutions that produced approximately 1000 colonies on nonselective (SCM) plates. The rate of plasmid loss per generation was calculated as described [G. M. Dani and V. A. Zakian, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3406 (1983)] after measurement of the number of cells containing the plasmid before and after growth in nonselective media. To determine the plasmid stabilities of pARS/LexA and pARS/LexA,798–805, strain SP1 was transformed simultaneously with pARS/LexA or pARS/LexA,798–805 and either pL50 or KL1027. Single transformants were streaked on SCM-Trp-Ura plates. Single transformants were each inoculated in 2 ml of SCM-Trp-Ura broth and grown at 30°C. Twenty-four–hour cultures were diluted to an OD_{600} of 0.0003 in 3 ml of YPD medium, grown

for 30 hours at 30°C. Cultures were diluted and plated on SCM-Trp and SCM-Trp-Ura plates.
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48. For the mobility shift DNA binding assay and DNA bending assay, whole-cell extracts were prepared from *S. cerevisiae* strain BJ926 (*24*) as described (*18*). Restriction fragments and double-stranded oligonucleotides were ^{32}P-end–labeled by standard procedures and extracted from a polyacrylamide gel [T. Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)]. Binding reactions were as follows. Whole-cell extract (1 μl) was mixed with 6.5 μl of buffer A [25 mM tris-HCl, pH 7.5, 1 mM EDTA, 10 percent glycerol, 0.01 percent NP-40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT)], 0.5 μl of bovine serum albumin (BSA) (10 mg/ml), 1.0 μl of a solution (1 mg/ml) of poly(dI·dC), and probe (10,000 cpm) and incubated for 15 minutes at 30°C. The samples were then analyzed on a low ionic strength polyacrylamide gel (*18*), which was then dried and exposed to Kodak XAR film for 4.5 hours. For monitoring DNA bending, *ARS1* fragments were excised from their plasmids with Eco RI and Hind III, subjected to electrophoresis in TBE buffer [0.089 M tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0] in an 8 percent polyacrylamide gel at 4°C and 1.7 v/cm for 60 hours, then stained with ethidium bromide, and photographed.
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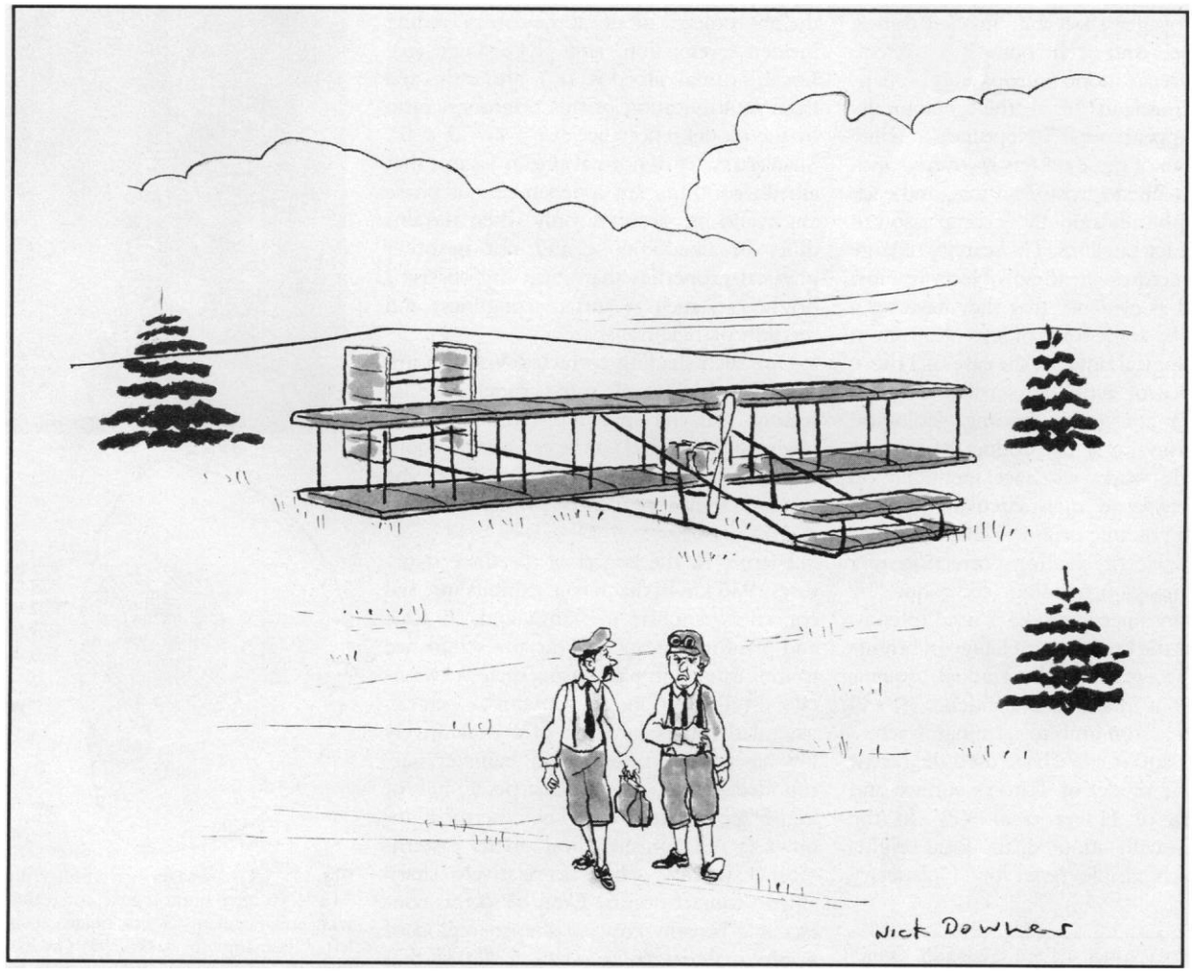

"The sandwich I had while up there was awful."