rar mutations which increase artificial chromosome stability in *Saccharomyces cerevisiae* identify transcription and recombination proteins

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Received February 8, 1991; Revised and Accepted March 7, 1991 GenBank accession no. M36725

ABSTRACT

In an attempt to identify *trans*-acting factors involved in replication origin function, we have characterized the *RAR3* and *RAR5* genes, identified by mutations which increase the mitotic stability of artificial chromosomes whose replication is dependent on the activity of weak ARS elements. Sequence analysis has shown that the *RAR3* gene is identical to *GAL11/SPT13*, which encodes a putative transcription factor involved in the expression of a wide range of genes. Change-of-function mutations that truncate the RAR3 protein appear to be required to enhance chromosome stability. In contrast, loss of the RAR5 protein results in enhanced chromosome stability, as if the protein is an inhibitor of ARS function. The *RAR5* gene encodes the 175 kDa DNA strand transfer protein β, an activity that can promote the transfer of a strand from a double-stranded DNA molecule to a complementary single strand. This observation implies that a presumed recombination activity can affect eukaryotic chromosomal replication.

INTRODUCTION

Initiating chromosomal replication is a key event in the eukaryotic cell cycle, and although generally regarded as a major regulatory step little is known of the biochemical events involved. Considerable progress has been made in the analysis of *in vitro* systems derived from mammalian cells that replicate eukaryotic viral DNA, and these have allowed the identification of host factors that are involved in chromosomal replication (1, 2, 3). In addition, a system based upon *Xenopus* egg extracts is becoming amenable to biochemical dissection (4).

As an adjunct to these studies using vertebrate cell extracts, a combination of genetic and biochemical analyses of the yeasts *S. cerevisiae* and *S. pombe* is making an important contribution to an understanding of the regulation and mechanism of S phase. Key proteins which appear to regulate the entry into S phase have been identified, and in addition a number of proteins involved in the elongation step of DNA replication have been biochemically and genetically characterized (5, 6). In addition, genetic assays in both yeasts have allowed the identification of ARS elements which have the genetic properties of origins of replication and, in *S. cerevisiae*, can correspond to sites of replication initiation (6, 7). By analogy with findings on eukaryotic viral and prokaryotic DNA replication, it is generally assumed that these ARS elements are recognized by initiator proteins that allow the assembly of proteins involved in the elongation phase of chromosome replication. So far, such key initiator proteins have not been identified, but a number of proteins that bind ARS-containing DNA fragments have been characterized, and these may have some influence on initiation. For example, the ABF1 factor binds a sequence present near some but not all ARS elements, and deletion of this binding site can reduce but does not abolish ARS activity. (8, 9, 10, 11, 12, 13, 14, 15). ABF1 appears related to another yeast protein, RAP1 (GRF1) (16); both proteins appear to be involved in various aspects of transcriptional regulation (8, 11, 17, 18, 19, 20, 21).

ARS elements have also been used in genetic strategies designed to identify factors influencing replication initiation in *S. cerevisiae*. Thus work by Tye and colleagues has identified a series of *MCM* genes that were identified by mutations which cause defects in plasmid replication or segregation (22, 23). The products of the *MCM1*, *2* and *3* genes appear to affect specifically ARS function (22, 23, 24). The *MCM1* gene encodes the transcription factor PRTF (25) but it is unclear whether changes in the activity of this protein affect replication directly or via a more circuitous route. In addition, Thrash-Bingham and Fangman (26) have shown that mutations in the *TUP1* gene result in a number of pleiotropic phenotypes including the stabilization of plasmids containing defective replication origins. The biochemical function of the TUP1 protein in unclear, but mutations in the *TUP1* gene can affect the transcription of a number of genes (27, 28). We have also isolated a series of mutations that appear to affect ARS function (29). These *rar* mutations have the phenotype of increasing the mitotic stability of certain weak ARS plasmids, as if the mutations in some way increase the probability that a defective ARS will function in S phase, perhaps by some change that affects the initiation mechanism directly.

We report here the genetic and molecular characterization of two *RAR* genes, *RAR3* and *RAR5*. The *RAR3* gene is identical

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to *GAL11/SPT13* and encodes a transcription factor required for the expression of a wide variety of yeast genes. The *RAR5* gene potentially behaves as a repressor of replication origin function, and encodes an activity that is presumed to be involved in DNA recombination.

MATERIALS AND METHODS

Plasmids

pF3 and pSE321 were isolated from a yeast library constructed in the cosmid pBT1-1 (29). pSE360 was derived from pSE321 by *Sal*I digestion, and contains a 9.2kb *Sal*I genomic fragment encompassing the *RAR3* gene. The structures of various subclones of pF3 in the vector YCp50 (30) are shown in Figure 1. pDK1 was constructed by inserting the 115bp *Hin*dIII-*Eco*RI fragment of plasmid M13-j48 (31), containing the inactive 1048 allele of the *HO* ARS, into the *Sal*I site of pSE296. pL55 and pSE375 were derived from pSE276-2 and pSE276-1 by deleting the *URA3* fragment. pDK427 was constructed by inserting the *URA3* gene into M13-j8 (*HO* deletion mutation; 31).

Other plasmids have been previously described: pSE276-1, pSE276-2, pSE295 (29); pSE296, pDK296-A17, pDK296-B12, pDK296-HO, pDK296-i56HO (32); pDK12 (33); M13se102 (34).

Nucleic acid methods

Nucleic acid methods were as described (35, 36).

Transposon insertion mutagenesis of pF4 using transposon Tn10LK, and pSE360 using Tn10LUK, was performed as described (37). pF4::Tn10LK mutant plasmids were introduced into the *rar5* strain R795-4A[pL55] to assess whether the plasmid stability phenotype was complemented. pSE360::Tn10LUK mutant plasmids were linearized with *Bam*HI and integrated into the *rar3* strain R742-1C[pSE375] to determine whether the *RAR3* gene was functional.

DNA was sequenced by the M13/dideoxynucleotide chain termination method using Klenow polymerase following cloning of random fragments obtained by sonication (38). DNA sequence analysis used the programs of Staden (39). The National Biomedical Research Foundation protein database (version 26), Swissprot protein database (version 14), and a translation of the EMBL nucleic acid database (version 24) were searched using FASTA and TFASTA programs (40). Software was implemented on the SERC SEQNET facility (Daresbury, UK).

Cloning the *RAR3* gene

A yeast gene library in the *LEU2*-containing cosmid vector pBT1-1 (29) was transformed into spheroplasts of a *rar3-1* strain (R742-1C). After incubation for 3 days at 30°C, Leu$^+$ transformants were extracted by macerating the top agar, allowing cells to be spread onto selective plates. The plates were replica plated onto YPD plates, which were incubated at 55°C for 150 minutes, then for 3 days at 30°C. Colonies showing growth were rechecked for heat-shock resistance. Heat-shock resistant transformants were transformed with the indicator plasmid pSE276-1, and this allowed the identification of a transformant where complementation of both the plasmid-stability and the heat-shock phenotypes was dependent on the *LEU2* plasmid. Plasmid pSE321, containing a 13.4kb insert, was isolated from this transformant.

Cloning the *RAR5* gene

Spheroplasts of the *rar5-1* strain R795-4A were transformed with a pBT1-1-based genomic library (29). Approximately 2,000 Leu$^+$ primary transformants were divided into 10 pools. Cells from each pool were used to inoculate a 10ml culture selecting for leucine prototrophy, and this culture was transformed with pSE276-2 using the lithium acetate method, yielding approximately 300−600 Leu$^+$ Ura$^+$ secondary transformants. These were screened for complementation of the Rar$^-$ phenotype by streaking on plates (lacking leucine and containing 10mg/l adenine) which select for the cosmid while allowing loss and visualization of the indicator minichromosome. This screen, of roughly 2 genome equivalents, yielded a single plasmid (pF3) that could complement the plasmid stability phenotype.

Yeast genetic methods

General growth media and genetic methods were as described (36). *Saccharomyces cerevisiae* strains are detailed in Table 1. Note that in future publications we shall rename *rar3* alleles as *gal11-3*; thus *rar3-1* becomes *gal11-31*. Yeasts were transformed using the sorbitol or lithium acetate (41) methods.

Disrupted versions of *RAR* genes were constructed by one-step gene disruption (42). Strains carrying a disrupted *RAR3* gene were constructed from pSE360::Tn10LUK plasmids. A *Bam*HI-*Sal*I restriction fragment containing the *RAR3* gene, disrupted by the Tn10LUK transposon, was used to transform a haploid yeast strain (W303-1B or R742-1C), selecting for complementation of the *ura3* marker. The predicted gene rearrangement was confirmed by Southern analysis (not shown). The transposons used disrupt the 964 codon *GAL11*(*RAR3*) reading frame at the following approximate positions: *rar3-38::URA3*, codon 350; *rar3-45::URA3*, codon 750 (coordinates from reference 43). Strains containing the *rar5::URA3* disruption were constructed by inserting the *URA3* gene into the reading frame. The 8.2kb

Table 1. Yeast strains used in this study

Strain	Genotype	Source or reference[a]
W303-1A	**a** *ade2-1 trp1-1 can1-100 leu2-3,112 ura3 his3-11,15*	*29*
W303-1B	α (other markers as in W303-1A)	29
R106	**a**/α (W303-1A×W303-1B)	
R199-1	α *rar3-1* (other markers as in W303-1B)	74
R340-5	**a** *rar3-2 trp5 his3 ura3 ade2-1*	74
R702	α *rar5-2* (other markers as in W303-1B)	
R703-2	α *rar5-3* (other markers as in W303-1B)	
R742	**a**/α (R199-1×W303-1A)	
R742-1C	α *rar3-1 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*	
R786-7A	α *rar5-4* (other markers as in W303-1B)	
R792-7C	**a** *rar5-7 ade2-1 his3 trp5 can1 ura3*	
R794-2C	α *rar5-5* (other markers as in W303-1B)	
R795-4A	α *rar5-1* (other markers as in W303-1B)	
R850	α *rar5-6* (other markers as in W303-1B)	
R892-1-5C	α *rar3-38::URA3* (other markers as in W303-1A)	
R893-7-1C	α *rar3-45::URA3* (other markers as in W303-1A)	
R900	α *rar3-38::URA3* (derived from R742-1C)	
DKY119	**a** *rar5::URA3* (other markers as in W303-1A)	
DKY120	**a**/α *RAR5/rar5::URA3* (other markers as in R106)	

[a]If none given, reference is this work.

*Bam*HI-*Xho*I fragment of pF4 was cloned into pUC8 to yield pDK513. The *URA3* gene was then inserted into the unique *Sac*I site of this plasmid, and the insert of the resulting plasmid (pDK522) was used to transform wild-type strains W303-1A and R106 to Ura$^+$, yielding strains DKY119 and DKY120 respectively. Integration at the *RAR5* locus was confirmed by Southern analysis (not shown). Growth rates of strains were determined by monitoring the OD_{600} of YPD cultures, which were scored as (+) if not significantly different from the wild-type rate and (−) if slower growing.

Measurement of plasmid stability and ARS function

The mitotic loss rates of centric plasmids were determined by quantitating plasmid loss rates during growth in rich (YPD) medium (32), and is given as loss rate per cell per generation. Complementation of the *rar* mutations was determined by monitoring the sectoring phenotype of colonies originally containing plasmids pSE276-1 or pSE276-2 on non-selective media as described previously (29). The ARS activity of acentric plasmids was determined by transformation (31); plasmids were defined as Ars$^+$ if transformants grew on selective medium and autonomous replication of unrearranged plasmid was detected by Southern analysis.

Scoring of Gal phenotype

Ability of strains to ferment galactose was assessed by replica plating onto EBGal plates (44). UDP-glucose-4-epimerase (*GAL10*) activity was assayed as described, using the two-step method (43, 45).

RESULTS

Isolation of *rar5* mutations

We obtained a further 12 independent Rar$^-$ mutants using plasmid pSE276-2 (Table 2), following the procedure previously described, using a sectoring assay to detect mutants showing enhanced mitotic stability of the minichromosome (29). The replication of pSE276-2 is dependent upon the weak ARS function associated with the *CEN3* element on the plasmid, and the plasmid is lost rapidly in wild-type strains grown under non-selective conditions. Seven mutants failed to complement each other, and since these mutants complement *rar1*, *2*, *3*, and *rar4* tester strains, they are assigned to a new complementation group, *rar5*. Of the remaining mutants, one failed to complement a *rar4* mutation, whereas the others complement all other *rar* mutations and have not been characterized further. The *rar5* mutations segregate 2$^+$:2$^-$ in backcrosses.

Phenotypic analysis of *rar3* and *rar5* mutations

We chose to analyze further mutations in the *rar3* and *rar5* complementation groups. This choice was based on pleiotropic phenotypes of *rar3* mutations that have facilitated subsequent genetic analysis (see below), and the observation that most mutations isolated with the pSE276-2 plasmid specifically identify the *RAR5* gene. A comparison of relative plasmid stabilities in wild-type, *rar3-1* and *rar5-1* strains is shown in Table 2. Both *rar3-1* and *rar5-1* mutations affect the mitotic stabilities of a range of weak ARS plasmids, although the magnitude of the effect varies between plasmids. The enhancement of plasmid stability appears to be independent of the vector system used or the other genetic elements (eg. *CEN* elements) present on the minichromosome. We also noticed that one acentric plasmid (pDK427) that is Ars$^-$ in a wild-type strain is capable of autonomous replication, albeit poorly, in the *rar5-7* mutant, implying that the phenotype is not dependent on the inclusion of a centromere in the plasmid (Table 2).

Cloning the *RAR3* and *RAR5* genes

Since the plasmid-stabilization phenotype of Rar$^-$ mutants is difficult to select against, we screened the mutants for another phenotype that would be useful for isolating the wild-type genes by complementation. Both the original *rar3* alleles were shown to confer sensitivity to heat-shock, and this phenotype co-segregates with the plasmid-stabilization phenotype in tetrad analysis, implying that both phenotypes are caused by the same mutation (not shown). We isolated the *RAR3* gene by transforming a *rar3-1* mutant with a *S. cerevisiae* gene library, constructed using a *LEU2*-containing cosmid, and selecting for resistance to heat-shock. This identified a transformant where the plasmid-stabilization phenotype was also complemented, and a cosmid (pSE321) isolated from this strain could complement the *rar3-1* phenotypes when reintroduced into the mutant.

We were unable to detect any convenient phenotypes of *rar5* mutants. Therefore, the *RAR5* gene was isolated by screening for complementation of the recessive plasmid-stabilization phenotype of the *rar5-1* mutation, following transformation with

Table 2. Plasmid loss rates in *rar3-1*, *rar5-1* and wild-type strains

Plasmid	Genotype	Plasmid loss rate in rich medium		
		Wild-type (W303-1A)	*rar3-1* (R742-1C)	*rar5-1* (R795-4A)
M13se102	M13mp9 *URA3*	Ars$^-$	Ars$^-$	Ars$^-$
pSE296	pBR322 *URA3 CEN4 SUP4-o*	Ars$^-$	Ars$^-$	Ars$^-$
pDK1	pBR322 *URA3 CEN4 SUP4-o* 1048 *HO* ARS	Ars$^-$	Ars$^-$	Ars$^-$
pDK427	M13mp9 *URA3* j8Δ *HO* ARS	Ars$^-$	Ars$^-$	Ars$^{\pm a}$
pDK296-HO	pBR322 *URA3 CEN4 SUP4-o HO* ARS	0.22 ± 0.01	nd	0.11 ± 0.01
pDK296-A17	pBR322 *URA3 CEN4 SUP4-o* A17 ARS	0.20 ± 0.01	nd	0.09 ± 0.01
pDK296-B12	pBR322 *URA3 CEN4 SUP4-o* B12 ARS	0.24 ± 0.02	0.11 ± 0.02	nd
pDK296-i56HO	pBR322 *URA3 CEN4 SUP4-o* 956 *HO* ARS	0.42 ± 0.02	0.34 ± 0.02	0.29 ± 0.01
pSE276-1	M13mp93 *URA3 CEN3 SUP4-o* 1062 *HO* ARS	0.21 ± 0.01	0.05 ± 0.005	nd
pSE276-2	M13mp93 *URA3 CEN3 SUP4-o* 918 *HO* ARS	0.36 ± 0.01	0.17 ± 0.04	0.12 ± 0.01
pDK12	pBR322 *URA3 CEN4 ARS1*	0.06 ± 0.015	0.02 ± 0.006	nd

nd, not determined.
[a], the strain used was R792-7C (*rar5-7*)

Table 3. Phenotypes of *rar3* mutations

Alleles Strain	*RAR3* W303-1B	*rar3-1* R742-1C	*rar3-2* R340-5	*rar3-38::URA3* R892-1-5C	*rar3-45::URA3* R893-7-1C
pSE276-1 loss rate	0.21 ± 0.01[a]	0.05 ± 0.005[a]	0.04 ± 0.01	0.13 ± 0.03[b]	0.16 ± 0.03
Heat-shock resistance	+	−	-	−	−
Growth rate	+	+	+	−	−
Galactose fermentation	+	−	-	−	−
UDP-galactose epimerase activity	17.0	3.3	3.0	3.0	nd
Galactose fermentation +*SUP4-o*[c]	+	+	+	−	−
UDP-galactose epimerase activity +*SUP4-o*[c]	nd	17.4	15.2	nd	nd

[a] Data also given in Table 2.
[b] Plasmid loss rate was 0.15 ± 0.04 in strain R900 (*rar3-38::URA3* in a R742-1C strain background)
nd, not determined
[c] Provided by pSE276-1 plasmid.

a yeast cosmid library (see Materials and Methods). This yielded a single cosmid which was able to complement the *rar5-1* mutation, which we designated pF3.

The pSE321 (*RAR3*) cosmid can complement the plasmid-stabilization phenotype of both *rar3-1* and *rar3-2* mutations, and similarly the pF3 (*RAR5*) cosmid can complement all seven *rar5* mutations. Neither plasmid can complement other *rar* mutations, nor does either plasmid affect the mitotic stability of the *ARS1* plasmid pSE295 in a wild-type strain.

Characterization of cosmids containing the *RAR3* and *RAR5* genes

The pSE321 (*RAR3*) and pF3 (*RAR5*) cosmids were subjected to transposon mutagenesis and subcloning in order to determine the extent of the complementing activity (Fig 1; data not shown for *RAR3*). In order to show that the regions with complementing activity corresponded to the *RAR3* and *RAR5* genes, and were not unlinked suppressors, the homologous yeast sequences were marked with either *URA3* or *LEU2* by directing the integration of appropriate plasmids. Tetrad analysis of these strains indicated that the cloned DNAs are tightly linked to the *RAR3* and *RAR5* loci (data not shown). In addition, by using chromosome blotting and tetrad analysis we have obtained mapping information for both genes. The *RAR3* gene is located on the left arm of chromosome XV, approximately 30cM from the centromere; this location is consistent with the mapping data for the *SPT13* gene (46; see below). Chromosome hybridization locates the *RAR5* gene to chromosome VII, and preliminary tetrad analysis indicates that the gene is linked to *cyh2* on the left arm (data not shown).

DNA sequencing of the complementing region from pSE321 indicated that the *RAR3* gene is identical to *GAL11/SPT13*, previously identified by mutations that affect transcription of a variety of genes. Our sequence is identical to that of Suzuki *et al* (43) in the region that they identify as the *GAL11* reading frame.

DNA sequencing of the *RAR5* gene indicated a single open reading frame of 4584bp, whose position and extent is consistent with the localization of the complementing region by subcloning and transposon mutagenesis, and a 3.2kb *Sac*I-*Kpn*I probe entirely internal to the open reading frame detects a 5.2kb transcript on Northern gels (not illustrated). The open reading frame potentially encodes a 175.3 kDa protein of predicted pI 7.50. By comparison with the EMBL database have established that *RAR5* is identical to the *S. cerevisiae DST2* gene, encoding DNA strand transferase β (STPβ) (47).

Disruption of the *GAL11(RAR3)* and *RAR5* gene

To determine whether the phenotype of the original *rar3* and *rar5* mutations is due to the absence of the gene products we disrupted the chromosomal copies of the *RAR* genes. The *GAL11(RAR3)* gene was disrupted using plasmids generated by transposon mutagenesis. Since the *GAL11(RAR3)* gene is known to be nonessential (43), restriction fragments containing the *rar3-38::URA3* and *rar3-45::URA3* mutations, which fail to complement the *rar3-1* mutation and may represent null alleles, were used to replace the wild-type gene or the *rar3-1* allele in a haploid strain. Plasmid stability is only marginally higher in these strains than in a wild-type strain (Table 3). Since the original *rar3* mutations enhance plasmid stability to a greater extent than these presumed null alleles, it is likely that the *rar3-1* and *rar3-2* mutants have partial or altered protein function. As expected strains bearing these gene disruptions show a range of pleiotropic phenotypes associated with *gal11* mutations, and are also heat-shock sensitive (Table 3).

By testing the Gal phenotypes of both *rar3-1* and *rar3-2* mutations in the presence of suppressor tRNAs, we have shown that the *SUP4-o* gene can suppress the transcription, but not the plasmid-stabilization phenotype associated with these mutations (Table 3). The suppressed mutation co-segregates with the *gal11(rar3)* locus, and thus both mutations that enhance plasmid

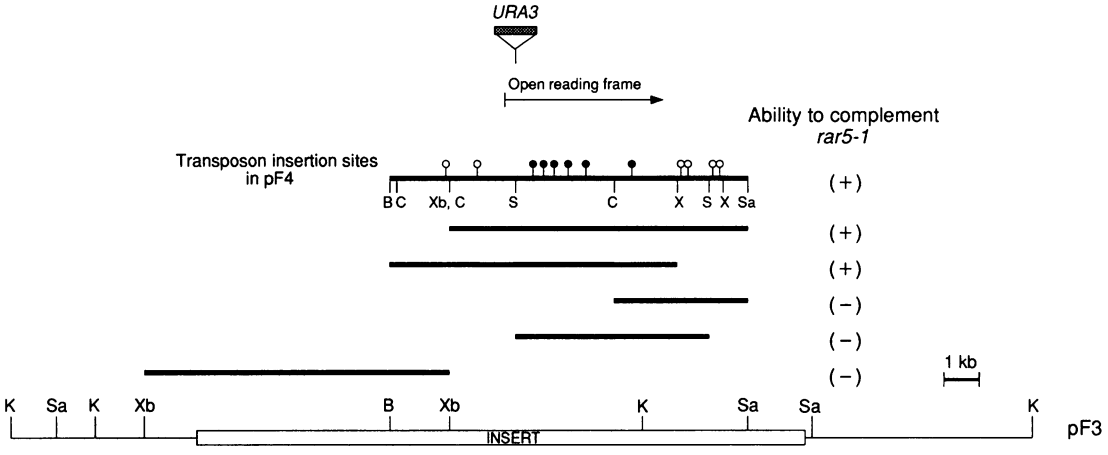

Figure 1. Localization of the *RAR5* gene by subcloning and transposon mutagenesis. The restriction map of pF3 and the relevant restriction sites in pF4 used for subcloning are shown. Indicated fragments were subcloned into YCp50 (30). The ability of each subclone to complement the *rar5-1* mutation is indicated. Sites of insertion of transposon Tn10LK into plasmid pF4, and the ability of the resultant clone to complement the *rar5-1* mutation, are indicated by # (unable to complement) or% (able to complement). The *Sac*I site used to create the *rar5::URA3* disruption is indicated. The nucleotide sequence over the region corresponding to the *RAR5* reading frame is identical to that of *DST2* (47). Restriction sites are: B, *Bam*HI; C, *Cla*I; K, *Kpn*I; S, *Sac*I; Sa, *Sal*I; X, *Xho*I; Xb, *Xba*I.

stability appear to create UAA nonsense mutations. Truncation of the GAL11(RAR3) protein may thus be required to affect plasmid replication, and we are at present constructing a series of deletion alleles to test this prediction. In the presence of the *SUP4-o* gene, some full-length GAL11(RAR3) protein will be produced, giving the observed suppression of the transcription phenotype (Table 3). However, the majority of the protein produced will continue to be truncated, as suppression is inefficient (48), and this may account for the inability of *SUP4-o* to suppress the plasmid stabilization phenotype. We have recent evidence that the plasmid-stabilization phenotype of *rar3-1* is semidominant, consistent with this interpretation (unpublished observations).

We have disrupted the chromosomal *RAR5* gene using a plasmid containing the *URA3* gene inserted at the *Sac*I site situated 97 residues into the *RAR5* coding region (see Figure 1 and Materials and Methods). This *rar5::URA3* disruption was used to replace one copy of the *RAR5* gene in the wild-type diploid strain R106 by integrative transformation. The resultant strain, DKY120, is genotypically *RAR5/rar5::URA3*. This strain was sporulated, and tetrads dissected. Most tetrads gave three or four viable spores, with 2:2 segregation of Ura^+:Ura^-, indicating that the *rar5::URA3* disruption is not lethal. However, strains carrying such a disruption show a reduction in growth rate. Thus the wild-type strain W303-1A has a doubling time of 110 minutes in log phase (YPD medium, 30°C), whereas DKY119, which is W303-1A containing the *rar5::URA3* disruption, has a doubling time of 130 minutes under identical conditions.

Disruption of the *RAR5* gene also confers a plasmid-stabilization phenotype similar to *rar5-1*. The loss rate of pSE276-2 in a *rar5::URA3* strain (0.13 ± 0.02) is almost identical to its loss rate in a *rar5-1* strain (0.12 ± 0.01). The plasmid-stabilization phenotype of strains carrying the *rar5::URA3* disruption is recessive and not complemented by crossing to a *rar5-1* strain. These results suggest that the original *rar5* mutations are null alleles, and that loss of the STPβ(RAR5) protein can in some way stimulate the function of defective ARSs. A strain bearing a deletion of the *RAR5* gene appears to show an identical plasmid-stabilization phenotype to the disruption mutation (not shown). We also find that a haploid strain bearing disruptions of both *RAR3* and *RAR5* genes is viable, and has a plasmid-stabilization phenotype similar to a *rar5* disruption strain alone (not shown).

DISCUSSION

The mitotic stability of weak ARS plasmids such as pSE276-2 can be increased either by inserting another ARS element in *cis*, or by mutations in *RAR3* or *RAR5* genes, implying that these genes encode *trans*-acting factors that have some effect on the initiation of DNA replication. The mutations may increase the function of weak ARSs, and it is also possible that new sequences may function as origins of replication in some Rar^- mutants. From analysis of two *RAR* genes described here, we find that both encode proteins affecting different DNA-related activities, raising the hope that genetic screens involving plasmid stability assays may be of general use for identifying proteins involved in chromosome function. The activity encoded by the *RAR3* gene is the putative transcription factor, GAL11, involved in the expression of a wide range of genes. In contrast, the *RAR5* gene product is implicated in DNA recombination, since this gene encodes an activity that can promote the transfer of a DNA strand from one complementary strand to another. We discuss here the possible relevance of these activities to the initiation of DNA replication.

The GAL11(SPT13/RAR3) protein: The *gal11* mutation was originally identified from its phenotype of reducing the expression of certain *GAL4*-dependent genes (44), but it has subsequently become clear that expression of a wide range of genes is reduced by this mutation, implying that the protein is a positive factor in transcription (43, 46). In some situations, however, mutations in the same gene can stimulate transcription, since *spt13* mutations suppress the transcriptional defect associated with some Ty insertions (46). Thus the gene product appears to be able to act as both a repressor and activator of transcription, depending on the promoter analyzed. The protein is highly basic (pI = 10.6),

is rich in polyglutamine tracts as observed for a number of DNA-binding regulatory proteins, and contains a high frequency of the (S/T)PXX motif postulated as a DNA-binding unit (49). It is likely that the GAL11(RAR3) protein encodes a transcription factor, and recent evidence suggests that the protein is an 'coactivator', transmitting the signal from UAS-binding proteins to general transcription factors at the transcription initiation site (50, 51).

Different mutant alleles of the *GAL11(RAR3)* gene have different effects on plasmid stability, and the simplest explanation of the results we have so far is that an alteration rather than simple loss of function is required for an apparent stimulation of ARS function. Indeed, a change of function allele of *GAL11* has been described recently that potentiates its ability to stimulate *GAL4*-dependent transcription (50). The two *gal11(rar3)* alleles that have the greatest stimulation of plasmid stability are caused by nonsense mutations, implying that truncation of the protein may alter its activity.

How do *gal11(rar3)* mutations apparently alter ARS function? This may occur as an result of changed transcription, either indirectly via changed levels of replication proteins, or more directly, since transcript formation has been shown to interfere with ARS function (52). However it is possible that the GAL11(RAR3) protein affects ARS function by a different mechanism, since transcription factors have been shown to affect replication origin function in a manner that does not involve transcription. Thus CTF/NF-I and oct-1/NF-III factors stimulate adenovirus replication in an *in vitro* system where transcription does not occur, and the DNA-binding, rather than the transcriptional activation, domains of the proteins are required for the effect (53, 54, 55, 56, 57). The mechanism of this effect is not clear, but may involve direct interaction between the transcription factor and replication proteins. The CTF factor can also stimulate SV40 origin function, but here the effect is dependent on chromatin structure, as if the factor ensures that DNA sequences required for initiation are not obscured by nucleosomes (58). In the absence of an *in vitro* system it will be difficult to determine whether the effect of *gal11(rar3)* mutations on plasmid stability is dependent on transcription or not. However, by analyzing the phenotype of a wider range of *gal11(rar3)* mutations, it may be possible to identify different functional domains of the protein.

The RAR5 protein: The *RAR5* gene encodes a biochemical activity characterized by A.Sugino and colleagues which they have designated DNA strand transfer protein β (STPβ). This protein was isolated as a mitotic factor that can promote the transfer of one strand of a double-stranded DNA molecule to a complementary single-stranded circle, and is obviously implicated in homologous recombination (59). Disruption and deletion mutations of the nonessential *RAR5* gene have the same effect on plasmid stability as the original *rar5* alleles, implying that loss of the activity can apparently stimulate ARS function. In this regard it is possible that the STPβ(RAR5) protein is analogous in function to *E.coli* specificity factors, which act to prevent initiation at sites other than *oriC* both *in vivo* and in *in vitro* replication systems (60, 61, 62).

We are currently investigating other phenotypes of *rar5* mutations, in particular the effect of this mutation on mitotic recombination. Deletion of the *RAR5* gene does not appear to confer marked sensitivity to DNA damage by UV light or X-rays (data not shown). The slow growth phenotype of the *rar5* mutation implies that the protein has an important cellular function, and this might not be expected of a protein uniquely involved in mitotic recombination. Mutations in the *RAR5* gene have recently been shown to confer a mild nuclear fusion defect (63). In addition, the observation that *rar5* mutations affecting STPβ can apparently enhance replication origin activity raises the question of the relationship between recombination and the replication of eukaryotic chromosomes.

Although recombination has an important role in the replication of some genomes, such as that of bacteriophage T4 (64), there is little evidence to suggest that it is involved in the replication of eukaryotic chromosomes. Mitotic recombination between circular plasmids may make a contribution to overall plasmid loss rate, but it is not significant for small plasmids (65), and nondisjunction is not the main mechanism whereby weak ARS plasmids are lost (66), so this is unlikely to be relevant to the phenotype of the *rar5* mutation. One speculative possibility is that the STPβ(RAR5) protein may influence replication by promoting the removal of D-loops in chromosomal DNA caused by the synthesis of short fragments that displace the other strand, structures that could otherwise serve as replication origins. In a recent model of initiation of replication proposed by Morrison *et al* (67), DNA polymerase I initiates the synthesis of short DNA strands at the replication origin, that are subsequently elongated by DNA polymerases II and III. If the STPβ(RAR5) protein were able to promote the removal of nascent DNA strands formed at replication origins, this might reduce the efficiency of origin utilization. Thus in the absence of the STPβ(RAR5) protein, subsequent elongation would be more efficient, and enhance the efficiency of origin use.

Proteins that promote such reactions similar to that promoted by the STPβ(RAR5) protein are the *E. coli* RecA protein, the bacteriophage T4 uvsX protein, and the rec1 protein of *Ustilago maydis* (68, 69, 70). The STPβ(RAR5) protein does not show any obvious similarity to these proteins, but as the action of these three proteins is ATP-dependent, unlike the STPβ(RAR5)-promoted reaction, the mechanism may be unrelated. In addition strand transferases that are not ATP-dependent have been isolated from human cell lines and *Drosophila* embryos (71, 72, 73), and it remains to be seen whether these are related in structure to STPβ.

ACKNOWLEDGEMENTS

We thank Jeanette Edwards for excellent technical assistance, Rick Singer for discussions, Sarah Bowen and Helen Ackford for comments on the manuscript, and James Micklem and Paul Coffer for contributions to the early stages of this work. We are grateful to Mark Goebl for informing us of the identity of *RAR5* and *KEM1*. We also thank N.Kleckner for strains. This work was supported by the Cancer Research Campaign. D.K. was the recipient of a Medical Research Council Studentship.

REFERENCES

1. Borowiec,J.A., Dean,F.B., Bullock,P.A. and Hurwitz,J. (1990) *Cell* **60**, 181–184.
2. Challberg,M.D. and Kelly,T.J. (1989) *Ann. Rev. Biochem.* **58**, 671–717.
3. Stillman,B. (1989) *Ann. Rev. Cell Biol.* **5**, 197–245.
4. Blow,J.J. and Nurse,P. (1990) *Cell* **62**, 855–862.
5. Bartlett,R. and Nurse,P. (1990) *BioEssays* **12**, 457–463.
6. Newlon,C.S. (1988) *Microbiol. Reviews* **52**, 568–601.
7. Umek,R.M., Linskens,M.H.K., Kowalski,D. and Huberman,J.A. (1989) *Biochem. Biophys. Acta* **1007**, 1–14.

8. Buchman,A.R., Kimmerly,W.J., Rine,J. and Kornberg,R.D. (1988). *Mol. Cell. Biol.* **8**, 210–225.
9. Diffley,J.F.X. and Stillman,B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2120–2124.
10. Francesconi,S.C. and Eisenberg,S. (1989) *Mol. Cell. Biol.* **9**, 2906–2913.
11. Shore,D. and Nasmyth,K.A. (1987) *Cell* **51**, 721–732.
12. Snyder,M., Buchman,A.R. and Davis,R.W. (1986) *Nature* **324**, 87–89.
13. Srienc,F., Bailey,J.E. and Campbell,J.L. (1985) *Mol. Cell. Biol.* **5**, 1676–1684.
14. Sweder,K.S., Rhode,P.R. and Campbell,J.L. (1988) *J. Biol. Chem.* **263**, 17270–17277.
15. Walker,S.S., Francesconi,S.C., Tye,B.-K. and Eisenberg,S. (1989) *Mol. Cell. Biol.* **9**, 2914–2921.
16. Diffley,J.F.X. and Stillman,B. (1989) *Science* **246**, 1034–1038.
17. Buchman,A.R., Lue,N.F. and Kornberg,R.D. (1988) *Mol. Cell. Biol.* **8**, 5086–5099.
18. Buchman,A.R. and Kornberg,R.D. (1990). *Mol. Cell. Biol.* **10**, 887–897.
19. Halfter,H., Kavety,B., Vandelkerckhove,J., Kiefer,F. and Gallwitz,D. (1989) *EMBO J.* **8**, 4265–4272.
20. Halfter,H., Muller,U., Winnacker,E.-L. and Gallwitz,D. (1989) *EMBO J.* **8**, 3029–3037.
21. Kimmerly,W., Buchman,A., Kornberg,R. and Rine,J. (1988) *EMBO J.* **7**, 2241–2253.
22. Maine,G.T., Sinha,P. and Tye,B.-K. (1984) *Genetics* **106**, 365–385.
23. Sinha,P., Chang,V. and Tye,B.-K. (1986) *J. Mol. Biol.* **192**, 805–814.
24. Gibson,S.I., Surosky,R.T. and Tye,B.-K. (1990) *Mol. Cell. Biol.* **10**, 5707–5720.
25. Jarvis,E.E., Clark,K.L. and Sprague,G.F. (1989) *Genes Dev.* **3**, 936–945.
26. Thrash-Bingham,C. and Fangman,W.L. (1989) *Mol. Cell. Biol.* **9**, 809–816.
27. Lemontt,J.F., Fugit,D.R. and Mackay,V.L. (1980) *Genetics* **94**, 899–920.
28. Trumbly,R.J. (1986) *J. Bacteriol.* **166**, 1123–112.
29. Kearsey,S.E. and Edwards,J. (1987) *Mol. Gen. Genet.* **210**, 509–517.
30. Stinchcomb,D.T., Mann,C. and Davies,R.W. (1982) *J. Mol. Biol.* **158**, 157–179.
31. Kearsey,S. (1984) *Cell* **37**, 299–307.
32. Kipling,D. and Kearsey,S.E. (1990) *Mol. Cell. Biol.* **10**, 265–272.
33. Kipling,D. and Kearsey,S.E. (1989) *Mol. Gen. Genet.* **218**, 531–535
34. Kearsey,S. (1983) *EMBO J.* **2**, 1571–1575.
35. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
36. Sherman,F., Fink,G. and Hicks,J.B. (1986) Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
37. Huisman,O., Raymond,W., Froehlich,K.-U., Errada,P., Kleckner,N., Botstein,D. and Hoyt,M.A. (1987) *Genetics* **116**, 191–199.
38. Bankier,A.T., Weston,K.M. and Barrell,B.G. (1987) *Methods Enzymol.* **155**, 51–93.
39. Staden,R. (1986) *Nucleic Acids Res.* **14**, 217–231.
40. Pearson,W.R. and Lipman,D.J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
41. Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.* **153**, 163–168.
42. Rothstein,R.J. (1983) *Methods Enzymol.* **101C**, 202–210.
43. Suzuki,Y., Nogi,Y., Abe,A. and Fukasawa,T. (1988) *Mol. Cell. Biol.* **8**, 4991–4999.
44. Nogi,Y. and Fukasawa,T. (1980) *Curr. Genet.* **2**, 115–120.
45. Fukasawa,T., Obonai,K., Segawa,T. and Nogi,Y. (1980) *J. Biol. Chem.* **255**, 2705–2707.
46. Fassler,J.S. and Winston,F. (1989) *Mol. Cell. Biol.* **9**, 5602–5609.
47. Dykstra,C.C., Kitada,K., Clark,A.B., Hamataka,R.K. and Sugino,A. (unpublished) M36725
48. Gilmore,R.A., Stewart,J.W. and Sherman,F. (1971) *J. Mol. Biol.* **61**:157–173
49. Suzuki,M. (1989) *J. Mol. Biol.* **207**, 61–84.
50. Himmelfarb,H.J., Pearlberg,J., Last,D.H. and Ptashne,M. (1990) *Cell* **63**, 1299–1309.
51. Nishizawa,M., Suzuki,Y., Nogi,Y., Matsumoto,K. and Fukasawa,T. (1990) *Proc. Natl. Acad. Sci. USA* 87, 5373–5377.
52. Snyder,M., Sapolsky,R.J. and Davies,R.W. (1988) *Mol. Cell. Biol.* **8**, 2184–2194.
53. Jones,K.A. Kadonaga,J.T., Rosenfeld,P.J., Kelly,T.J. and Tjian,R. (1987) *Cell* **48**, 79–89.
54. Mermod,N., O'Neill,E.A., Kelly,T.J. and Tjian,R. (1989) *Cell* **58**, 741–753.
55. O'Neill,E.A., Fletcher,C., Burrow,C.R., Heintz,N., Roeder,R.G. and Kelly,T.J. (1988) *Science* **241**, 1210–1213.
56. Pruijn,G.J.M., Van der Vliet,P.C., Dathan,N.A. and Mattaj,I.W. (1989) *Nucleic Acids Res.* **17**, 1845–1863.
57. Verrijzer,C.P., Kal,A.J. and Van der Vliet,P.C. (1990) *EMBO J.* **9**, 1883–1888.
58. Cheng,L. and Kelly,T.J. (1989) *Cell* **59**, 541–551.
59. Dykstra,C.C., Hamatake,R.K. and Sugino,A. (1990) *J. Biol. Chem.* **265**, 10968–10973.
60. Kaguni,J.M. and Kornberg,A. (1984) *J. Biol. Chem.* **259**, 8578–8583.
61. Louarn,J., Bouche,J.-P., Patte,J. amd Louarn,J.-M. (1984) *Mol. Gen. Genet.* **195**, 170–174.
62. Ogawa,T., Pickett,G.G., Kogoma,T. and Kornberg,A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1040–1044.
63. Kim,J., Ljungdahl,P.O. and Fink,G.R. (1990) *Genetics* **126**, 799–812.
64. Mosig,G. (1987) *Ann. Rev. Genet.* **21**, 347–371.
65. Hieter,P., Mann,C., Snyder,M. and Davies,R.W. (1985) *Cell* **40**, 381–392.
66. Koshland,D., Kent,J.C. and Hartwell,L.H. (1985) *Cell* **40**, 393–403.
67. Morrison,A., Araki,H., Clark,A.B., Hamatake,R.K. and Sugino,A. (1990) *Cell* **62**, 1143–1151.
68. Cox,M.M. and Lehman,I.R. (1987) *Ann. Rev. Biochem.* **56**, 229–262.
69. Fujisawa,H., Yonesaki,T. and Minagawa,T. (1985) *Nucleic Acids Res.* **13**, 7473–7481.
70. Holden,D.W., Spanos,A. and Banks,G.R. (1989) *Nucleic Acids Res.* **17**, 10489–10489.
71. Eisen,A. and Camerini-Otero,R.D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7481–7485.
72. Hsieh,P., Meyn,M.S. and Camerini-Otero,R.D. (1986) *Cell* **44**, 885–894.
73. Lowenhaupt,K., Sander,M., Hauser,C. and Rich,A. (1989) *J. Biol. Chem.* **264**, 20568–20575.
74. Kearsey,S.E. (1987) In McKaken,R. and Kelly,T. (eds.), DNA replication and recombination. UCLA Symposia on Molecular and Cellular Biology, Vol.47, pp. 335–365.