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Fine-Structure Analysis of the DNA Sequence Requirements for Autonomous Replication of *Saccharomyces cerevisiae* Plasmids

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An autonomously replicating segment, *ARS*, is located 293 base pairs downstream from the histone H4 gene at the copy-I H3-H4 locus. The sequences needed for autonomous replication were defined by deletion analysis to include an *ARS* consensus sequence and an additional 3′-flanking region. External deletions into the 3′-flanking yeast sequences resulted in a loss of replication function. However, disruptions of the required 3′-flanking domain by either 10-base-pair linker-scanning substitutions or larger internal deletions did not impair autonomous replication. Thus, replication is dependent upon a flanking chromosome domain, but not an exact DNA sequence. The extent of the yeast sequences required in the 3′-flanking domain is variable depending on the nature of neighboring plasmid vector sequences. That is, there are certain vector sequences that prohibit replication when they are placed too close to the *ARS* consensus. These results suggest that the functional 3′-flanking domain of the H4 *ARS* is a specific DNA or chromatin structure or both.

Fragments of DNA that allow autonomous replication of circular plasmids have been isolated from the yeast *Saccharomyces cerevisiae*. A large number of biochemical and genetic experiments have demonstrated that such autonomously replicating segments (*ARS*) have many of the properties expected of an origin of DNA replication (10, 40). Although direct proof for this role in the chromosome is lacking, it is still of great interest to determine the sequence and structural elements required for *ARS* function in an attempt to understand the process of DNA replication.

The complete DNA sequence requirements for autonomous replication have not yet been established. All *ARS* sequences examined to date have contained an 11-base-pair core consensus sequence 5′-$^{T}_{A}$TTTAT$^{A}_{G}$TTT$^{T}_{A}$-3′ (4, 34). Deletion studies of two independent *ARS* have established the requirement for this consensus sequence in promoting autonomous replication (6, 15). This single element is not sufficient for autonomous replication, however. Additional structural or functional elements or both must be required for efficient replication.

Aside from the core consensus sequence, a comparison of the available yeast *ARS* DNA sequences has failed to identify any other specific sequence motif. Therefore, the simple notion that several independent conserved regions of DNA combine to produce a fully functional *ARS* seems to be incorrect. Preliminary evidence that at least part of the additional information required for autonomous replication resides in the DNA flanking the core consensus has been provided by deletion experiments on *ARS1* (6, 33) and the *ARS* linked to the *HO* gene (14, 15). In the case of *ARS1*, a large 46- to 109-base-pair flanking region on one side of the consensus sequence was shown to be required for efficient replication. On the other hand, only a 24-base-pair flanking region was required for efficient functioning of the *HO ARS*. However, in both of these studies the vector sequences positioned at the deletion endpoints were different in each derivative, precluding an unambiguous interpretation of the role of the flanking domain.

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This paper describes the results of a systematic fine-structure analysis of a yeast *ARS* located at the histone H3-H4 copy-I locus 3′ of the H4 gene (31). A series of progressive deletion, linker-scanning, and linker deletion mutations in the histone H4 *ARS* are described. The experiments were specifically designed to test the role of flanking DNA sequences in replication. In addition, the interactions between the yeast *ARS* and the flanking vector DNA were also studied in an attempt to determine the role of structural elements in autonomous replication.

MATERIALS AND METHODS

Yeast and bacterial strains. The *Escherichia coli* host strain for all bacterial transformations was ED8654 (*supE supF hsdR hsdM*$^{+}$ *met trpR*) (25). Transfections by recombinant M13 phages were performed on strain JM101 [Δ(*lac pro*) *thi supE* F′ *traD36 proAB lacI*^{q} Δ*lacZM15*] (23). The *S. cerevisiae* host strain used for analyzing deletion endpoints, internal deletions, and linker substitution derivatives was DBY747 (*MAT***a** *his3Δ1 leu2-3 leu2-112 trp1-289 ura3-52*) (D. Botstein). *S. cerevisiae* YN217 (*MAT***a**/α *ura3-52/ura3-52 lys2-801/lys2-801 ade1-101/ade2-101*) that was used for the segregation assays was kindly provided by P. Hieter.

Reagents and media. The media used for bacterial and yeast growth have been described previously (29, 31). All restriction enzymes, *E. coli* exonuclease III, and *E. coli* DNA polymerase large fragment were obtained from New England BioLabs (Beverly, Mass.). Nuclease S1 was obtained from Sigma Chemical Co. (St. Louis, Mo.).

The M13 phage vectors M13mp8 and M13mp9, as well as the corresponding plasmids pUC8 and pUC9, were obtained from New England BioLabs. The parental plasmid used for the segregation assay described in the results was constructed by isolating *CEN4* as an *Xho*I restriction fragment from YCp19 (R. Davis) and cloning it into the *Sal*I site of the plasmid YRp14 (35). The resulting plasmid is analogous to YRp14CEN4 described by Hieter et al. (11).

Generation of deletions. The 374-base-pair *Sau*3AI restriction fragment that contained the *ARS* was isolated from the

plasmid pMS191 (31) by cloning into the *Bam*HI site of the yeast vector YIp5 (2) to give the new recombinant, pAB4. For further manipulation, the *Sau*3AI *ARS* fragment was subcloned into the *Bam*HI site of pUC8 to produce the plasmid pAB9. As a result of this construction the *ARS* fragment in pAB9 was flanked on one side by *Eco*RI and on the other side by *Hin*dIII restriction target sites contributed by the polylinker cloning site of pUC8.

Deletions originating from the H4-proximal side of the restriction fragment were made by first digesting 20 μg of pAB9 with *Hin*dIII to linearize the plasmid. The DNA was then treated with 25 U of exonuclease III at 37°C for 15-s intervals lasting from 15 to 90 s. The enzyme was killed by heating at 65°C for 10 min. The DNA was treated with approximately 1 U of S1 nuclease for 30 min at 37°C and precipitated in ethanol. After ligation with synthetic *Bam*HI linkers (New England BioLabs and Boerhinger Mannheim Biochemicals, Indianapolis, Ind.), the DNA was digested with the restriction enzymes *Eco*RI and *Bam*HI. After electrophoresis on an 8% polyacrylamide gel, fractions of approximately 100 base pairs were eluted and purified. These pools were then cloned into the M13 vector M13mp8. Individual plaques, representing independent deletion derivatives, were then grown up for DNA sequencing of the deletion endpoints. Dideoxynucleotide sequencing was performed as described previously (27).

The set of deletions originating from the other side of the *Sau*3AI restriction fragment was made in an analogous fashion. However, in this case the plasmid pAB9 was first treated with *Eco*RI before treatment with exonuclease III and S1 nuclease. After ligation of synthetic *Bam*HI linkers to the deleted material, the DNA was treated with the restriction enzymes *Bam*HI and *Hin*dIII to produce a series of deletions with common *Hin*dIII ends but with a *Bam*HI site at the deletion endpoint in every case. Discrete size classes of *Bam*HI-*Hin*dIII deletion derivatives were then eluted from polyacrylamide and cloned into the M13 vector M13mp9.

Functional analysis of derivatives. For the progressive deletion constructions, the *Hin*dIII-*Bam*HI and *Bam*HI-*Eco*RI restriction fragments of potentially interesting deletions were isolated from the M13 replicative forms on 8% polyacrylamide gels and cloned into the corresponding restriction sites of the yeast vector YIp5. Recombinant plasmids were selected in *E. coli* and purified on CsCl gradients for subsequent analysis in a yeast transformation assay.

For the linker substitution and internal deletion constructions, *Hin*dIII-*Bam*HI and *Bam*HI-*Eco*RI restriction fragments of appropriate deletions were separated from their corresponding vector sequences by 8% polyacrylamide gel electrophoresis. The two purified restriction fragments were then ligated between the *Eco*RI and *Hin*dIII sites of YIp5, and recombinant plasmids were isolated and purified in *E. coli*. Transformation of strains DBY747 and YN217 by recombinant plasmids was performed essentially by the method of Hinnen et al. (12) and Beggs (1).

Segregation vector assay. The segregation assay was performed essentially as described by Hieter et al. (11). The only major alteration in this procedure was the maintenance of selective pressure on the transformed cells until the final few generations of growth. Individual colonies of transformed cells were isolated on complete synthetic medium lacking uracil. Pink colonies were picked into 25 ml of YPD, and samples were removed at 4 h. Appropriate dilutions were made and spread on indicator plates containing complete synthetic medium lacking uracil and supplemented with adenine at 2 mg/liter. After 7 days at 28°C, the colonies on these plates were scored for sectoring and color phenotype.

RESULTS

The histone H4 *ARS* was cloned from the yeast copy-I H3-H4 *Hin*dIII fragment Sc191(31). It was identified by subcloning *Sau*3AI restriction fragments into the *Bam*HI site of the chimeric plasmid YIp5, which is composed of the bacterial plasmid pBR322 and the yeast *URA3* selectable gene (2). The nonreplicating plasmid YIp5 transformed yeast strains containing the *ura3-52* allele to URA^+ prototrophy at a frequency that was less than 0.1 transformants per μg of DNA. When a 374-base-pair *Sau*3AI restriction fragment from Sc191 was present on the plasmid, it transformed the same yeast strains to URA^+ at a frequency of 80 to 1,000 colonies per μg of DNA. Subsequent restriction mapping and DNA sequence analysis established the position of the *Sau*3AI fragment to be on the 3′ downstream side of the histone H4 gene in Sc191. The start of the *Sau*3AI fragment corresponds to position 1786 in the published copy-I H3-H4 DNA sequence, 293 base pairs from the end of the histone H4 gene (30).

The YIp5 derivative containing the 374-base-pair *Sau*3AI restriction fragment was designated pAB4. It demonstrated all of the properties expected of a replication-proficient *ARS* plasmid (10, 11, 24). The plasmid was shown to exist as extrachromosomal circular molecules in the transformed cells by Southern blot analysis of genomic DNA (32). Total yeast DNA from these yeast cells transformed *E. coli* to ampicillin resistance, and the plasmid recovered in *E. coli* had the same structure as pAB4 as judged by restriction map analysis. Under selective pressure, the plasmid was maintained at 30 to 300 copies per cell in approximately 10 to 30% of the population, while in the absence of selective pressure the plasmid was rapidly lost from the culture. Finally, DNA sequence analysis demonstrated the presence of the *ARS* core consensus sequence, beginning at position 81 of the *Sau*3AI restriction fragment. Thus, this relatively small *ARS* fragment provided a useful system in which to examine the molecular genetics of autonomous replication.

Mapping *ARS* function by progressive external deletions. The left and right boundaries of the DNA sequences required for *ARS* function were first defined by a series of progressive deletions generated from both ends of the fragment. The deletions were made by exonuclease III and nuclease S1 digestion as described in Materials and Methods, and the deletion endpoints were sequenced. Potentially interesting deletions were then subcloned into the chimeric vector YIp5. The main advantage of this protocol is that the vector sequences juxtaposed at the deletion breakpoints are identical for each construct in the series.

These plasmids were then tested for *ARS* function by determining the frequency of transformation of a yeast strain containing the *ura3-52* allele to prototrophy. Autonomous replication of the deletion plasmids was then confirmed by plasmid stability studies and Southern blot analysis. For replication-deficient deletions, the plasmid sequences in the rare transformants were confirmed to be exclusively integrated.

A portion of the DNA sequence of each deletion derivative tested in the transformation assay is shown in Fig. 1. The derivatives shown in Fig. 1A represent deletions coming from the histone H4-proximal side of the restriction fragment. These righward deletions (R series deletions) were cloned into YIp5 as *Bam*HI-*Eco*RI restriction fragments. In

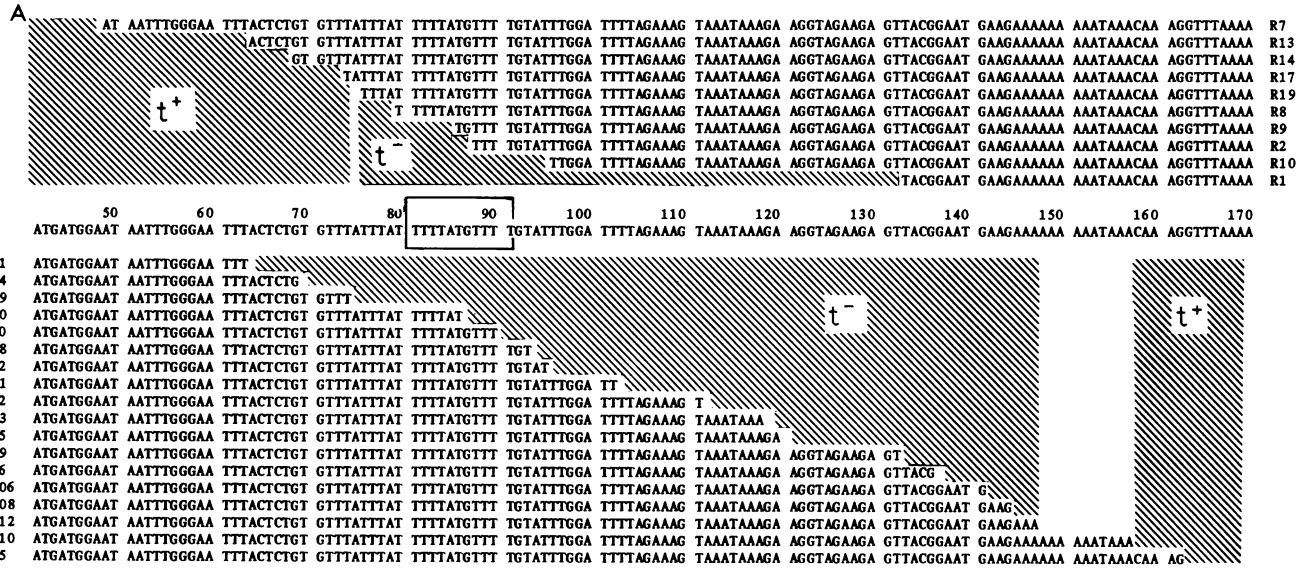


FIG. 1. DNA sequence of external deletions. The DNA sequences of the deletions that were tested in the transformation assay are shown. The wild-type sequence from nucleotides 41 to 170 of the 374-base-pair *Sau*3AI histone *ARS* fragment is presented in the center. The consensus sequence is boxed. Nucleotide number 1 of the fragment corresponds to position 1786 in the published DNA sequence of the copy-I H3-H4 locus (28). (A) DNA sequences of the rightward or R set of deletions. In addition to the sequences shown, these deletion derivatives contain DNA up to the *Eco*RI site after nucleotide 374. (B) DNA sequences of the leftward or L set of deletions. These contain DNA from the *Hin*dIII site before nucleotide 1 to the deletion endpoints shown here. The behavior of each of these deletions in the transformation assay is illustrated by shading. The t^+ group of deletions were able to promote a high frequency of transformation. In contrast, the t^- group was unable to promote a high frequency of transformation of yeast auxotrophs.

each case, the *Bam*HI linker marked the deletion endpoint shown in the figure, and the *Eco*RI site was located at the end of the restriction fragment at nucleotide 374. Deleting up to and including nucleotide 73 (deletion R17) had no effect on the ability of the restriction fragment to promote a typical high frequency of transformation. However, removing an additional two nucleotides (deletion R19) resulted in a substantial loss of *ARS* function, and the succeeding deletion tested (deletion R8) was unable to promote transformation at a frequency that was any greater than that achieved by the parental vector YIp5. This deletion possesses only one nucleotide in addition to the *ARS* consensus sequence.

The leftward set of deletions (L series deletions) shown in Fig. 1B are progressive deletions from the histone H4-distal side of the restriction fragment. These were cloned into YIp5 as *Hin*dIII-*Bam*HI restriction fragments. In these constructions the *Bam*HI linker marked the deletion breakpoint and the *Hin*dIII site was located at nucleotide position 1. It was possible to delete up to and including nucleotide 158 from this direction without observing any loss of *ARS* function. That is, the YIp5 derivative containing deletion L110 was able to transform the yeast host to URA^+ at high frequency. Removing an additional 10 nucleotides had a marked effect on *ARS* function. The plasmid containing deletion L112 was unable to transform cells to URA^+ at high frequency. This was the case for all of the succeeding deletions that were tested and illustrated in Fig. 1B. In summary, progressive external deletions defined a region of at least 74 base pairs, delimited by constructs R19 and L112, that is required to produce viable URA^+ transformants by autonomous replication.

Defective *ARS* deletions are impaired in replication. The experiments described above used high-frequency yeast transformation as an assay of *ARS* function. While the assay is simple and convenient, it is obviously not a direct measure of replication. For example, in addition to the requirement for autonomous replication of the plasmid in the transformed cell, it must be able to segregate to the daughter cell during cell division. In fact, plasmid segregation during mitosis is extremely asymmetric even with fully functional *ARS* plasmids (16, 24, 41). A mutational defect that further affects this process could show a dramatic change in phenotype in the transformation assay. It was therefore important to test the *ARS*-defective derivatives in a second assay that could more easily differentiate between these two types of defects.

The assay described by Hieter et al. (11) enables one to follow the fate of specific plasmids and their progeny through several generations. Yeast cells carrying the ochre allele *ade2-101* give red colonies owing to the accumulation of 5-aminoimidazole ribonucleotide. The mutation may be suppressed by the ochre suppressor *SUP11*. Complete suppression results in white colonies, and partial suppression is expressed phenotypically as colonies of intermediate color. Thus, a diploid strain homozygous for the *ade2-101* mutation gives red colonies in the absence of the *SUP11* gene (unsuppressed), pink colonies in the presence of one copy of the *SUP11* gene (partially suppressed), and white colonies in the presence of two or more copies of the suppressor (fully suppressed). This sensitivity to the copy number of the *SUP11* marker has been exploited by cloning the gene on a *CEN-ARS* plasmid. A diploid tester strain originally transformed by one copy of the plasmid is pink, and the fate of the plasmid copy number can be followed by the colony color. A failure to replicate the plasmid results in pink:red sectored colonies while proper replication but defective segregation results in white:red sectors (11).

A vector that contained pBR322 sequences as well as the selectable yeast *URA3* gene, the *SUP11* gene, and *CEN4* was used for these studies. The entire wild-type *Sau*3AI *ARS* restriction fragment was cloned between the *Eco*RI-

TABLE 1. Relative stabilities of YRp14CEN4 minichromosomes containing various H4 *ARS* derivatives

Construction	1:0 events (%)	2:0 events (%)	Total (%)
Complete ARS1	1.6	0.7	2.3
L35	2.4	0.7	3.1
L96	>50	<0.1	>50
L55	>50	<0.1	>50
L63	>50	<0.1	>50
L71	>50	<0.1	>50
L50	>50	<0.1	>50
L60	>50	<0.1	>50
YRp14CEN4	>50	<0.1	>50
L63/R33	1.4	0.7	2.1

*Hin*dIII sites of this vector. In addition, several of the leftward deletions were cloned between the *Hin*dIII-*Bam*HI sites of the vector. These plasmids were used to transform a diploid host homozygous for the *ade2-101* and *ura3-52* alleles. The fate of the plasmid was then analyzed by observing the color of transformed colonies after growth in nonselective medium for variable amounts of time.

The results of these experiments are summarized in Table 1. After approximately two generations in nonselective medium, cells transformed with the plasmid that contained the intact 374-base-pair restriction fragment produced predominantly pink colonies composed of cells containing one copy of the plasmid. Pink:red half-sectored colonies were observed in 1.6% of the colonies. These half-sectored colonies represent instances in which the plasmid was lost from either the mother or the daughter cell during the first cell division after plating. This can result from either a failure to replicate the plasmid or a loss of one copy of the replicated plasmid during division. In contrast, white:red half-sectored colonies, indicating a defect in plasmid segregation, were observed in only 0.7% of the colonies.

Deletion L35, which demonstrated normal *ARS* function when tested in the high-frequency transformation assay, showed a slight increase in the frequency of pink:red half-sectored colonies. Thus, this assay is considerably more sensitive than transformation efficiency in detecting defects in *ARS* function. The remaining deletions tested in this assay were L96, L63, L55, L79, L60, and L50. In each case, the plasmids containing these deletions were very unstable in the transformed cells. Even under continual selective pressure, the bulk of these transformed cells grew into completely red colonies when plated on indicator plates. The frequency of 1:0 events was over 50% for these deletion mutation plasmids. More importantly, the frequencies of 2:0 events were less than 0.1% for each of the deletions. The parental vector, which contains a very low efficiency *ARS* closely linked to the *SUP11* gene (34), was identical in its stability to all of the defective *ARS* deletions tested. These results confirm that the defect in the L-series deletion mutations must at least involve replication and cannot be solely in the segregation of replicated molecules.

Role of specific DNA sequences in *ARS* function. As described above, the sets of progressive *ARS* deletions shown in Fig. 1 were analyzed for *ARS* activity in two independent assays, and both indicated the importance of a region of approximately 74 base pairs for replication. Linker substitution mutations were therefore constructed across the region as a series to define those internal sequences essential for *ARS* function (21, 22). Full-length *Hin*dIII-*Eco*RI restriction fragments containing 10-base-pair linker substitutions were generated by combining appropriate leftward and rightward deletions at the synthetic *Bam*HI linker. With the exception of this small linker substitution, the 374-base-pair restriction fragment was identical in size and DNA sequence to the original wild-type restriction fragment. These substitution derivatives were tested for *ARS* function by cloning them into the *Eco*RI-*Hin*dIII site of YIp5 and using the resulting plasmids to transform yeast auxotrophs to prototrophy.

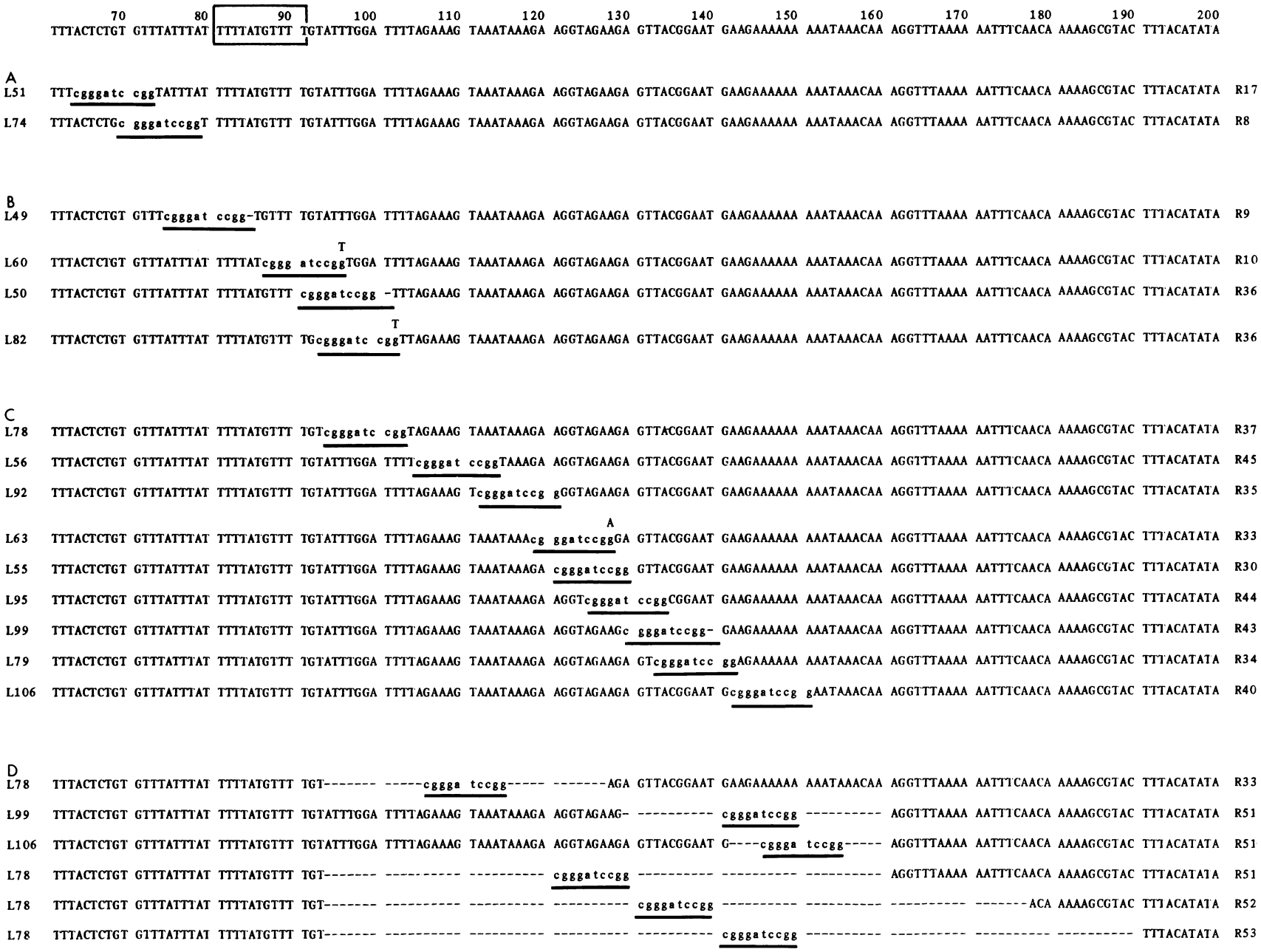
A portion of the DNA sequence of these linker substitutions is shown in Fig. 2. The two mutations shown in Fig. 2A demonstrated that substituting 10 base pairs on the left side of the consensus sequence had no effect on *ARS* function. Interestingly, only one nucleotide to the left of the consensus sequence remained unchanged in isolate L74/R8, and this mutation retained full *ARS* function.

The next three linker substitution mutations shown in Fig. 2B demonstrate the absolute requirement for an intact core consensus sequence. The linker substitutions in derivatives L49/R9, L60/R10, and L50/R36 disrupted the integrity of the consensus sequence, and these derivatives were defective in promoting high-frequency transformation. In each case, the frequency of transformation was well below one viable transformant per μg of DNA. The fourth mutation shown in Fig. 2B, clone L82/R36, maintained the integrity of the complete 11-base-pair consensus as well as the subsequent G residue, but disrupted the adjacent 10 nucleotides. This mutation was also defective in *ARS* function.

The remaining linker substitution mutations (Fig. 2C) were constructed to specifically identify those DNA sequences in the flanking 66 base pairs that were required for *ARS* activity. All of these mutations exhibited completely normal *ARS* function when cloned into YIp5 and assayed for transformation frequency. One of the linker substitutions in the middle of this region, L63/R33, was also subcloned into the YRp14CEN4 vector and tested for stability in the colony color assay. This construction had wild-type stability with no increase in either 1:0 or 2:0 events (Table 1). These results present a surprising paradox. The set of external leftward deletions indicated that approximately 66 base pairs flanking the core consensus were required for *ARS* function. Yet the progressive placement of 10-base-pair substitutions in this flanking region had no observable effect on *ARS* function.

It was reasoned that perhaps the DNA flanking the core consensus contained a specific required nucleotide sequence but that it was larger than the mutagenic linker and was not significantly disrupted by the substitution. Therefore, internal deletions were constructed to remove all or part of the flanking region in an attempt to resolve the actual sequence requirements of the region. These internal deletions were constructed similarly to the linker substitution mutations except that the leftward and rightward deletions chosen created deletions at the site of the linker instead of simple base substitutions. A portion of the DNA sequence of these deletions is illustrated in Fig. 2D. It is important to note that with the exception of the deleted nucleotides and the presence of the 10-base-pair linker at the site of the deletion, the remaining DNA was identical to the original 374-base-pair restriction fragment. These deletions were then cloned as *Hin*dIII-*Eco*RI restriction fragments into YIp5 and subsequently tested in the transformation assay. All six of the internal deletion derivatives shown in Fig. 2D were able to transform auxotrophic yeasts to URA^+ at a frequency that was much greater than 100 transformants per μg of DNA.

Effect of flanking vector sequences on *ARS* function. In



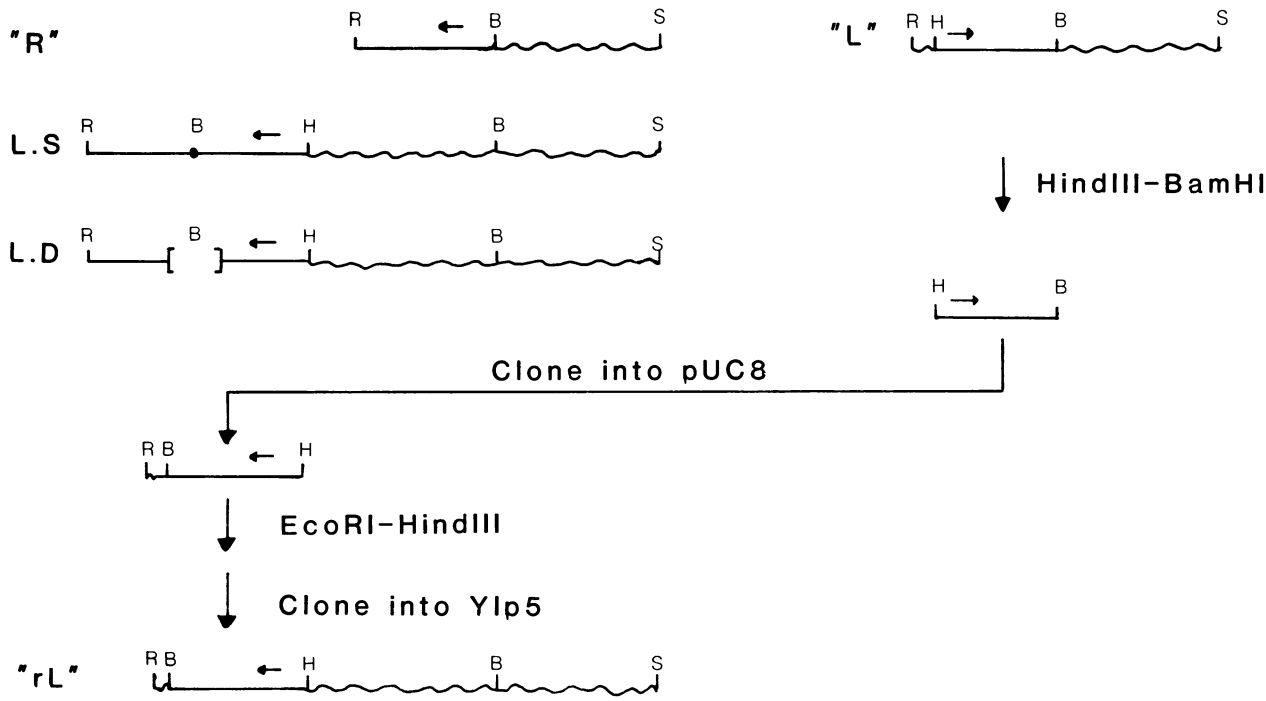


FIG. 3. Orientation of deletions with respect to vector sequences. The orientations of the rightward (R) and leftward (L) external deletions, the linker substitution (L.S) mutations, and the linker deletions (L.D) in the vector YIp5 are shown in this figure. The vector sequences are depicted as wavy lines, and the yeast sequences are shown as straight lines. The relative orientation of the 11-base-pair consensus sequence is depicted with an arrow. The direction of the arrow represents the 5′ to 3′ orientation of the sequence 5′-TTTTATGTTTT-3′. The method used to reverse the L-series of deletions with respect to vector sequences is also shown. By passing the *Hin*dIII-*Bam*HI restriction fragments through pUC8, the rL set of deletion derivatives was generated. R, *Eco*RI; B, *Bam*HI; S, *Sal*I; H, *Hin*dIII.

assessing the ability of particular external deletions, linker substitutions, and internal deletions to behave as autonomously replicating segments in yeasts, the vector sequences adjacent to the yeast DNA were identical within a given set of mutations. However, the orientation of the yeast DNA with respect to flanking vector DNA in the leftward series of external deletions was different from the orientation of the rightward external deletions, the linker substitutions, and the internal deletion mutations. This difference is illustrated in Fig. 3. It was possible that the different relative orientations may have contributed to the apparent paradox that a 66-base-pair region of DNA was required flanking the consensus sequence but that a specific DNA sequence within the region was not essential.

To resolve this question, certain of the L-series deletions were reversed with respect to the flanking vector sequences and tested for *ARS* activity. This reversal was accomplished by cloning the *Bam*HI-*Hin*dIII restriction fragment of an L-series deletion into pUC8 and subsequently placing the resulting *Hin*dIII-*Eco*RI restriction fragment into YIp5. The end products of this scheme were identical to the L-series of deletions with the exception of a few additional base pairs of DNA derived from the linker system in pUC8. The orientation of a typical rL deletion with respect to its flanking YIp5 sequences is shown in Fig. 3.

When the replication-competent deletion L35 was reversed, the YIp5 derivative containing rL35 was also replication competent. It was comparable to its L35 counterpart both in its ability to promote a high frequency of transformation and in the growth rate of the resulting transformed cells. The generation time of both strains was 3.2 h in complete synthetic medium lacking uracil.

Next, the replication-defective deletions L96, L79, and L55 were tested. When these fragments were reversed, they unexpectedly became replication proficient; deletions rL96, rL79, and rL55 were all able to promote high-frequency transformation. The growth rates of cells transformed with rL96 and rL79 were, in fact, similar to that of cells transformed with a fully functional *ARS* plasmid. Cells transformed by rL55 demonstrated a somewhat depressed growth rate with a doubling time of 4.8 h. Placing these leftward deletions in a different vector environment therefore restored *ARS* function.

Finally, reversal of the replication-negative deletions L71, L78, L50, and L60 did not suppress the defect. Unlike the orientation-dependent phenotype described above, deletions rL71, rL78, rL50, and rL60 behaved identically to their L-series counterparts. The YIp5 derivatives containing these deletion derivatives did not produce viable transformed colonies. These results indicate that at least 12 base pairs on

FIG. 2. DNA sequences of linker mutations. The DNA sequences of the linker substitution and linker deletion mutations from nucleotides 61 to 200 are shown in this figure. These sequences are portions of the complete 374-base-pair *Hin*dIII-*Eco*RI restriction fragment. The linker is shown in each case by underscoring. Panels A, C, and D contain mutation derivatives that demonstrated completely wild-type *ARS* function in the transformation assay. The four mutations shown in panel B were unable to promote a high frequency of transformation. The consensus sequence is boxed.

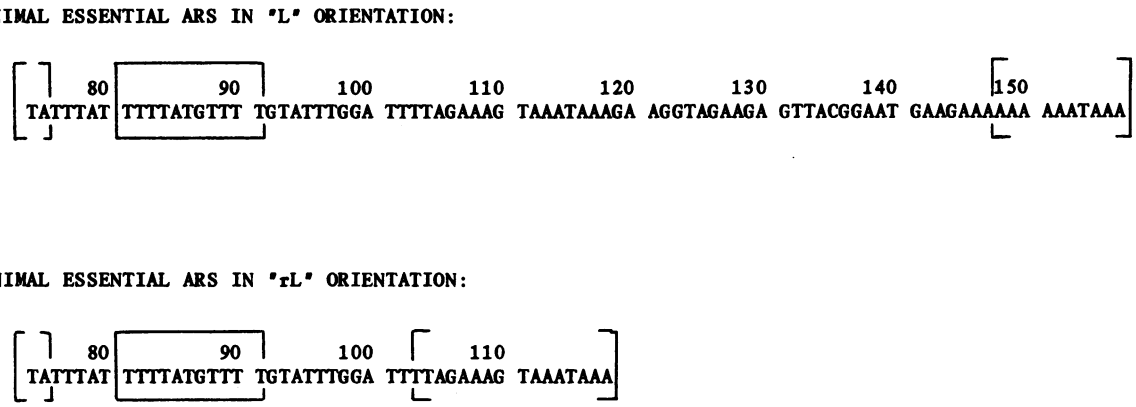


FIG. 4. Functional boundaries of the *ARS*. The minimal essential DNA squences necessary for *ARS* function in both the L and rL orientations are shown. The consensus sequence is presented within a box. Those sequences within brackets represent sequences that may be required but that have not been directly tested.

the 3′ side of the core consensus are required for *ARS* function in either of the two vector environments tested.

DISCUSSION

***ARS* core consensus.** The 11-base-pair *ARS* core consensus sequence has been shown to be necessary for autonomous replication of *ARS1* and the *HO* gene *ARS* (6, 15). The linker-scanning analysis of the H4 *ARS* reported here confirms the absolute requirement of this sequence-specific element for plasmid replication. The functional 5′ end of this core sequence agrees well with the standard consensus notation since the *Bam*HI linker in construction L74/R8 comes to within one nucleotide of the 11-base-pair consensus box (Fig. 2A). This substitution generates mutations in 8 of the 10 nucleotides at this position and yet retains full *ARS* function.

The linker substitutions on the immediate 3′ side of the *ARS* core sequence are particularly interesting. The *Bam*HI linker in isolate L82/R36 is positioned two nucleotides past the end of the *ARS* consensus box and is replication defective, while in L78/R37 it is positioned three nucleotides past the end of the consensus and is fully *ARS* positive. Both linkers result in the mutation of all 10 base pairs at these positions. These results suggest the importance of the nucleotides present immediately past the end of the standard 11-base-pair consensus. However, the defect in L82/R36 is apparently more complex than the T-to-C change two nucleotides past the consensus since the DNA sequence of the yeast 2μm *ARS* naturally contains a C residue at this position.

The 3′ flanking domain. Deletions into a region flanking the 3′ side of the core consensus element produced a defect in plasmid DNA replication as assayed by both high-frequency transformation and the colony color assay for mitotic chromosome transmission (11). The vector plasmid sequences positioned at the deletion breakpoints were identical for all of the deletions in a series. Therefore, a change in *ARS* function could be unambiguously assigned to a change in the deletion endpoint and not to an artifact caused by the local DNA context of that endpoint. These results provide the best evidence to date that mutations close to the *ARS* consensus actually impair DNA replication and not simply mother-daughter segregation. In addition, the 3′-flanking domain deletions were tested in two different vector environments, and the two series of constructions clearly demonstrate that context effects are an important consideration in interpreting experiments with *ARS* fragments. The 3′ deletion boundary for *ARS* function was different in the two orientations in YIp5 (Fig. 4).

These results may reconcile an apparent discrepancy between the sizes of the 3′-flanking domains reported for *ARS1* (6, 33) and for the *HO* gene *ARS* (14, 15). The plasmid vector environments for these two studies were entirely different. The size of the reported 3′ domain flanking *ARS1* is very similar to the L-series H4 *ARS* deletions, while the size of the *HO* gene *ARS* is very similar to the rL-series deletions. Thus, the size of the 3′-flanking domain is likely to be determined by both the flanking yeast DNA sequences and the vector sequences to which they are fused.

Unlike the sequence specificity of the *ARS* core consensus, the function of the 3′-flanking domain of the H4 *ARS* does not have an exact DNA sequence requirement. With the possible exception of L82/R36, linker substitutions of 10 base pairs placed serially throughout the 3′-flanking domain had no effect on *ARS* activity. Despite this lack of a specific DNA sequence consensus, there are nevertheless certain chromosomal environments that are unacceptable in the 3′-flanking domain and abolish *ARS* function when they replace the normal DNA sequences. The vector sequences near the *Bam*HI site of YIp5 are one example of these inhibitory sequences.

The deletion mutations described in this paper have been useful for identifying both the 5′ and 3′ boundaries of *ARS* function. However, it is not possible to determine the absolute extent of the minimal functional DNA from these experiments. The R-series deletions all retain at least 300 base pairs of 3′ sequences to the end of the 374-base-pair yeast fragment, while L-series deletions all retain at least 90 base pairs of 5′ sequence to the beginning of the 374-base-pair yeast fragment. If redundant functional elements were present outside each of these boundaries, the minimal limits of the H4 *ARS* could be larger than that defined by the external deletions. Minimal functional units, based on the deletion endpoints, are currently being constructed to address this question.

H4 *ARS* function. There are several potential explanations for the unusual properties of the 3′-flanking domains. One possibility is that the yeast DNA sequences normally flanking the *ARS* core consensus box encode multiple examples of a function necessary for replication. Internal deletions and linker-scanning mutations that destroy one of the

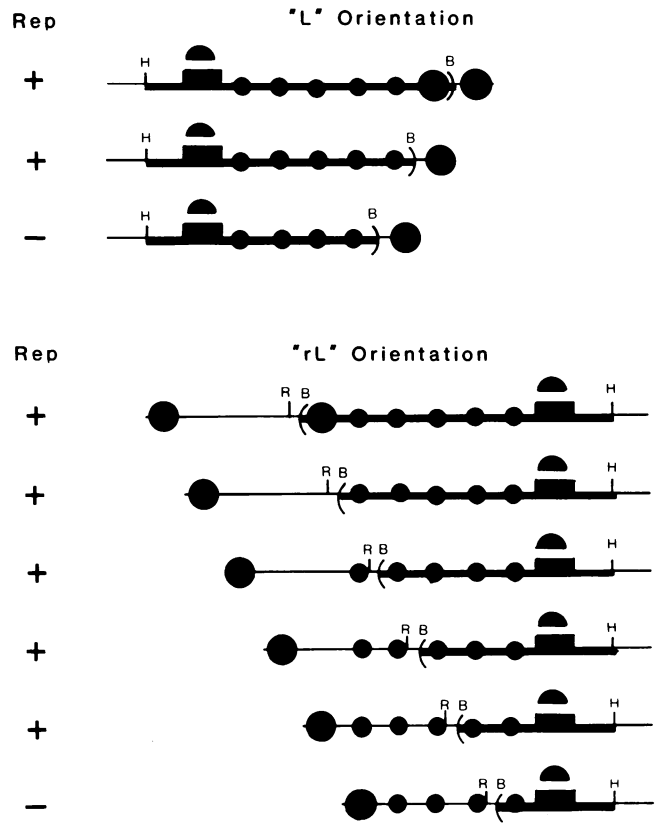


FIG. 5. Structural model for *ARS* function. The proposed effects of deletions in the L-series and rL-series constructions are illustrated diagrammatically. Yeast DNA sequences are represented by thick lines, and vector sequences are shown by thin lines. The deletion endpoint in each case is indicated by a curved bracket. The box represents the *ARS* core consensus sequence, and the half-circle covering the box represents a hypothetical consensus-recognition protein. The small circles adjacent to the consensus represent less-specific DNA-binding proteins, and the larger circles represent nucleosomes assembled on the flanking yeast and vector regions. The ability of the construction to support autonomous replication is indicated as either Rep + or Rep −. In the examples it is assumed arbitrarily that at least five units of the DNA-binding protein (small circles) must assemble to be replication positive. The net result of the model is that the deletion boundaries are different in the two cases and that different amounts of yeast DNA appear to be needed for *ARS* activity. H, *Hin*dIII; R, *Eco*RI; B, *Bam*HI.

repetitive elements would have no effect on replication, but external deletions that remove the last of the repetitive elements would destroy *ARS* activity. To explain the differences observed in *ARS* function resulting from different vector environments, YIp5 sequences would have to complement this function in only one orientation. Transcriptional promotion close to the *ARS* is a possible candidate for such a function. Multiple promoters in the yeast DNA adjacent to the core consensus might be present to assure initiation of replication at the *ARS*. For the rL-series deletions lacking these promoters, transcription through the *ARS* could be provided by a fortuitous promoter in the YIp5 vector sequences (3). This model does not adequately explain the need for the 12 to 27 base pairs of yeast DNA in the rL-series constructions. In addition, nuclease S1 mapping has so far failed to identify RNA transcripts from the *ARS* region, although small or highly unstable transcripts could not have been detected.

Alternatively, the 3′-flanking domain may contribute to autonomous replication in a structural capacity. A number of observations are consistent with this hypothesis. For example, the base composition of the DNA around known *ARS* elements is aproximately 70% A+T and led Broach et al. (4) to propose that *ARS* function is dependent on this AT-rich base composition. The results described in this report are consistent with the involvement of base composition in autonomous replication. The 66 base pairs of yeast DNA that normally flank the *ARS* consensus are 75% A+T. The 280 base pairs adjacent to the *Bam*HI site in YIp5, in the *tet*^{r} gene direction, average 35% A+T. In contrast, the region of DNA adjacent to the *Eco*RI site, in the *amp*^{r} gene direction, averages 62% A+T, and there are regions within this segment in which the average content for 100 nucleotides exceeds 70% A+T.

An AT-rich segment may in itself provide a secondary structure adjacent to the consensus that is required for DNA replication. It may also contribute to the formation of a chromatin structure that facilitates autonomous replication. For example, it is known that poly(dA)-poly(dT) sequences tend to exclude the reconstitution of nucleosomes in vitro (18, 26). There are a large number of observations suggesting the presence of a special chromatin structure at replication origins. In yeasts, a unique chromatin structure has been shown to exist around the consensus of *ARS1* (20, 36, 37), and 2μm plasmid origin (8, 19, 39), and the *ARS* associated with the histone H4 gene studied here (J. Brown and M. M. Smith, unpublished observations). There is also strong evidence for a nucleosome-free domain near the simian virus 40 origin of replication (13, 28, 38). These biochemical observations together with the present genetic experiments strongly suggest that structural constraints play a major role in the initiation of DNA replication.

The model illustrated in Fig. 5 incorporates these structural components to explain how a particular *ARS* deletion can be replication defective in one orientation and replication proficient in the other. The model is made up of two components. The first is the *ARS* core consensus sequence, and it is proposed to serve as a specific protein-binding site or enzyme recognition sequence necessary for replication. The second component consists of less-specific DNA-binding proteins that bind to the adjacent 3′-flanking region to form a complete initiation-replication complex. Finally, it is proposed that the formation of this complex is dependent on a unique DNA-chromatin structure. For example, a nucleosome in the flanking DNA may prevent association of these binding proteins with the DNA and block the formation of a functional replication complex.

Linker substitutions throughout the 3′-flanking domain do not affect *ARS* function since they do not change the structure of the region. In the case of the external progressive deletions, at some point the first nucleosome present on vector DNA would be positioned so close to the consensus that formation of the complete initiation complex would be prevented. The apparent size of the 3′-flanking domain reflects the position of the first blocking nucleosome in the vector DNA and is therefore different for the alternate orientations.

Although this model evolved from attempts to explain the behavior of *ARS* derivatives on an episomal plasmid, a similar mechanism may also be active in the yeast genome. Not all core consensus sequences can promote autonomous replication even when cloned with relatively large regions of flanking yeast DNA (unpublished observations). This implies that there are apparently normal yeast DNA sequences

that can act like YIp5 vector sequences to suppress *ARS* function.

The idea that chromatin structure can both absolutely and perhaps selectively prevent replication from a specific origin has important implications. First, such a mechanism may explain how mutations relatively distant from the *ARS* core consensus could affect the efficiency of autonomous replication (17). Second, the model provides for the temporal control of replication origins during the S phase. Dynamic alterations in the chromatin structure of the 3′-flanking domain might allow certain origins to function early while others are turned on late (5, 7, 9). Finally, the model could ensure that a particular origin of replication would be used just once per division cycle if the chromatin structure of the 3′-flanking domain in the newly replicated chromatids was different from that of the prereplication *ARS*.

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