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Drosophila Scaffold-Attached Regions Bind Nuclear Scaffolds and Can Function as ARS Elements in Both Budding and Fission Yeasts

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Histone-depleted nuclei maintain sequence-specific interactions with genomic DNA at sites known as scaffold attachment regions (SARs) or matrix attachment regions. We have previously shown that in *Saccharomyces cerevisiae*, autonomously replicating sequence elements bind the nuclear scaffold. Here, we extend these observations to the fission yeast *Schizosaccharomyces pombe*. In addition, we show that four SARs previously mapped in the genomic DNA of *Drosophila melanogaster* bind in vitro to nuclear scaffolds from both yeast species. In view of these results, we have assayed the ability of the *Drosophilia* SARs to promote autonomous replication of plasmids in the two yeast species. Two of the *Drosophila* SARs have autonomously replicating sequence activity in budding yeast, and three function in fission yeast, while four flanking non-SAR sequences are totally inactive in both.

The eucaryotic chromosome must be organized into both functional and structural domains in order to facilitate the proper expression, replication, and partitioning of DNA through the cell cycle. It has been shown that chromosomal DNA is organized into relatively independent units of replication, or replicons, which allow its complete duplication within the length of an S phase (17). Other studies suggest that the basic chromatin fiber, which results from the interaction of histones with chromosomal DNA, is organized at a higher-order level into topologically constrained looped domains (reviewed in reference 71). That functional units of chromosome organization may correlate with structural ones has been proposed and discussed in many instances (reviewed in references 18, 20, 21, 52). One proposal for the coordination of structure and function is that replication origins might coincide with or flank sequences that anchor chromatin loops to nonhistone structural proteins.

Residual nuclear structures devoid of histones and soluble nuclear proteins can be obtained from eucaryotic cells following the extraction of isolated nuclei by either high salt concentrations (nuclear matrices) or lithium diiodosalicylate (nuclear scaffolds). Scaffold-attached regions (49) are DNA sequences selectively retained by scaffolds after the digestion of chromosomal DNA with restriction endonucleases. They represent likely candidates for anchorage sites of chromatin loops in vivo. Such attachment sites have been mapped in the genomic DNA of diverse organisms, including *Drosophila melanogaster*, chick cells, a variety of mammalian cells, and budding yeast (reviewed in reference 18). In *Saccharomyces cerevisiae*, scaffold attachment regions (SARs) include both centromeres and autonomously replicating sequence (ARS) elements (4). ARS elements of *S. cerevisiae* have recently been shown to function as origins of replication on plasmids (6, 30). Some of them also serve as genomic origins of replication (7, 31, 42). In this paper, we show that nuclear scaffolds can be isolated from *S. pombe* nuclei and that the endogenous ARS elements of this species are also scaffold bound.

Although scaffold-bound regions do not share extensive sequence homology within or among species, they do display a number of significant similarities, the most obvious of which is an elevated A+T content. Also of interest is the recurrence of homologies to different short sequence motifs (18, 19), of which we present a closer inspection in this work. These similarities suggest that SARs represent a particular class of sequences, among which structural and functional characteristics are conserved to varying degrees. Supporting this idea, it has been shown that scaffolds from human, rat, mouse, and chick cells bind *Drosophila* SARs (dSARs) (12, 35, 48, 58) and that *Drosophila* scaffolds and mammalian matrices bind the yeast ARS1 element (4, 67). The binding of the *Drosophila* histone locus SAR to yeast scaffolds and of the matrix attachment region from the mouse immunoglobulin κ chain gene to yeast nuclear matrices and chick nuclear scaffolds has also been reported (4, 13, 58).

To extend our analysis of the relationship between SARs and ARS elements, we have examined four well-characterized SARs from *D. melanogaster* for interaction with nuclear scaffolds and for ARS activity in the two yeast species *S. cerevisiae* and *Schizosaccharomyces pombe*. Analysis of a different set of dSARs in *S. cerevisiae* has been carried out by Miassod and co-workers and is presented in the accompanying paper (9). Together the data suggest that the specific SAR-scaffold interaction observed in various species relies on the structural features shared by the diverse SAR elements and that these features are related to those promoting ARS activity in both yeasts.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* strains 62-5c (*Mata leu2 his3 lys2 pep4-3*) (a gift from H. Riezman) and BJ2168 (*Mata leu2 trp1 ura3-52 pep4-3 prb1-1122 prc1-407 gal2*) (a gift from D. Shore) and an (*ura4-D18 leu1-32 end1 h-*) *S. pombe* strain (from J. Kohli) were used for nuclear isolation and binding studies. For transformations, we used a (*Mata ura3-52*) derivative of *S. cerevisiae* X21-80 constructed by H. Riezman and the *ura4-D18* strain of *S. pombe* (23). Complete medium for nuclear isolation was YPAD (62) for *S. cerevi-*

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siae and YE (3% glucose, 0.5% yeast extract, with 100 mg each of adenine, uracil, leucine, lysine, and histidine per liter) for *S. pombe*. Selective plates and media were prepared as described previously (62).

DNA methods. Plasmid constructions, isolation, and analysis, as well as Southern analysis of transformant yeast DNA were performed with standard methods (44). For the Southern analysis, DNA immobilized on Hybond-N nylon membranes (Amersham Corp.) was hybridized to ^{32}P-labeled probes (random priming kit; Amersham). Blots were washed under high-stringency conditions (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% sodium dodecyl sulfate, at 65°C for 2 to 3 h). Published methods were used to isolate DNA from *S. cerevisiae* (28) or *S. pombe* (27).

Plasmid constructions. The plasmid pIU contains the 1.17-kilobase (kb) *Hin*dIII *URA3* gene fragment of *S. cerevisiae* D4+ (60) in the *Hin*dIII site of pBR322. The direction of *URA3* transcription is clockwise with respect to the map of pBR322 (see reference 44). Plasmids pF9 and pF12 were constructed by subcloning the 1.45-kb *Eco*RI TRP1-ARS1 fragment from *S. cerevisiae* (68), blunt ended with Klenow polymerase (Boehringer Mannheim Biochemicals), in both orientations into the unique *Nru*I site of pIU (in the pBR322 Tet^r region).

SAR and non-SAR *D. melanogaster* DNA fragments (19, 49) were subcloned into pIU after their isolation from plasmids containing the *ftz* regulatory element, the *Sgs-4* gene locus, or the large histone gene repeat unit. The plasmids containing fragments from the *Drosophila hsp70* 87A7 locus were derived by subcloning the *URA3* gene into the *Hin*dIII site of plasmids 122×14 and 122×4 (22), yielding pIU143 and pIU41, respectively. These plasmids are equivalent to insertion of the *hsp70* DNA fragments into the *Sal*I site of pIU. Plasmid pFL20 has been previously described (27, 43). It contains two independent *Eco*RI inserts from *S. pombe* genomic DNA inserted in a vector identical to pIU. pAS1 was derived from pFL20 by insertion of the *S. pombe ade6*$^+$ gene (a gift of J. Kohli).

Transformation methods. *Escherichia coli* was transformed as described previously (24). Intact *S. pombe* and *S. cerevisiae* cells were transformed by the lithium acetate protocol of Ito et al. (33), with several modifications suggested to us by J. Kohli. Briefly, exponentially growing cells (ca. 2×10^7 cells per ml) were collected by centrifugation, washed once in water and once in TEL (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA-NaOH, 0.1 M lithium acetate), resuspended in TEL at 10^9 cells per ml, and incubated for 30 min at 30°C with gentle agitation. A 100-μl portion of cells was combined with 1 μg of plasmid DNA in 50 μl of TE (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA-NaOH) and incubated for 30 min at 30°C. A 700-μl portion of 40% polyethylene glycol 4000 in TEL was added, and incubation was continued for 60 min. After a 42°C heat shock of 5 min, cells were centrifuged briefly in a microcentrifuge, washed two times, suspended in minimal medium, and plated on uracil-free selective plates.

Our transformation frequencies (colonies per microgram of DNA) for ARS$^+$ plasmids in *S. pombe* were high, comparable with those described in the literature (27, 46, 73). Frequencies lower by about 2 orders of magnitude were obtained if either the number of cells per transformation (10^8 in 100 μl) or the amount of plasmid DNA (1 μg) was decreased 10-fold. Thus, the concentrations of competent cells and of plasmid DNA can both be limiting factors in this protocol. The addition of 50 μg of pBR322 DNA as carrier did not change transformation frequencies in our standard conditions. This was true for both yeast species.

Isolation of nuclei and scaffolds. Nuclear isolation from *S. cerevisiae* was as described previously (4), with some modifications (11a).

S. pombe nuclei were isolated as follows. Usually two 2-liter cultures on YE media, at a cell density between 5×10^6 and 1×10^7 cells per ml, were harvested by centrifugation for 5 min at 2,000 × *g*. The pellet was washed once in 40 to 80 ml of distilled water at room temperature, and the cell pellet was weighed. A typical yield was around 2 g of culture per liter. Cells were suspended (10 ml/g [wet weight] of cells) in 0.1 M Tris hydrochloride (pH 8.0)–0.1 M EDTA-KOH (pH 8.0)–10 mM dithiothreitol and incubated with gentle agitation at 30°C for 10 min. Cells were recovered by centrifugation (1,200 × *g*, 5 min) and were suspended in 10 ml of 1.1 M sorbitol–0.1 mM $CaCl_2$–20 mM KPO_4 (pH 6.5) per g (SP). Spheroplast formation was started by the addition of 2,000 U of lyticase per ml and 2 mg of Novozyme (Novo Industries) per ml. After 15 or 20 min of gentle agitation at 30°C, cells were checked for complete spheroplasting by microscopic observation. Spheroplasts were collected at 1,400 × *g* for 10 min and gently washed twice by suspension in 1.1 M sorbitol–20 mM KPO_4 (pH 6.5) with 0.5 mM phenylmethylsulfonyl fluoride. The final spheroplast pellet was transferred to ice, suspended in 40 ml of cold breaking buffer {15% Ficoll-400 [Sigma Chemical Co.] in 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–KOH [pH 6.8], 0.25 mM spermidine, 2 mM KCl, 2 mM K-EDTA, 1% Trasylol [Bayer], 0.5 mM phenylmethylsulfonyl fluoride, 1% thiodiglycol, 1 μg pepstatin per ml}. This suspension was homogenized with 10 vigorous strokes of a tight Dounce homogenizer on ice. Complete cell lysis was checked by light microscopy.

The homogenate was centrifuged in two tubes in a Sorvall HB4 rotor at 300 × *g* for 5 min to remove large debris. This and all subsequent steps were done at 4°C. Supernatants were transferred to precooled polycarbonate tubes and centrifuged in the HB4 rotor at 9,000 rpm (13,400 × *g*) for 15 min. The postnuclear supernatant was discarded, and the pellet containing crude nuclei was suspended and rehomogenized with 10 strokes of the Dounce in 10 ml/g (starting cell weight) of 10 mM PIPES-KOH (pH 6.8), 2 mM spermidine, 0.8 mM spermine, 2 mM KCl, 2 mM K-EDTA, 0.5% Trasylol (Bayer), 0.5 mM phenylmethylsulfonyl fluoride, 1% thiodiglycol, 1 μg of pepstatin per ml, 12% glycerol. The resuspension was mixed with an equal volume of 66% (wt/vol) Percoll in the same solution, rehomogenized, and transferred to 40-ml polycarbonate tubes. Percoll gradients were formed in a Sorvall SS34 rotor at 18,000 rpm (39,000 × *g*) for 45 min. A band consisting of clean nuclei, as judged by optical as well as biochemical criteria, was collected from the top of the gradient. This was diluted at least three times with 0.25× A (5 mM PIPES-KOH [pH 6.8], 0.125 mM spermidine, 0.05 mM spermine, 20 mM KCl, 2 mM K-EDTA, 10% glycerol, 0.5% Trasylol [Bayer], 0.5 mM phenylmethylsulfonyl fluoride, 1% thiodiglycol, 1 μg of pepstatin per ml) and centrifuged (27,000 × *g*, 15 min). The supernatant was carefully removed by aspiration, and nuclei were washed again in 0.25× A and recovered by centrifugation in a swing-out rotor at 5,000 × *g* for 10 min. This final pellet was either used directly or resuspended in the same buffer containing 60% glycerol and stored at −20°C. The yield in nuclei (measured in A_{260} U in 1% sodium dodecyl sulfate) varied from 50 to 200 A_{260} from a 4-liter culture.

Scaffold-binding assays. Preparation of scaffolds, the map-

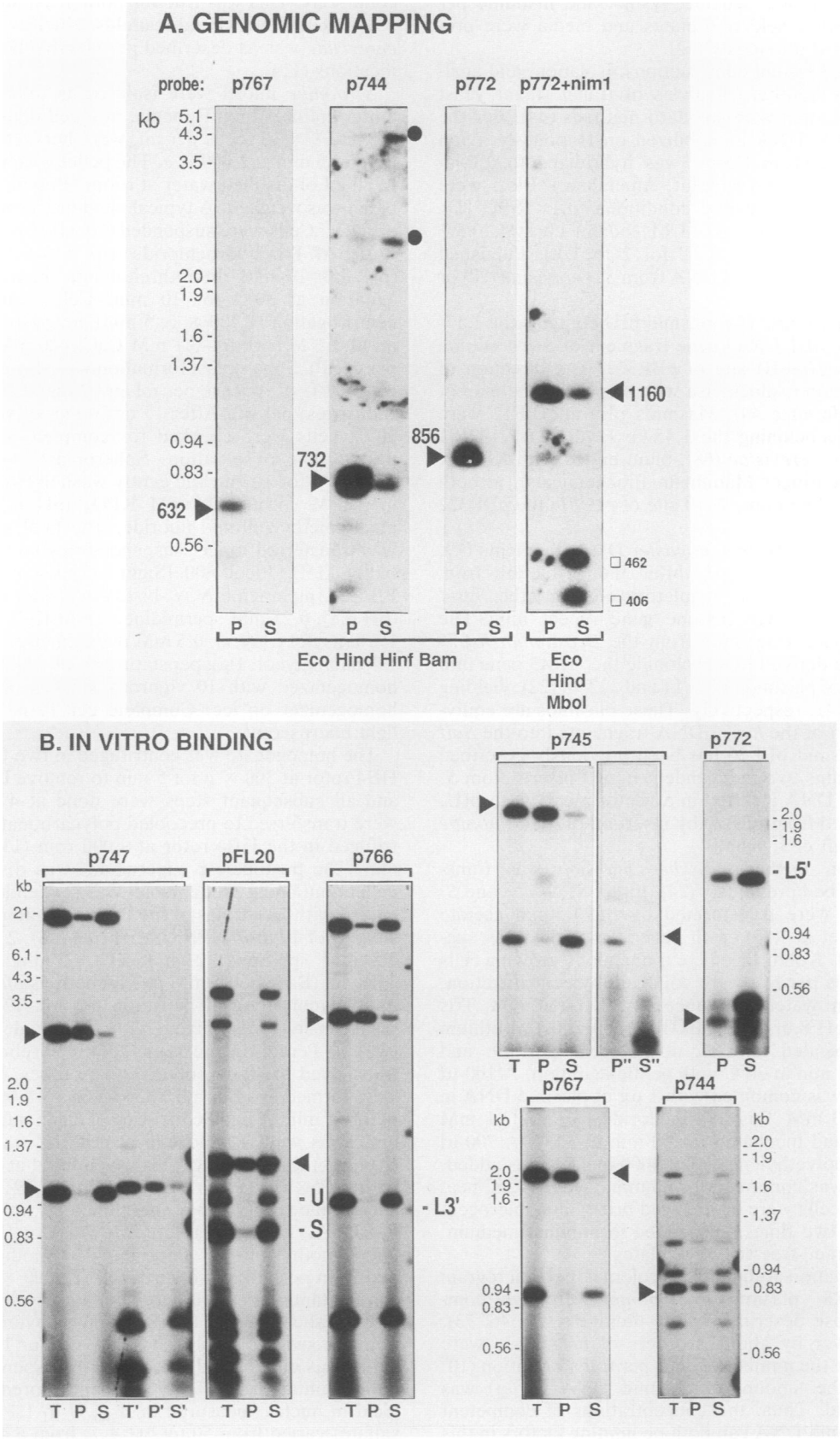

A. GENOMIC MAPPING
probe: p767 p744 p772 p772+nim1
kb
5.1
4.3
3.5
2.0
1.9
1.6
1.37
0.94
0.83
0.56
632
732
856
1160
462
406
P S P S P S P S
Eco Hind Hinf Bam
Eco Hind MboI
B. IN VITRO BINDING
p747
pFL20
p766
p745
p772
kb
21
6.1
4.3
3.5
2.0
1.9
1.6
1.37
0.94
0.83
0.56
- U
- S
- L3'
- L5'
T P S T' P' S'
T P S
T P S
T P S P'' S''
P S
p767
p744
T P S
T P S

TABLE 1. Transformation of *S. cerevisiae* and *S. pombe*

Replicating plasmids[a]		Transformation of:					
		S. cerevisiae			*S. pombe*		
Insert (kb)	Plasmid	Transf.[b]	Mitotic stability[c,e]	Plasmid loss[d,e]	Transf.[b]	Mitotic stability[c,e]	Plasmid loss[d,e]
S. c. *TRP1-ARS1* (1.45 kb)	pF9	$>10^4$	23–45	11–18	0		
	pF12	$>10^4$	20–30	7–15	0		
S. p. ARS + STB (1.0 + 1.2 kb)	pFL20	$>10^4$	11–28	3–15	10^3	65–95	30–60
	pAS1	10^4	13–15	2–3	3×10^3 to 10×10^3	60–70	40–60
ftz SAR (1.2 kb)	pIUF122	$>5 \times 10^3$	1.5–5	<0.4–0.6	<200	55–80	25–50
	pIUF121	5×10^3	7–9	0.5–2	4×10^3 to 10×10^3	45–75	30–60
Histone SAR (1.31 kb)	pIUC11	0			5×10^3 to 10×10^3	25–90	10–35
	pIUC17	0			$>10^4$	30–60	5–25
hsp70 SAR (1.27 kb)	pIU41	10^3	2.5	0.5–1	2×10^3 to 5×10^3	40–50	10–15
hsp70 SAR + gene (1.27 + 3.25 kb)	pIU143	1×10^3 to 2×10^3	0.5–5.5	<0.5	1×10^3 to 2×10^3	15–30	<0.5–2

[a] The cloning of *Sgs-4* SAR and non-SAR fragments that flank the dSARs in the *Drosophila* genome resulted in nonreplicating plasmids as follows. pIU, vector; pIUS82, *Sgs-4* SAR (0.87 kb); pIUS167,*Sgs-4* non-SAR (1.7 kb); pIUS83,*Sgs-4* non-SAR (0.9 kb); pIUF191 + pIUF192,*ftz* non-SAR (1.9 kb); pIUD17, histone non-SAR (1.6 kb) (see Results).

[b] Transf., Transformation efficiency, in colonies per microgram of DNA.

[c] Percentage of plasmid-containing cells after 10 generations of growth under selective conditions.

[d] Same as footnote *c* after 7 (*S. cerevisiae*) or 10 (*S. pombe*) generations of nonselective growth.

[e] Numbers represent the extremes of 3 to 4 independent measurements taken on independent transformants for each plasmid. Given the considerable scattering of these values, this presentation, rather than the mean and standard deviation, accurately reflects the collected data.

ping of genomic attachment sites, and in vitro binding assays were performed as described previously (4), except that 7 μg of *Hin*fI-digested pBR322 or pAT153 (44) was used for each 3 A_{260} U of *S. pombe* nuclei as nonspecific competitor in the in vitro binding assay. In some cases, non-pBR322-derived bacterial vectors were used as competitor DNA with no effect on the results.

Mitotic segregation of plasmids. Mitotic stability and plasmid loss are defined as the percentage of plasmid-containing cells in a clonal transformant population grown under selective and nonselective conditions, respectively (55), the latter being best defined in a per-generation basis (loss rate). These values, which are correlated both at the theoretical and experimental levels (50, 55), are influenced by at least three variables: the efficiency with which a given ARS element acts as an origin of replication, the bias in mitotic segregation of the plasmid, and the number of divisions a cell can undergo after having lost its plasmid. Our plasmid vector (pIU) permits transformation of both yeast species with identical constructs. The plasmid has no centromere, since this function is not conserved cross-species. In the absence of a centromere, the segregation bias becomes a major parameter in the assessment of mitotic stability and plasmid loss. As a consequence, the percentage of plasmid-containing cells in a population becomes very low, even under selective conditions, particularly with the SAR-containing plasmids, making accurate kinetic measurements of loss rates virtually impossible. We therefore measured the above-defined values by plating equal dilutions of transformant cells grown in liquid culture for a defined number of generations on selective and nonselective plates in parallel. Replica plating of the nonselective plates yielded comparable results.

Sequence analysis. For the sequence comparisons we chose five scaffold-binding sequences each from *S. pombe*, *S. cerevisiae*, and *D. melanogaster* and grouped these as SARs. As non-SAR fragments, we used flanking regions of the identified SARs when sequence was available and the *URA3* gene and *LEU2* genes from *S. cerevisiae*, since these are shown in this study (and additional unpublished data) not

FIG. 1. (A) Endogenous ARS elements in *S. pombe* are attached to the nuclear scaffold. In panel A, four Southern blots of scaffold-bound (P) and scaffold-released (S) DNA are shown after probing with pARS767 (p767), pARS744 (p744), pARS772 (p772), or this last sequence plus the *nim1* gene of *S. pombe*. For each sample, 2.5 optical density at 260 nm U of isolated *S. pombe* nuclei was stabilized and extracted with LIS as described in Materials and Methods. The histone-free nuclei were digested to completion with combinations of restriction enzymes: *Eco*RI (Eco), *Hin*dIII (Hind), *Bam*HI (Bam), and *Hin*fI (Hinf) or *Eco*RI, *Hin*dIII, and *Mbo*I (last panel). Depending on the enzymes used, 60 to 85% of the genomic DNA was released. Bands containing ARS sequences are recovered primarily in the scaffold-bound fraction and are indicated by the filled triangles (size indicated in base pairs). Open squares represent *nim*1 fragments, and the closed circles indicate unidentified *S. pombe* fragments that apparently cross-hybridize with the *S. cerevisiae LEU2* gene. These provide a control for supernatant fragments. (B) *S. pombe* ARS sequences bind to nuclear scaffolds in vitro. The pFL20 plasmid or six M13 vectors carrying the *S. cerevisiae LEU2* gene and each a different *S. pombe* ARS were end labeled after digestion with *Eco*RI and *Hin*dIII (probes p747, p767, p766, and p744); *Eco*RI, *Hin*dIII, and *Xho*I for p745; *Pst*I, *Sal*I, *Eco*RI, and *Hin*dIII for pFL20; and *Acc*I, *Eco*RI, and *Hin*dIII for p772. To allow closer mapping of the binding site, the p744 probe was redigested with *Dpn*I and the p747 (T′, P′, S′) and p745 (P″, S″) probes were redigested with *Hin*fI. All were bound to *S. pombe* scaffolds during digestion with *Eco*RI, *Bam*HI, and *Hin*dIII. T, Total labeled probe; P, scaffold-bound DNA; S, nonbound DNA. The closed triangles indicate bands containing the ARS sequences recovered in the scaffold-bound fraction. Other yeast sequences are labeled as follows: L3′ or L5′ for the 3′ or 5′ halves of the *LEU2* gene, U for *URA3*, and S for the *S. pombe* stability locus on pFL20 (27). In lanes p745, p772, p767, and p744, the largest bands containing M13 sequence are not shown, but lanes p747, p766, and pFL20 show that neither pBR322 nor M13 phage sequences bind the *S. pombe* nuclear scaffold.

to bind nuclear scaffolds. The 15 SAR sequences covered 13.2 kb, and the 7 non-SARs came to 10.4 kb.

The exact sequences used are as follows. For dSARs the fragments were a 657-base-pair (bp) *Hin*fI-*Eco*RI fragment from the large histone repeat found upstream of the H1 gene, the 1.17-kb *Eco*RI fragment found 4.8 kb upstream of *ftz*, the *Eco*RI-*Bam*HI fragment of 757 bp immediately upstream of *Sgs-4*, the 970-bp *Bam*HI-*Xho*I fragment found between the two divergent *hsp70* genes at locus 87A7, and the 1.32-kb fragment upstream of the *Xho*I site within the promoter of the first of the three distal *hsp70* genes at the 87C1 locus. For *S. pombe*, SAR-ARS fragments were as follows: the 1.49-kb *Eco*RI-*Mbo*I fragment of pARS766 (Fig. 2), the 1.05-kb *Mbo*I fragment from pARS767, 490 bp from the flanking *Mbo*I site to the second *Acc*I site of pARS772 (see Fig. 2), the 1.2-kb *Eco*RI ARS fragment from pFL20 (43), and the 1.7-kb ARS fragment from pARS756 (45). For *S. cerevisiae* SARs, we have used the 843-bp *Hin*dIII-*Eco*RI fragment containing the ARS1, a 725-bp fragment containing the HMR-E silencer and ARS (1), the 120 bp of available sequence from the HO ARS (37), the minimal 160-bp *Sau*3A-*Rsa*I fragment containing the ARS element downstream of the histone H4 gene (5), and the 629-bp *Sau*3A fragment containing centromere III (11).

The control non-SAR DNA from *S. cerevisiae* includes the 614-bp *Eco*RI-*Hin*dIII fragment containing the *TRP1* gene, a 1.6-kb fragment containing copy 1 of the histone H3 and H4 genes, a 1.17-bp *Hin*dIII fragment containing the *URA3* gene (ARS consenses checked only on 970-bp *Pst*I-*Hin*dIII fragment), and 939 bp of the available sequence for the *LEU2* gene. From *D. melanogaster* we used the 1.4-kb *Eco*RI-*Hin*dIII fragment immediately downstream of the *ftz* SAR; 3.75 kb from the 87C1 locus of *hsp70*, containing the coding regions and spacer of *hsp70* genes; and a 944-bp fragment 3′ of the histone SAR, including the 5′ end of the histone H1 gene. All sequences were obtained from the EMBL sequence database.

Sequences were screened on both strands for the presence of perfect or one-error matches to the *S. cerevisiae* ARS consensus (8, 66), the *S. pombe* ARS consensus (45), the A box, and the two most frequent variants of the T-box: TTATTTTTT or TTTTATTATT (19). On the average, the SAR sequences are 71% A+T and the non-SARs used in this study were 59% A+T. The statistical occurrence of these sequences in random DNA that is either 71 or 59% A+T is given in the legend to Table 2.

RESULTS

ARS elements bind to the nuclear scaffold in *S. pombe*. In order to assess the generality of the observation that ARS elements are bound to the nuclear scaffold in budding yeast (4), we have tested ARS elements from the fission yeast *S. pombe* for association with nuclear scaffolds. To do this, we extracted histones and a subset of nonhistone proteins from isolated *S. pombe* nuclei with lithium diiodosalicylate and subsequently digested the genomic DNA with several restriction endonucleases. The nuclear scaffolds are easily sedimented and maintain interaction with genomic DNA. Between 15 and 40% of the genomic DNA sediments with the scaffold, depending on the number of enzymes used in the digestion. The association of specific sequences can be analyzed by comparative Southern hybridization to scaffold-bound (pellet) and scaffold-released (supernatant) DNA. Figure 1A shows that following digestion of genomic DNA with *Eco*RI, *Hin*dIII, *Bam*HI, and *Hin*fI, fragments of 632, 732, and 856 bp containing the *S. pombe* ARS elements ARS767, ARS744, and ARS772 (45), respectively, are recovered with the scaffold pellet. Because no flanking regions from these ARS elements were available, we also probed these blots and similar *Eco*RI-*Hin*dIII-*Mbo*I digests with the coding region of the *nim1* gene of *S. pombe* (61) in order to control for fragments in the unbound or supernatant fraction. This latter digestion gives two *nim1* gene fragments of 406 and 462 bp, both recovered in the supernatant (Fig. 1A, last panel). Here the ARS772 fragment is 1,160 bp and is again recovered in scaffold-bound DNA.

Selective binding of a given DNA fragment to nuclear scaffolds can also be demonstrated by incubation of scaffolds

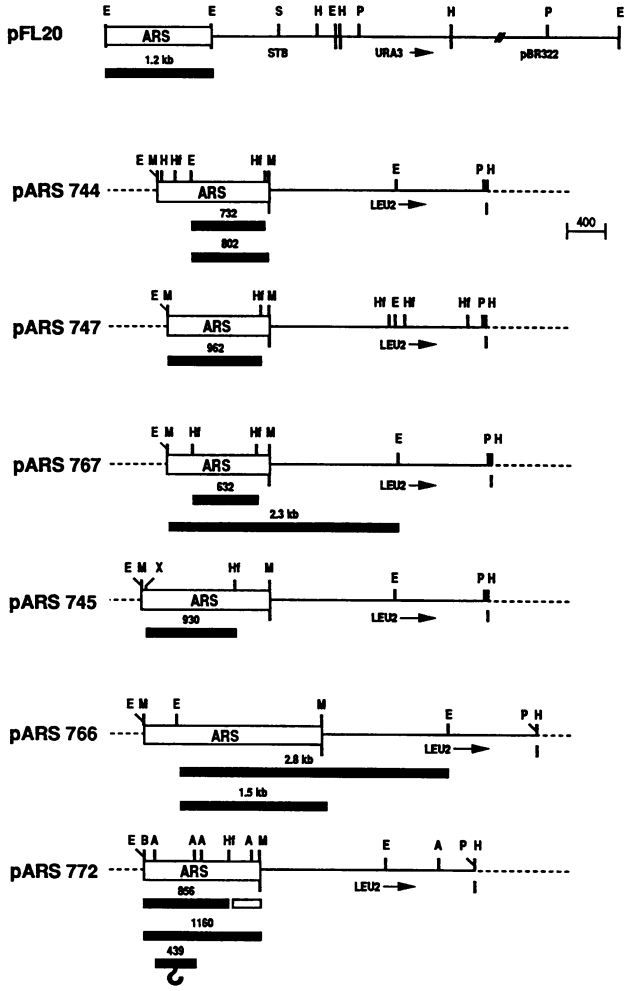


FIG. 2. Maps of *S. pombe* ARS plasmids and the position of scaffold-bound fragments. Schematic representations of the plasmid pFL20 (43) and the six ARS-containing M13 phages (pARS744, pARS745, pARS747, pARS767, pARS772, and pARS766; see reference 45) are shown here. Below the maps, the scaffold-bound fragments from either in vitro rebinding (Fig. 1B) or from genomic mapping (Fig. 1A) are indicated by filled bars (one with a hook). Sizes are indicated in base pairs or kilobases above the SAR. A 300-bp fragment from within the ARS that does not bind the scaffold is indicated by an open box. Restriction enzymes were abbreviated as follows: *Eco*RI (E), *Sal*I (S), *Hin*dIII (H), *Bam*HI (B), *Mbo*I (M), *Xho*I (X), *Hin*fI (Hf), *Pst*I (P), and *Acc*I (A). Only the sites relevant for the interpretation of the scaffold-binding data are given. The box indicates the original cloned ARS fragments. The dotted line indicates phage M13 sequences.

with an end-labeled probe containing different restriction fragments and nonspecific competitor DNA (12, 19, 35; see Materials and Methods). Generally it has been found that this assay for SAR binding in vitro accurately reflects the association of chromosomal DNA sequences with the nuclear scaffold. In addition, this assay allows the study of cross-species interactions and requires fewer nuclei. Figure 1B shows that the *S. pombe* ARS elements carried on the plasmids pARS747, pARS745, pARS766, pARS772, pARS767, and pARS744 (45) and the pFL20ARS (43) have a strong affinity for the nuclear scaffold of fission yeast ARS fragments (indicated by filled triangles). Neither the vector sequence, *URA3*, nor *LEU2* of *S. cerevisiae* binds the *S. pombe* scaffolds. This is not simply due to dilution of the vector by competitor DNA, since the ARS probes are in M13 and the competitor is pBR322. Moreover, neither *URA3* nor *LEU2* is present in the competitor and pBR322 end-labeled at *Hin*fI sites shows no scaffold association in the same rebinding assay (data not shown).

Due to the organization of the plasmids (see Fig. 2), the ARS-binding fragment sometimes contains part of the adjacent *LEU2* gene from *S. cerevisiae*. However, the 3′ part of the *LEU2* gene itself has no affinity for the scaffold (see fragments labeled *LEU2* 3′, Fig. 1B), and the 5′ region has only weak affinity, as evidenced by the weak scaffold association of the fragment labeled *LEU2* 5′ in the p772 binding (Fig. 1B). Flanking non-ARS sequences from *S. pombe* and the *S. pombe* stability sequences (STB) also do not bind to the *S. pombe* scaffold (see Fig. 1B). Figure 2 schematically shows the regions within the *S. pombe* ARS elements that we have mapped as scaffold bound.

***Drosophila* SARs bind nuclear scaffolds from *S. cerevisiae* and *S. pombe*.** Plasmids containing cloned genomic DNA inserts from the *ftz*, *hsp70*, *Sgs-4*, and the histone gene repeat loci from *D. melanogaster* were digested with the appropriate restriction enzymes, end labeled, and used as probes in an in vitro binding assay with scaffolds from *S. cerevisiae* (Fig. 3A) or *S. pombe* (Fig. 3B), as described previously (4; Materials and Methods). The same plasmids have been used previously to map SARs at these loci in *D. melanogaster* (19, 49), and relevant maps can be found in Fig. 3C. In our analysis using yeast nuclear scaffolds, only the dSAR-containing restriction fragments quantitatively partition with scaffold-bound DNA, while non-SAR fragments are recovered in the supernatant. With the exception of the 0.87-kb SAR fragment from the *Sgs-4* locus, the dSARs bind efficiently to yeast scaffolds (Fig. 3A and B). The *Sgs-4* SAR was shown to have a somewhat lower affinity for nuclear and chromosomal scaffolds from *Drosophila* and HeLa cells as well (35, 48).

ARS activity of dSARs in the two yeast species. The existence of specific SAR-scaffold interactions across species, as well as the similarities in sequence among yeast ARS-SAR and *D. melanogaster* SAR elements (4, 19; see below; Table 2), prompted us to test the ability of dSARs to promote autonomous replication of plasmids in yeast cells. The four dSAR fragments shown above and four flanking non-SAR fragments were subcloned in the plasmid pIU (see Materials and Methods and Fig. 3C). The *S. cerevisiae URA3* gene contained in this nonreplicating yeast vector allows complementation of *S. cerevisiae ura3* or *S. pombe ura4* (43) auxotrophic mutants. As controls in transformation experiments, we used plasmids containing known ARS elements or pIU alone.

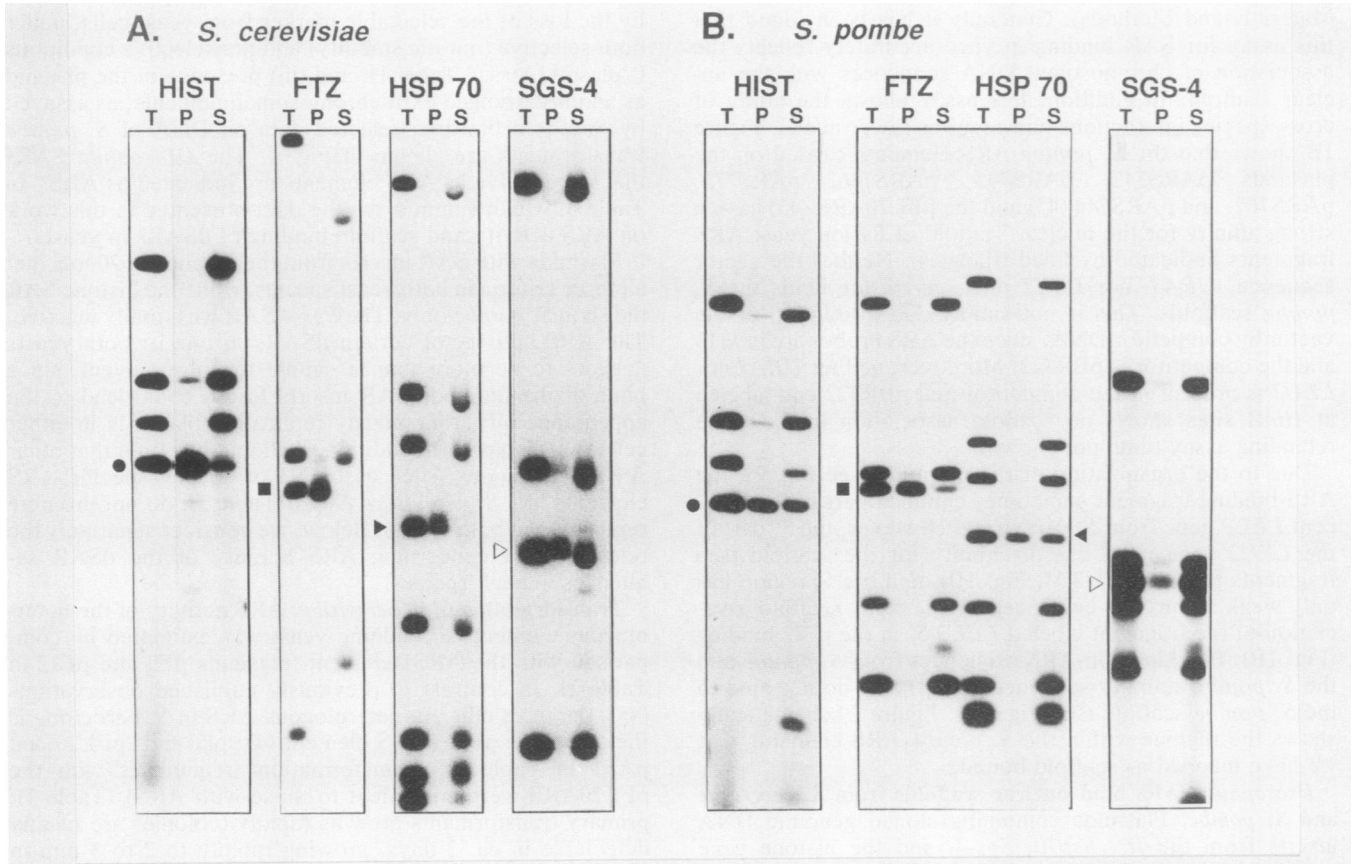
Three criteria were used to assign ARS activity to a DNA insert: (i) a high frequency of transformation (Table 1), expressed as the number of transformant colonies obtained with 1 μg of plasmid DNA; (ii) mitotic segregation, assayed by the loss of the selectable marker from yeast cells, under both selective (mitotic stability) and nonselective conditions ("plasmid loss," Table 1); and (iii) presence of the plasmid as an unrearranged extrachromosomal molecule, as assayed by Southern blotting; selected data on DNA of *S. pombe* transformants are shown in Fig. 4. The *Drosophila* SARs that were active as ARS elements are indicated as ARS^+ in Table 3, which summarizes the data presented in this work on ARS activity and scaffold binding of dSARs in yeasts.

Plasmids with SAR inserts from the *ftz* and *hsp70* loci met all three criteria in both yeast species, while the histone SAR did so in *S. pombe* only. The *Sgs-4* SAR was totally inactive. The ARS activity of certain dSARs in one or both yeasts appears to be more than a simple fortuitous event, since none of the four non-SAR inserts tested could lead to the appearance of autonomously replicating plasmids in either cell type (Table 1). In addition, it should be noted that other A+T-rich regions, such as CEN3 or species-specific ARS elements like *S. cerevisiae* ARS1 (Table 1), do not promote replication in both yeasts. Below, we consider separately the relevant data concerning ARS activity of the dSAR sequences in each species.

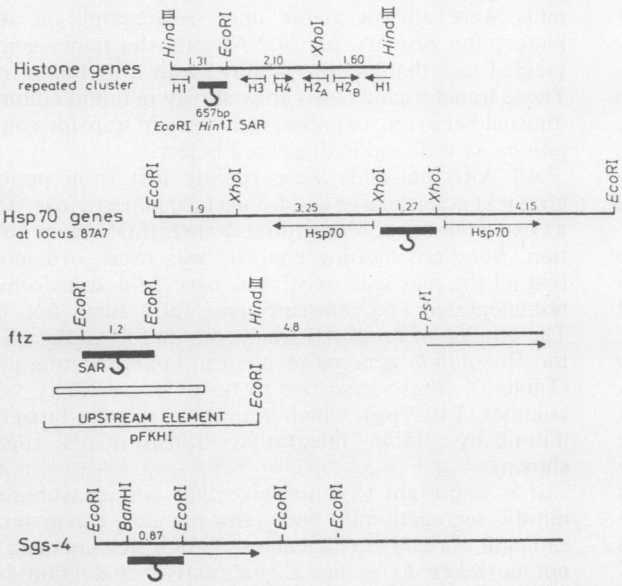
Transformation of *S. cerevisiae*. ARS activity of the heterologous elements in budding yeast was estimated by comparison with the ARS1 element (plasmids pF9 and pF12 in Table 1). In contrast to previously published observations (45), the most efficient heterologous ARS in *S. cerevisiae* is the *S. pombe* pFL20ARS element (43; plasmids pFL20 and pAS1 in Table 1). Transformation frequencies with the pFL20ARS were equivalent to those with ARS1 (Table 1); primary transformants grew as rapidly (colonies are readily detectable in ca. 2 days, growing rapidly to 2 to 3 mm in diameter), and generation times were similar (data not shown). The *ftz* and *hsp70* SARs also transformed *S. cerevisiae* with relatively high frequencies of about 1/2 and 1/10 those of the controls, respectively. The *hsp70* SAR, however, gave rise to very slow-growing colonies, which never reached large sizes (below 1 mm in diameter), even after prolonged incubation. While transformants with other plasmids were always viable upon restreaking on selective plates, the primary *hsp70* SAR transformants sometimes yielded less than 100% viability, with a minimum of 30%. These transformants also grew slowly in liquid culture. This unusual behavior was observed in *hsp70* transformants of *S. pombe* as well and is discussed below.

All ARS plasmids were rapidly lost from populations grown in nonselective conditions (see plasmid loss, Table 1), as expected for nonintegrative transformation. In confirmation, Southern blotting analysis was used to demonstrate that all the plasmids exist in *S. cerevisiae* transformants in nonintegrated and nonrearranged form (data not shown). The cloning of non-SAR fragments that flank the dSARs in the *Drosophila* genome resulted in nonreplicating plasmids (Table 1). These gave rise to no or occasionally very few colonies (1 to 3/μg), which in every case were fast-growing, mitotically stable integrative transformants (data not shown).

It is important to emphasize that our measurements of mitotic segregation (in both yeast species) can give only an estimate of relative efficiencies of ARS elements and should not be taken as a direct quantitative evaluation of ARS activity (see Materials and Methods). Our data on mitotic stability nonetheless clearly show significant differences in the levels of function of the various ARS elements identified



C.



Drosophila locus	inserted fragment	site in pIU	Orient.	Plasmid name
Histone repeat	HindIII-EcoRI SAR^k	NruI	B	pIUC11
			A	pIUC17
	XhoI-HindIII non-SAR^k	NruI	A	pIUD17
Hsp70 87A7	XhoI, SAR	SalI	B	pIU41
	XhoI, SAR+gene	SalI	A	pIU143
ftzUSE	EcoRI, SAR	EcoRI	A	pIUF122
			B	pIUF121
	EcoRI(-Hind) non-SAR	EcoRI	A	pIUF191
			B	pIUF192
Sgs-4	(Bam)-EcoRI SAR	EcoRI	A	pIUS82
	EcoRI (gene) non-SAR	EcoRI	A	pIUS167
	EcoRI 3'non-SAR	EcoRI	A	pIUS83

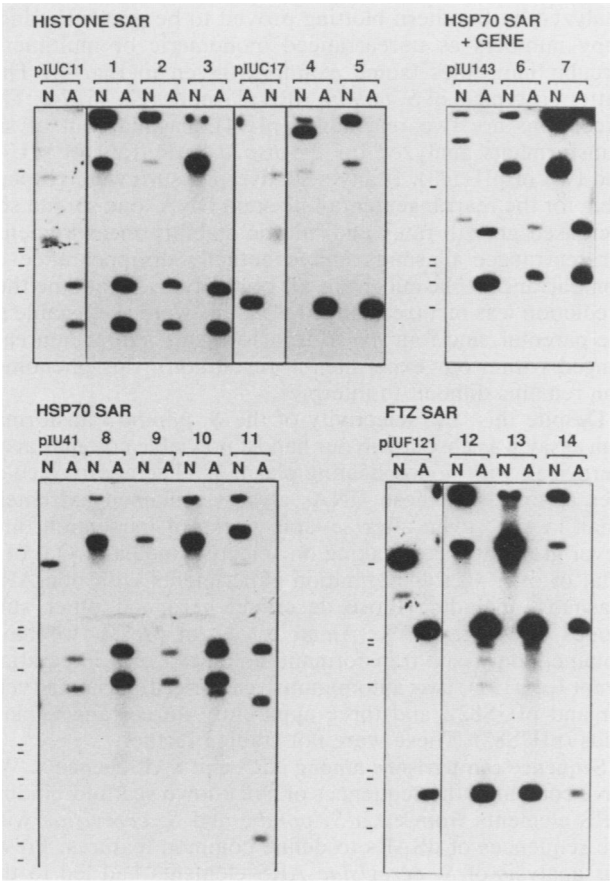


FIG. 4. Southern blot analysis of *S. pombe* transformant DNAs. About 50 pg of plasmid DNAs as markers (indicated by their names) and 0.1 to 0.5 μg of DNA from various *S. pombe* cells transformed with the same plasmids (numbered lanes to the right of each plasmid) were run on 0.8% agarose gels, transferred onto Nylon membranes, and hybridized to radiolabeled pIU (see Materials and Methods). Titles above the panels refer to the dSAR inserts of the plasmids. N, Undigested DNA; A, digested with *Ase*I (New England BioLabs, Inc.). The size markers indicated on the left are λ DNA cut with *Hin*dIII and *Eco*RI; sizes starting from the top are 21.4, 5.15, 5.0, 4.27, 3.48, 1.98, 1.9, 1.59, 1.37, 0.83, and 0.74 kb. No cross-hybridization of the probe to *S. pombe* DNA was detected at the stringency used (data not shown). Undigested genomic DNA runs slightly above the 21.3-kb marker. Undigested *S. pombe* transformant DNAs reveal two major forms of the plasmid, one of equivalent and one of much lower mobility than genomic DNA, as observed by others (27, 46, 73). Both forms are resolved upon digestion to the bands expected for monomeric or head-to-tail multimeric unrearranged plasmid, except for transformants no. 10 and 11 (see text). Unrearranged patterns were observed with over 45 ARS-plasmid transformants of *S. pombe* analyzed and were confirmed in several cases by digestion with other restriction enzymes. Copy numbers of the plasmids are high (above 10 to 50 as a lower estimate), as judged from comparison with parallel blots with *S. cerevisiae* transformant DNAs (data not shown) and from the relative intensities of the marker plasmids and transformant DNAs shown here. The additional bands of intermediate sizes observed in lanes 12 and 13, which are identical for all the transformants with each plasmid, are due to aberrant (star) activity of the enzyme used, a problem we have often encountered with *S. pombe* transformant DNA preparations.

(Table 1). The same relative hierarchy of ARS activity is seen in the transformation efficiencies and the growth rate of colonies, that is: ARS1 = pFL20ARS > *ftz* SAR > *hsp70* SAR. To provide more quantitative evidence for these differences, we have measured plasmid stability for ARS1 and the *ftz* SAR in a centromeric plasmid, which yielded values of 95 and 24%, respectively (see also B. Amati et al., EMBO J., in press).

Transformation of *S. pombe*. In the case of fission yeast, we used as control plasmids pFL20 and pAS1, which both contain the pFL20ARS element (43), and a sequence (STB) reported to confer increased mitotic stability to autonomously replicating plasmids (27). In contrast to what was seen in *S. cerevisiae*, transformation frequencies do not correlate with the replicative activity of given ARS elements (see Table 1). For example, the histone SAR (plasmids pIUC11 and pIUC17) transforms at somewhat higher frequencies than either the pFL20ARS or the *ftz* SAR but is clearly less efficient when evaluated in terms of mitotic stability. Additionally, the *ftz* SAR is strongly orientation dependent in terms of transformation efficiencies (compare plasmids pIUF121 and pIUF122), although both types of *ftz* SAR transformants display growth rates and mitotic stabilities equivalent to those of the pFL20ARS.

Simply on the basis of colony growth rate and mitotic stability, we can estimate relative ARS activity in *S. pombe* as pFL20ARS = *ftz* SAR > histone SAR > *hsp70* SAR. The percentages of plasmid-containing cells are generally higher in *S. pombe* than in the *S. cerevisiae* transformant popula-

FIG. 3. dSARs bind to nuclear scaffolds from both *S. cerevisiae* (A) and *S. pombe* (B). Plasmids containing the indicated *Drosophila* loci were restriction digested and end labeled as follows. HIST, Large histone gene repeat (see reference 49), digested with *Eco*RI-*Hin*dIII-*Xho*I; FTZ, *ftz* upstream region, plasmid pFKH2 (see reference 35), *Eco*RI-*Hin*dIII; HSP70, Plasmid 122X14 (22), *Xba*I-*Bam*HI-*Hin*DIII; SGS4, in panel A, plasmid pIUS82 (this work); in panel B, plasmid p3.8 (see reference 19), both *Eco*RI-*Bam*HI-*Hin*DIII. Note that in the latter, the 0.87-kb SAR comigrates with a 0.9-kb non-SAR fragment. Binding of the subcloned *Sgs-4* SAR alone to *S. pombe* scaffolds led to identical conclusions (data not shown). The probes (lanes T) were added to yeast nuclear scaffolds digested with either *Eco*RI, *Bam*HI, and *Hin*dIII (HIST, HSP70, SGS4, and FTZ in panel B) or *Eco*RI, *Hin*dIII, and *Pst*I (FTZ in panel A). Digestion of the *ftz* probe with *Pst*I shortens the uppermost pBR322 vector by roughly 670 bp. The resulting fragments are visible in the supernatant fraction but not in the total, since the total was loaded without *Pst*I digestion in this case. Binding and separation of scaffold-bound (lanes P) and soluble (lanes S) DNA were performed as described previously (4). The SAR fragments are 1.31-kb histone SAR (closed circles), 1.17-kb *ftz* SAR (closed squares), 1.27-kb *hsp70* SAR (closed triangles), and 0.87-kb *Sgs-4* SAR (open triangles). All adjacent non-SAR and vector fragments are recovered in the supernatants and are not schematically indicated. (C) Maps of the dSAR loci studied in this work, with a summary of the DNA fragments subcloned in the yeast nonreplicating vector pIU. The four *Drosophila* loci studied are shown schematically to the left (maps from reference 35). Scaffold attachment regions are indicated by the black hooked bar below the maps. The table lists the plasmids used for the ARS assay. The restriction enzymes used to generate the fragments inserted into pIU are given; parentheses indicate sites in the *Drosophila* fragments for which adjacent sites in the polylinker were actually used. [k], The fragment was filled in with Klenow polymerase prior to ligation. Note that insert orientation A runs clockwise and B runs counterclockwise related to the pBR322 map. Orientation A is defined from the standard maps of the loci (presented here) from which the inserts were derived.

TABLE 2. Sequence analysis of SAR and non-SAR sequences from yeasts and *D. melanogaster*[a]

Sequence	Frequency in[b]:		Ratio of occurrence[c]
	SARS (71% A+T)	Non-SARS (59% A+T)	SAR/ non-SAR
S. cerevisiae ARS (A/T)TTTAT(G/A)TTT(A/T)	0.53	0.05	10.6 (1.8)
S. pombe ARS (A/T)(A/G)TTTATTTA(A/T)	0.61	0.04	15.3 (2.6)
A box AATAAA(T/C)AAA	0.56	0.12	4.7 (0.8)
T box TTATTTTTTT or TT TTATTATT	0.74	0.08	9.3 (1.6)

[a] SAR and non-SAR fragments included in the sequence analysis come from *S. cerevisiae*, *S. pombe*, and *D. melanogaster* and are described in detail in Materials and Methods.

[b] The numbers are the frequencies of occurrence per 100 bp of the given sequence motif or the motif with a 1-bp error. The frequency at which they would be found in a random sequence with the base composition characteristic of either SARs (71%) or non-SARs (59%) is as follows: either ARS consensus or the T box variant would occur at 0.018/100 bp for 71% A+T and 0.003/100 bp in 59% A+T. The A box would occur at 0.013/100 bp and 0.003/100 bp in 71 and 59% A+T DNA, respectively. The values, even in non-SAR DNA, are thus higher than the frequency expected in random sequences.

[c] The first set of numbers indicate the ratios of occurrence in SAR versus non-SAR DNA. Numbers in parentheses are the same ratios, after normalizing the frequencies of occurrence to the frequency expected in random sequences of the same base composition.

TABLE 3. Summary of ARS activity and scaffold association of dSARs in yeasts

D. melanogaster SAR	Presence or absence of activity in:			
	S. cerevisiae		*S. pombe*	
	ARS	SAR	ARS	SAR
HIST	−	+	+	+
HSP70	±	+	±	+
FTZ	+	+	+	+
SGS4	−	+	−	±

tion (Table 1), in perfect agreement with previously published values (27, 45). Since this is observed under both selective and nonselective conditions, it implies that mitotic loss, and possibly unequal mitotic segregation, is less frequent in fission yeast.

The phenotypes of *hsp70* SAR transformants (plasmids pIU41 and pIU143) can be described in the same terms as for *S. cerevisiae*, i.e., frequent but very unstable, slow-growing transformants giving rise to very small and highly lethal sectored colonies. These are clearly not abortive transformants, however, as they can be restreaked and grown on selective media. The very poor growth characteristics of *hsp70* SAR transformants in both yeast species suggest that there might be a general counterselection for these plasmids at high copy numbers, perhaps due to their binding to and titration of an essential cellular protein. This could derive from the presence in the *hsp70* SAR insert of the entire *Drosophila hsp70* upstream regulatory region, which can bind the *S. cerevisiae* (and probably *S. pombe*) heat shock-responsive transcription factor. This protein, indeed, has been shown by gene disruption experiments to be essential for growth at all temperatures in *S. cerevisiae* (65) and is constitutively capable of binding its target site in vivo in budding yeast cells (64).

Almost all the transformant ARS plasmid DNAs we have analyzed by Southern blotting proved to be present at high copy numbers as unrearranged monomeric or multimeric circular molecules (some examples given in Fig. 4). This pattern is typical of *S. pombe* ARS plasmids (27, 45, 74). The exceptions are two rearranged pIU41 plasmids out of six transformants analyzed for the *hsp70* SAR (four of pIU41 and two of pIU143). If any selective pressure were responsible for the rearrangement of plasmid DNA, one should see increased growth rates and mitotic stability measurements for rearranged plasmids and eventually disappearance of nonrearranged plasmid from all populations. Since neither prediction was met (rearranged plasmids were as unstable as the parental, and four *hsp70* transformants still are unrearranged within our experimental resolution), this phenomenon remains difficult to interpret.

Despite this, the selectivity of the *S. pombe* transformation assay was absolute in our hands: no viable colonies at all were seen with nonreplicating plasmids. The abortive colonies arising with these DNAs always remained extremely small in size, even after several weeks of incubation, and never grew after restreaking on selective medium. Out of a total of over 40 transformation experiments with non-ARS plasmids, including those described here and other subclones of the *ftz* SAR (Amati et al., in press), we have obtained only six transformant colonies: one integration event (pIU191), two autonomous rearranged plasmids (vector and pIUS82), and three apparently unrearranged plasmids (pIUS82). These were not studied further.

Sequence comparisons among ARS and SAR elements. We have compared the sequences of five known scaffold-binding ARS elements from each *S. pombe* and *S. cerevisiae* with the sequences of dSARs to define common features. Previous analysis of *S. cerevisiae* ARS elements had led to the definition of an 11-bp ARS consensus sequence, 5′-(A/T)TTTATPuTTT(A/T)-3′ (8, 66), which has been clearly implicated in ARS function through mutagenesis experiments (reviewed in reference 53). Inspection of the dSARs studied here reveals that two (*ftz* and histone SARs) contain a perfect match to the ARS consensus sequence, while two others (*hsp70* and *Sgs-4*) do not. One dSAR with a perfect ARS consensus and one without are able to promote plasmid replication (see Table 3).

A more general search for the occurrence of 10 of 11 or 11 of 11 matches to the *S. cerevisiae* ARS consensus in 15 SAR and 7 non-SAR fragments shows a 10-fold higher frequency of the ARS consensus per unit length in SAR versus non-SAR DNA (Table 2). This is not simply a reflection of the higher A+T content of SAR fragments (ca. 71%) over these non-SAR DNA (ca. 59%; see the legend to Table 2), which could lead at most to a fivefold enrichment on a random statistical basis. Strikingly, we find 10 perfect matches to the ARS consensus among SARs and none among non-SARs.

Comparison of the nine sequenced ARS elements of *S. pombe* revealed the presence of an 11-bp conserved consensus 5′-(A/T)(A/G)TTTATTTA(A/T)-3′ (45). Despite a superficial similarity to the *S. cerevisiae* consensus, it differs from it both in sequence and by the fact that its deletion does not systematically eliminate ARS activity (45). Perfect and near-perfect (10 of 11) *S. pombe* consenses were found with a 15-fold enrichment in SAR versus non-SAR elements (Table 2). If all functional ARS elements (including dSARs) are compared with nonfunctional sequences, rather than SARs versus non-SARs, a weaker enrichment is seen (sevenfold; data not shown). Both numbers are significantly above the enrichment expected from the difference in percentage of A+T.

Two sequence motifs were previously defined by a general matrix comparison of a number of dSAR sequences: the so called A box, 5′-AATAAA(T/C)AAA-3′, and the T-box, 5′-TT(A/T)T(T/A)TT(A/T)T-3′ (19). Again, both of these can be found in yeast ARS regions (4); the relative frequency of A and T boxes in SARs versus non-SAR regions is given in Table 2. The A box is roughly five times more abundant in SARs than in non-SAR fragments, which corresponds to the enrichment expected on a random statistical basis. Although its occurrence as repeated clusters within SARs speaks to the contrary, this may mean that the A box is not a relevant diagnostic feature of SARs.

The T box, on the other hand, is ninefold more abundant in SARs versus non-SAR fragments, nearly twice the enrichment one might expect on the basis of base composition. A third motif found in SARs of many species in the putative consensus sequence for cleavage by *Drosophila* topoisomerase II (19). This was omitted from our analysis here, since it is fairly degenerate and does not coincide with preferred cleavage sites of *S. cerevisiae* topoisomerase II (R. Walter and S.M.G., unpublished data).

DISCUSSION

ARS elements and nuclear scaffold binding in *S. pombe*. We demonstrate in this work that as seen previously in *S. cerevisiae* (4), *S. pombe* ARS elements have a selective affinity for the nuclear scaffold. The scaffold association of ARS elements is thus not unique to budding yeast. We find the observation particularly significant because *S. cerevisiae* and *S. pombe* have evolved separately since very early phylogenetic times (e.g., see reference 32). This divergence is characterized by many molecular and physiological differences in the life cycles of the two yeasts, including different sequence requirements for the replication of extrachromosomal plasmids. Indeed, only a minority of the *S. pombe* ARS elements have ARS function in *S. cerevisiae*, and those *S. pombe* sequences which promote replication in budding yeast are not necessarily ARS elements in *S. pombe* (45, 46). Similarly, the ARS1 of *S. cerevisiae* is not functional as an ARS in *S. pombe*.

Due to a low level of transformation with non-ARS plasmids, earlier reports suggested that *S. pombe* might permit autonomous replication of virtually any plasmid DNA (23, 27, 46, 74). Variable transformation efficiencies and various forms of the plasmid DNA in *S. pombe* cells were reported (integrated, autonomous, with or without rearrangements). In contrast, we observe a clear-cut ARS-dependent transformation of *S. pombe*, autonomous replication of extrachromosomal plasmids in every transformant, and very rare rearrangement events restricted to the weakest ARS plasmid (pIU41). Our results are very similar to those of Maundrell et al. (45), who have performed deletion studies on eight *S. pombe* ARS elements, finding clear differences between ARS^+ and ARS^- plasmids. Our work demonstrates a requirement for ARS elements for efficient plasmid maintenance in *S. pombe* nearly identical to that observed in *S. cerevisiae*. It remains to be tested by two-dimensional gel electrophoresis whether or not *S. pombe* ARS elements themselves are origins of replication in vivo.

Nuclear scaffold-DNA interactions across species. Four previously identified dSARs bind selectively to nuclear scaffolds from both budding and fission yeasts. Non-SAR fragments from the respective *Drosophila* loci do not show this tight interaction with yeast nuclear scaffolds. In the accompanying paper (9), Brun et al. show that 13 of 16 dSARs tested also bind nuclear scaffolds from *S. cerevisiae*, while non-SAR regions do not. These observations extend the previously reported cross-species interactions to a more significant number. We have also observed selective binding of four *S. pombe* ARS elements (ARS745, ARS747, ARS772, and pFL20ARS) to nuclear scaffolds from HeLa cells (data not shown) and find that similar regions mediate the association to the scaffolds in both systems. Although cross-species interactions are not conserved in all possible combinations (9, 48), the available data, together with the general sequence similarities among ARS-SAR elements, suggest that SAR-scaffold interactions are mediated by similar molecular features in all species. In view of the apparent lack of histone H1 (reviewed in reference 57) and a nuclear lamina-like structure in *S. cerevisiae* (11a), it is remarkable that scaffold-binding sites appear to be a conserved element of chromosomal organization.

Replication activity of dSARs in yeasts. We reasoned that the evolutionary conservation in binding specificity of the four dSARs might be paralleled by a conservation of ARS function in yeasts, which we have shown for two of the dSARs in *S. cerevisiae* and three in *S. pombe*. The non-SAR sequences tested are completely inactive. Our sample number being small, these observations could admittedly represent purely serendipitous events. However, the study of 85 SAR fragments that were mapped over an 800-kb segment of the *Drosophila* first chromosome demonstrates a statistically significant enrichment of ARS elements among dSAR sequences (9, 66a). A total of 19 of 54 SARs (binding >50% in *D. melanogaster*), but only 2 of 35 non-SAR fragments, have ARS activity in *S. cerevisiae*. Together with our data, we can define a significant subclass (40%) of dSARs having ARS activity in yeasts, while few non-SAR fragments do (5%).

Two earlier reports present data on heterologous ARSs consistent with the comapping of ARSs and SARs. By using synchronized HeLa cells, Aguinaga et al. (3) isolated a matrix-bound DNA fraction apparently enriched in ARS elements. Similarly, Sykes et al. (67) have identified a fragment from the human *HPRT* locus that both replicates in *S. cerevisiae* and binds with high affinity to nuclear matrices from several mammalian cell types.

The cloning of heterologous DNA with ARS function in *S. cerevisiae* has been an approach used by many laboratories in recent years, leading to an impressive list of heterologous ARSs, including sequences from 23 different eucaryotic organisms, two animal viruses, and a bacterium (reviewed in reference 53). The results from such studies suggest that functional ARS elements can be found in virtually all eucaryotic chromosomal DNAs at roughly the frequencies expected for origins of replication. However, they also provide clear evidence that most heterologous ARS elements in yeasts are not a priori likely to be origins of replication in their respective organism.

What then is the biological significance of the observed correlation between scaffold binding and ARS activity? At the very least, it might derive from a fortuitous similarity of the relatively A+T-rich DNA sequences necessary to constitute functional ARS and detectable SAR elements. These elements, however, are not composed of totally random sequences, and closer inspection reveals that the certain sequences characteristic of ARSs are generally encountered in higher eucaryotic SARs and vice-versa (see Table 2). For instance, both the *S. pombe* and the *S. cerevisiae* ARS consenses are enriched in eucaryotic SARs. Mutagenesis and reversion studies confirm the importance of the ARS consensus for autonomous replication in *S. cerevisiae* (39,

53), although in some cases partial matches (10 out of 11) to the consensus are sufficient (HO ARS, see reference 38; *hsp70* SAR, this work; 39). Further evidence for the importance of this conserved consensus is the purification of a protein from the scaffold fraction that recognizes the *S. cerevisiae* ARS consensus (J. Hofmann and S.M.G., unpublished data).

Possible roles for DNA conformation in SARs and ARS elements. It is noteworthy that the two conserved yeast ARS consenses contain runs of oligo(dT). Indeed, oligo(dT) stretches also characterize the third motif that we find consistently enriched in SAR versus non-SAR DNA, i.e., the T box. Runs of thymidine residues are known to confer a distinct narrow minor groove structure to the DNA double helix (51). Recent work by Laemmli and coworkers has experimentally implicated oligo(dA) · (dT) stretches in the selective binding of dSARs to *Drosophila* nuclear scaffolds and of artificially constructed polymers to two purified proteins, histone H1 and topoisomerase II (2, 34, 36). Deletion studies of the *ftz* SAR in our own laboratory (Amati et al., in press) suggest that binding to the yeast scaffold correlates best with this motif as well. The extended flanking regions of A+T-rich DNA that are characteristic of functional ARS elements may contribute a characteristic secondary structure, such as the narrow minor groove, that could participate in the assembly of initiation complexes rather than providing strictly sequence-specific protein-binding sites.

The repetition of oligo(dT) stretches spaced at intervals of 10 to 11 nucleotides along the DNA helix can also induce DNA bending. Bent DNA has been detected experimentally in six SARs of *D. melanogaster* (29) and in the human *HPRT* ARS-matrix attachment region MAR (67) and several yeast ARS regions (63, 72). Stretches of more than 50 bp of bent DNA are also found in the two regions mapped by two-dimensional gel electrophoresis as origins of replication in the *Drosophila* chorion locus (26). While the bent region appears to enhance ARS efficiency (72), DNA containing oligo(dT) stretches that are not bent bind efficiently in vitro to both *Drosophila* and yeast scaffolds (Amati et al., in press). Thus, other studies (2, 36) as well as our own suggest that the more significant common feature shared by ARS and SAR sequences may be the narrow minor groove rather than the bending of the DNA helix.

Are SARs chromosomal origins of replication? At least three ARS elements in *S. cerevisiae* are known to function as origins of replication (6, 7, 30, 31, 42; for reviews, see references 53 and 69). The fact that ARSs are essential for the maintenance of a circular derivative of chromosome III suggests that there are no cryptic non-ARS origins in yeast chromosomes (54). Here we show that the frequency of heterologous ARS elements from *Drosophila* genomic DNA is much greater among SAR than among non-SAR fragments. It seems unlikely that DNA elements with the structural and functional properties common to SARs and ARS elements would have evolved purely by chance in eucaryotic genomes without having specific functions. Thus, one is led to question whether SARs, or at least some of them, may be chromosomal origins of replication in higher eucaryotic cells.

The idea of an association of replication origins with a karyoskeletal structure in vivo is not new (16, 56; reviewed in references 52 and 70), but it is also controversial. Other models propose that origins are located away from the skeleton in chromatin loops and attach transiently upon initiation of replication (e.g., see reference 47). Our assay for scaffold attachment may not be able to differentiate between permanent and transient association if the transient binding depends on soluble inhibitors that block the site in vivo. The previous evidence in favor of the permanent attachment of replication origins is based on (i) the correlation between the size of the topologically constrained looped domains in chromosomes or nuclei and the estimated size of replicons (10) and (ii) pulse-labeled studies with tritiated thymidine during S phase that suggest that replication origins (identified as the early replicating fraction) may be permanently attached to the high-salt-extracted matrix. Late-replicating regions, which can be chased in vivo to the soluble fraction, appear to be transiently attached during replication (15). This is generally consistent with our data.

The work of Razin et al. (59) also lends support to the idea that origins are scaffold or matrix attached. These authors isolated active replication origins (oriDNA) from a chicken erythroblast cell line by using a method developed by Zannis-Hadjopoulos et al. (75). With hybridization and renaturation studies, they have demonstrated that within an error size margin of 5 kb, all oriDNA sequences were contained in an independently isolated nuclear matrix DNA fraction. Since nuclear matrix DNA itself was only a subset of total (unique) DNA, this observation suggested that all of the oriDNA fraction was recovered on the matrix.

One of the mammalian origins mapped to date is found in the amplified *DHFR* domain of the methothrexate-resistant CHOC 400 cell line. This has been initially mapped downstream of the *DHFR* gene to a 28-kb region containing two origins, spaced by ca. 22 kb (41). By measuring the direction of replication fork movement, Handeli et al. (25) have confirmed this arrangement and have proposed that a replication termination site is roughly centered between the two origins. Dijkwel et al. (14) have mapped a SAR in a 3.4-kb fragment which coincides with the termination site. At a functional level, it is not yet known whether or not the SAR contributes to either origin or termination function in this region.

The identification of the protein(s) binding to SARs and demonstration of their role in DNA replication will address the role of SARs as origins directly. Other approaches, such as transient transfection experiments with cultured *Drosophila* cells using SAR-containing plasmids, have failed to reveal any ARS activity (data not shown). The same negative result has been obtained for other heterologous yeast ARSs from *Drosophila* DNA (40). Since origin activity in these cells may require a particular chromosomal context, the most direct means of testing whether SARs function as origins of replication will be by biochemical mapping in *Drosophila* genomic DNA with either the recently developed two-dimensional electrophoretic methods (6, 30) or other available techniques (25, 75).

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