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Genetics

Yeast calmodulin and a conserved nuclear protein participate in the *in vivo* binding of a matrix association region

(scaffold-attached region/nuclear matrix–scaffold/gene regulation)

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ABSTRACT **Chromatin becomes reorganized during mitosis each cell cycle. To identify genes potentially involved in these supramolecular events, we have used a colony-color assay to screen temperature-sensitive mutants of *Saccharomyces cerevisiae*. When a sequence that mediates attachment to the nuclear matrix *in vitro* was inserted into the *GAL1* promoter of a *lacZ* fusion gene, β-galactosidase synthesis was inhibited. This observation permitted screening for temperature-sensitive-inducible mutants on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates. Only 1 of 20 complementation groups of newly isolated mutants exhibited temperature-sensitive inducibility for the matrix association region but not for control *CEN3* or *STE6* inserts—a *cmd1* mutant in which the last 7 amino acids of calmodulin were truncated by an ochre termination codon. Another mutant (*smi1*) exhibited a rare phenotype at the nonpermissive condition, which included S phase and budding arrest. We cloned and sequenced the *SMI1* gene, which encodes a 57-kDa polypeptide with evolutionarily conserved epitope(s) found in mammalian cell nuclei. Thus, we provide evidence for involvement of calmodulin and another conserved protein in the *in vivo* binding of a matrix association region.**

DNA within interphase nuclei and mitotic chromosomes is believed to be organized into topologically constrained looped domains ranging from 5 to 200 kb in length (for review, see ref. 1). *In vitro* DNA-binding assays have identified sequences termed MARs (for matrix-association regions), also called SARs (for scaffold-attached regions), that have been proposed to mediate loop attachment *in vivo* (1–3). These sequences are at least 200 bp long, are A+T-rich (≈70%), and often contain topoisomerase II and other A+T-rich consensus motifs. MARs and their matrix-binding sites exhibit cross–organism specificities of interactions suggesting strong evolutionary conservation (2, 4, 5). Particularly intriguing is the observation that MARs often reside within or near functionally important sequences. In the yeast *Saccharomyces cerevisiae*, autonomously replicating sequences (ARS), *CEN* sequences, and certain silencer regions also contain MARs (5–7).

Little is known regarding the mechanics of chromosomal loop reorganization during the cell cycle. Attachment sites would need to be propagated, presumably during S phase, in concert with DNA replication. In addition, these structures would need to become temporarily disrupted and significantly reorganized during mitotic chromosome formation. Developing an understanding of these dynamic and complex supramolecular assembly processes would be experimentally intractable using only classical biochemical techniques. Therefore, we decided to use a genetic approach to dissect these processes. We have taken advantage of *S. cerevisiae* for genetic screening of the putative integrity of chromosomal loop attachment *in vivo* and provide evidence for the involvement of calmodulin and an evolutionarily conserved protein† in MAR sequence interactions.

MATERIALS AND METHODS

Media, Genetic Manipulations, and Complementation Cloning. Cells were grown under nonselective conditions in YPD medium (1% yeast extract/2% bactopeptone/2% glucose) and under selective conditions in YNB minimal medium (0.67% yeast nitrogen base minus amino acids, supplemented with the appropriate sugar and nutritional requirements). β-Galactosidase activity indicator plates were made by buffering YNB/galactose minimal medium with 70 mM potassium phosphate (pH 7.0) and adding 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-GAL) to 40 μg/ml (8). β-Galactosidase assays were done after permeabilizing cells with chloroform and SDS, as described (9). Yeast were transformed by using the lithium acetate procedure (10). Genomic DNA isolation, site-directed integration, gap-repair mapping, allele rescue, and other genetic manipulations were done as reported (8, 11). Complementation cloning was done as described (12).

Colony-Color Assay, Genetic Screening, and Mutagenesis. Replicas on X-GAL plates were grown at the permissive temperature of 24°C to establish the colonies and were then shifted to 37°C for 8–16 hr to screen for conditional β-galactosidase expression. Ethyl methanesulfonate mutagenesis of W303-1B transformed with MAR test plasmids was done as described (13). Cells which were temperature-sensitive (ts) for both growth and expression of β-galactosidase were cured of plasmids and then retransformed to ensure that β-galactosidase activity was not from mutagenesis of the test plasmid. Mutations that affect plasmid copy number, *GAL* gene expression, or, even possibly, X-GAL permeability were controlled for by assessing expression patterns in cells transformed with pLR1Δ20B (14). (It should be noted that the colony-color assay is simply a genetic screen and is not quantitative. The assay gives the cumulative representation of β-galactosidase induction, rather than the level of enzyme in cells at the time of plate visual inspection. This aspect is important because cells die during induction and the enzyme

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Abbreviations: MAR, matrix association region; X-GAL, 5-bromo-4-chloro-3-indolyl β-D-galactoside; UAS_G, upstream activating sequence in the *GAL1* promoter; ts, temperature-sensitive; ARS, autonomously replicating sequence.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L15423).

turns over.) Mutants were outcrossed once to F809 and backcrossed twice to the parent strain, W303-1A. Cosegregation of ts lethality and ts β-galactosidase activity was demonstrated by scoring tetrads from the crosses. Other applications and controls for the colony-color assay are as described (15).

Immunological Techniques. Anti-SMI1 peptide antibodies were generated against keyhold limpet hemocyanin-coupled peptide corresponding to amino acids 436–453 (CK-QESERKEDEKQPKVEEK) in rabbits by Immuno Dynamics (La Jolla, CA). Antisera or IgGs were cleared before use on nitrocellulose-filter discs saturated with whole-cell yeast extracts from a *smi*1-null mutant strain and used on immunoblots at 1:250 dilution (50 μg/ml). For immunofluoresence, the IgG fraction was affinity purified (16), and fluorescein isothiocyanate-labeled goat anti-rabbit IgG was used (17). Immunoblot analysis was done as described (18, 19).

RESULTS

An *in Vivo* Assay for MAR Sequence Attachment in Yeast. To develop an *in vivo* assay for attachment of MAR sequences in yeast, we took advantage of the observation of Brent and Ptashne (20) that transcription is blocked when a DNA-binding protein interacts with a sequence placed in the *GAL1* promoter between the upstream activating sequence (UAS_G) and the TATA box. We used a YEp vector containing a *GAL1–lacZ* fusion gene with an *Xho* I linker replacing a 158-bp deletion between the UAS_G and TATA sequences (14). Because we had found previously that nuclear matrices isolated from *S. cerevisiae* exhibit specific *in vitro* binding of a MAR from the mouse κ immunoglobulin gene (4), we decided to use this sequence in an effort to identify evolutionary conserved processes. Interestingly, when we inserted a 254-bp fragment of this MAR (2) in either orientation into the *Xho* I test site, an ≈27-fold reduction of β-galactosidase activity occurred compared with control constructs containing pBR322 sequences (Fig. 1). Furthermore, the same MAR was not inhibitory when inserted upstream of the UAS_G. These results suggest that yeast possess protein(s) that recognize the mouse κ gene MAR *in vivo*.

Genetic Screening for Suppression of MAR Inhibition. We used a colony-color assay on X-GAL plates as a primary genetic screen to identify ts conditional mutants for inducibility of β-galactosidase synthesis at 37°C in cells carrying the MAR plasmid. In addition, we chose to study further only those ts-inducible mutants that were also ts lethal, thereby readily permitting cloning of complementing genes. *A priori*, such an approach could reveal genes encoding defective MAR sequence-binding proteins or genes encoding defective regulators of the transcription, steady-state level, or activity of such proteins. In addition, the screen might reveal genes of more remotely involved proteins, such as cell-cycle-dependent MAR attachment components, which might allow the *GAL1* promoter to become transiently accessible to the transcription machinery, or enzymes that play a role in the synthesis and modification of nuclear structures.

We mutagenized yeast carrying the MAR–reporter plasmid with ethyl methanesulfonate and, by replica plating on X-GAL indicator plates, identified 20 mutants that were ts for both induction of β-galactosidase and growth. These strains, which *s*uppress *M*AR *i*nhibition, are designated *smi* mutants. The 20 mutants fell into 20 different complementation groups, indicating that numerous gene defects were involved.

To subdivide these mutants, we used reporter constructs in which either a *S. cerevisiae CEN3* fragment (21) or *STE6* operator (22) replaced the MAR insert for a secondary colony-color-assay screen. The *CEN3* fragment is a yeast MAR (5), whereas the *STE6* sequence binds the α2 repressor

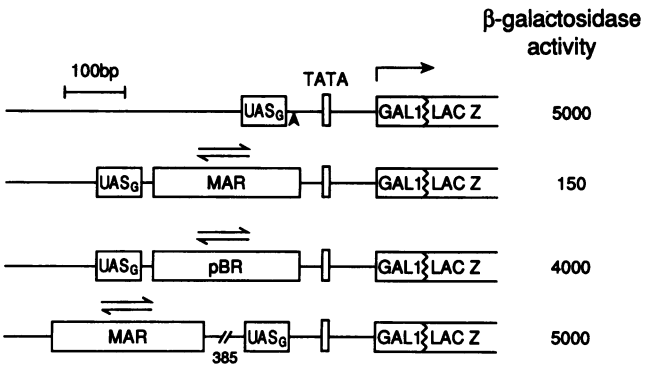

FIG. 1. Position-dependent inhibition of the *GAL1* promoter in transformed yeast by a mouse MAR sequence. The upper construct shows the initial plasmid [pLR1Δ20B (14)] into which the MAR was inserted at the *Xho* I site in either orientation (at the arrowhead) between the UAS_G and TATA box to form the second constructs, termed pBF20B-Ma and -b. The MAR sequence corresponded to a 254-bp *Alu* I fragment (2). As a control, pBR322 sequences from either position 1451–1708 or 54–311 of pBluescript were inserted in either orientation (at arrowhead). In the fourth constructs the MAR was inserted upstream of the UAS_G. Cells were shifted from glucose- to galactose-containing medium, and β-galactosidase activity was assayed after five generations.

but requires the *SSN6* product for function (23), which contains a tetratricopeptide repeat domain found in some matrix proteins (24). These inserts, like the MAR, were found to efficiently inhibit β-galactosidase synthesis in wild-type cells. Introduction of the *CEN3* and *STE6*–reporter constructs into the *smi* mutants revealed that 1 of the 20 isolates, *smi19*, gave strong ts induction only with the MAR insert and another isolate, *smi69*, gave a pattern of ts induction with the MAR and *CEN3* inserts but did not give such a pattern with *STE6* (Fig. 2; data not shown). Most of the other mutants gave ts induction of the MAR and *STE6* constructs but not of *CEN3*, except for *smi1*, which showed induction with all three constructs (Fig. 2; data not shown). We describe below identification of the defective genes in *smi19* and *smi1* mutants.

Cloning and Sequencing the *SMI19* Gene. We cloned the *SMI19* gene by complementation and found that a 2-kb fragment contained in a YCp vector both complemented the ts lethality and restored repression of β-galactosidase synthesis at 37°C of mutant *smi19*. Determination of the complete sequence of the 2-kb fragment revealed that *SMI19* was the yeast calmodulin gene *CMD1* because its sequence showed 100% identity (25). The mutant *smi19* calmodulin gene was cloned and sequenced and also sequenced after PCR amplification from genomic DNA. Both approaches revealed that only one base change had occurred to generate the mutant, a C → T change at nt 601 in the GenBank sequence (411 nt from the ATG), leading to the generation of an ochre termination codon that truncated the native protein by only 7 amino acids at its C terminus (data not shown).

Cloning and Sequencing the *SMI1* Gene. *smi1* cells exhibited a distinctive terminal phenotype at 37°C: ≈80% arrest with a tiny bud and a DNA content between 1N and 2N (where N is haploid number) (Fig. 3). Because cell size did not increase, both cell growth and division were arrested. We cloned the *SMI1* gene by complementation and found that a 2.75-kb *Pst* I fragment contained in a YCp vector both complemented the ts lethality and restored repression of β-galactosidase synthesis at 37°C of mutant *smi1* (data not shown). Proof that this complementing fragment is allelic to *smi1* was obtained by a cis–trans complementation test (data not shown). In addition, RNA analysis revealed a 2.2-kb transcript complementary to the *SMI1* sequence, pulse field gel analysis placed the gene on the right arm of chromosome

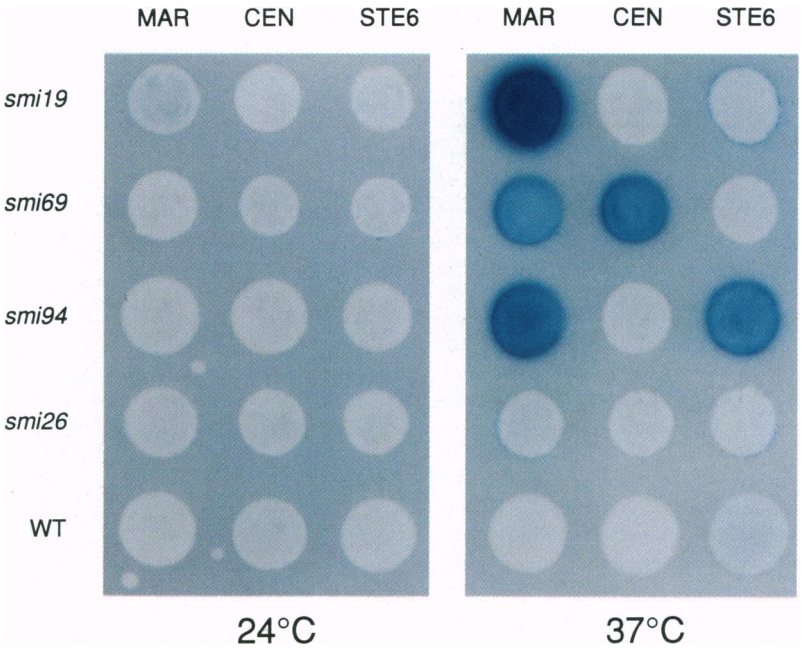

FIG. 2. Colony-color assay of selected *smi* ts mutants. Yeast strains (indicated at left; WT, wild type) were transformed with plasmids carrying either MAR, *CEN*, or *STE6* inserts within the *GAL1* promoter of a *lacZ* reporter gene. Transformants were replica-plated at 24°C on X-GAL indicator plates and after growth one duplicate was shifted to 37°C for 16 hr. Fragments were each inserted into the *Xho* I site of pLR1Δ20B (14). To control for copy number, we replaced the 2μ sequences in pBF20B-Ma (Fig. 1) with a *CEN4*–ARS1 fragment, yielding a low-copy-number MAR plasmid (pBF3). The *CEN* plasmid (pRN8D) carries a functional 139-bp *Bam*HI–*Bgl* II fragment of *CEN3* (21). The *STE6* construct (pBFS6) contains a tandem pentamer of the 32-bp α2 repressor-binding site (22).

7, and disruption of *SMI1* recreated the ts phenotype (unpublished results).

The complete sequence of the 2.75-kb *Pst* I fragment containing the *SMI1* gene revealed an uninterrupted open reading frame beginning at nt 666 encoding a putative protein of 505 amino acids with a calculated M_r of 57,079 (Fig. 4). The protein sequence has 31% charged amino acids with internal patches rich in asparagine/glutamine at residues 39–53 and 340–359. It is primarily hydrophilic, being composed of 18.5% acidic and 9% basic amino acids, with 24 of the 63 C-terminal residues being acidic. There is a putative nuclear localization signal (KRKVK) at residues 5–9 (27), a potential weak leucine zipper/heptad repeat between residues 286 and 314 (28), and a region enriched in proline, glutamate, serine, and threonine at residues 405–426 (29). No other known motifs or consensus sequences correlated with DNA-binding activities were found. Neither the nucleic acid nor the amino acid sequences share any extended homology with sequences presently in GenBank or National Biomedical Research Foundation data bases.

***SMI1* Homolog(s) Are Evolutionarily Conserved Nuclear Proteins.** Immunoblot analysis using SMI1-antipeptide antibodies detected a major protein band with a similar electrophoretic mobility to the *S. cerevisiae* protein from several mammalian sources and *Schizosaccharomyces pombe* (Fig. 5, lower arrowhead). The protein migrated with an apparent size 18 kDa larger than its predicted mass. Other cross-reacting bands were sometimes apparent (Fig. 5, upper arrowhead). Although the antipeptide antibodies were not useful for indirect immunofluorescent experiments with *S. cerevisiae*, we found strong and highly specific intranuclear staining with nucleolar exclusion when immunofluorescent analysis was performed on CV-1 cells with affinity-purified antibodies (Fig. 6). Similar results were obtained with hamster, HeLa cells, and normal human fibroblasts (data not shown). We conclude that SMI1 homolog(s) are conserved nuclear proteins.

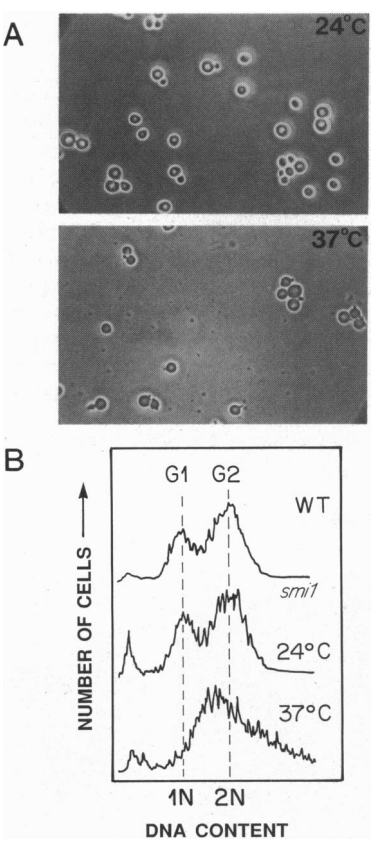

FIG. 3. Morphology and DNA content of *smi1* cells after being shifted to 37°C. (*A*) Haploid cells were visualized by phase-contrast microscopy after either exponential growth at 24°C or being shifted to 37°C for 10 hr. (*B*) Homozygous diploid cells were grown similarly at 24°C or 37°C and analyzed by flow cytometry for DNA content (26). WT, wild type; N, haploid number.

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                GGCTTAACAT GCATTGCACC ACCGTTTGTA CATCATAAGT TAATTACATT CCTTTTTAGG    60
                ATAGTCTACT ATTTTATTTT TCCCGTATCT GGAAAACCCC CTTAGATCCT TGTCACATAA   120
                TCATAAAAAT AAAGGCACCA AGCCCTAAAG CACGTGACAT ATGTCATTAC CCTAGATTAC   180
                ATATAGGGCT TCCAAAGCCT ATTGGAGGTC GAAAAAAAGA AAAAAGAAAA TAAAAAAGCG   240
                AAAAAAAGAA ATGCATAATT CGCAGATTGT TTTTTTTTTC TTTTTCTAAT GTCAGTTTTC   300
                CTACTTCGAG ATCAAAAGAA TGTTTTTGCA CCGCAACTGA AAAGGTTGTG TTTTCTTTCC   360
                AGAGTAAATT ACATTTTAAT CAATTCTTTG CGCTGCTACG CAATCTTCAA ACGCAAGTCC   420
                ATTCTTAGAA ATTTTAGCTT CTCTCAATAT TGAAATATTT GCAAATACGC CATTCAAGAA   480
                TCAAGCCTGT TGAAAATAAA GGTACAAAAT TAAATCCGCT AATTATTTTT TTTCGTGTTC   540
                GACTAAAGTT ATCGAAAAAT TGAAAATATA CAAGGACTAG CTATACTATC AATACGCAAA   600
                GAAAATACAA AATAAACGCA CATTCGTTCT ATAGGCGACA ACTTGGCAGA ATTTTTTAGT   660
                ATAAAATGGA TCTATTCAAA AGAAAAGTTA AAGAATGGGT ATACTCCCTC AGCACTGACG   720
1                M  D  L  F  K  R  K  V  K  E  W  V  Y  S  L  S  T  D  D
                ACCATTATGC AGAGTATAAC CCCGATGAAA CGCCTACTTT TAACATGGGT AAACGTTTAA   780
20               H  Y  A  E  Y  N  P  D  E  T  P  T  F  N  M  G  K  R  L  N
                ACAGCAACAA TGGTCAGGTA AATCCCTCTC AAATGCATTT GAATAGTGTA GATGAGGAAA   840
40               S  N  N  G  Q  V  N  P  S  Q  M  H  L  N  S  V  D  E  E  M
                TGAGCATGGG ATTTCAAAAT GGCGTGCCAT CTAATGAAGA CATAAATATT GATGAATTTA   900
60               S  M  G  F  Q  N  G  V  P  S  N  E  D  I  N  I  D  E  F  T
                CGTCCACGGA GTCAAACGAT GGTGTCTCTG AAACCCTCTT AGCTTGGAGA CACATCGATT   960
80               S  T  E  S  N  D  G  V  S  E  T  L  L  A  W  R  H  I  D  F
                TTTGGACCAG TGAACATAAT CCAGATTTAA ATGCAACTTT GAGTGATCCT TGCACCCAAA  1020
100              W  T  S  E  H  N  P  D  L  N  A  T  L  S  D  P  C  T  Q  N
                ACGATATCAC TCACGCAGAG GAAGACTTGG AAGTTAGCTT TCCCAACCCA GTAAAAGCGT  1080
120              D  I  T  H  A  E  E  D  L  E  V  S  F  P  N  P  V  K  A  S
                CTTTCAAAAT TCATGATGGC CAAGAAGATT TAGAATCGAT GACCGGTACT TCTGGCTTGT  1140
140              F  K  I  H  D  G  Q  E  D  L  E  S  M  T  G  T  S  G  L  F
                TTTATGGCTT CCACTAATG ACACTAGATC AGGTTGTAGC TATGACTCAG GCCTGGAGAA  1200
160              Y  G  F  Q  L  M  T  L  D  Q  V  V  A  M  T  Q  A  W  R  N
                ACGTCGCAAA GAACCTAAAC AAAAGATCTC AACAAGGTTT ATCCCATGTT ACATCTACTG  1260
180              V  A  K  N  L  N  K  R  S  Q  Q  G  L  S  H  V  T  S  T  G
                GCTCTTCTTC ATCTATGGAA AGACTAAATG GTAACAAGTT CAAACTGCCA AATATCCCAG  1320
200              S  S  S  S  M  E  R  L  N  G  N  K  F  K  L  P  N  I  P  D
                ATCAAAAATC TATTCCTCCA AATGCAGTGC AACCGGTATA TGCACATCCT GCTTGGATTC  1380
220              Q  K  S  I  P  P  N  A  V  Q  P  V  Y  A  H  P  A  W  I  P
                CTTTAATAAC GGACAATGCC GGTAACCATA TCGGTGTTGA CTTGGCGCCT GGTCCAAATG  1440
240              L  I  T  D  N  A  G  N  H  I  G  V  D  L  A  P  G  P  N  G
                GTAAATATGC TCAAATTATA ACATTTGGGA GGGACTTCGA TACGAAATTT GTCATTGCTG  1500
260              K  Y  A  Q  I  I  T  F  G  R  D  F  D  T  K  F  V  I  A  E
                AAAATTGGGG TGAATTTCTG TTATCGTTTG CCAACGATTT GGAAGCTGGT AATTGGTATT  1560
280              N  W  G  E  F  L  L  S  F  A  N  D  L  E  A  G  N  W  Y  L
                TAGTAGATGA CAATGACGAC TACTTTAGCG GTGATGGTGA ATTGGTCTTC AGGGATAAGA  1620
300              V  D  D  N  D  D  Y  F  S  G  D  G  E  L  V  F  R  D  K  K
                AATCTAATGG TCCTATACAA GATTATTTCG AAGTTTTAAA AAGAAGAACG TGGATCAAGT  1680
320              S  N  G  P  I  Q  D  Y  F  E  V  L  K  R  R  T  W  I  K  Y
                ATCAAGAAAA CTTGAGATCA CAACAGCAAA AATCTCAACC TGACACATCC TTGCAGGAGC  1740
340              Q  E  N  L  R  S  Q  Q  Q  K  S  Q  P  D  T  S  L  Q  E  Q
                AGAAATACGT GCCTGCCTCG CAAAAGAAAG TGGCAGCTGA ACAGCCTTCT ACCCTCAACG  1800
360              K  Y  V  P  A  S  Q  K  K  V  A  A  E  Q  P  S  T  L  N  A
                CAGAATCCAT AAAGGGCGAA GATAGTGGTA GTGCAGATGT ACAATCTGTT CAAGATCACG  1860
380              E  S  I  K  G  E  D  S  G  S  A  D  V  Q  S  V  Q  D  H  E
                AATCTGTCAA AATTGTGAAA ACAGAACCTA GCGAAGCGGA GACTACAACT GTAAATACAG  1920
400              S  V  K  I  V  K  T  E  P  S  E  A  E  T  T  T  V  N  T  E
                AAAGCCTTGG ACAAGCAGAG CATGAAATCA AAGCTGATAA TGTAGACATT AAACAAGAAA  1980
420              S  L  G  Q  A  E  H  E  I  K  A  D  N  V  D  I  K  Q  E  S
                GCGAGAGGAA AGAAGACGAA AAGCAACCAA AGGTTGAAGA AAAAGAACAC GTTGAAAACG  2040
440              E  R  K  E  D  E  K  Q  P  K  V  E  E  K  E  H  V  E  N  E
                AACATGTCAC AGAATCAGCA AAAAAAGACG ATGATGTAAA TAAGCAAACA GAAGAAATGA  2100
460              H  V  T  E  S  A  K  K  D  D  D  V  N  K  Q  T  E  E  M  N
                ACAAAAAGGA AGAAAATGAA ATAAGAAGTG ATGATGCTAA AGTGGAAGAA GCGAGAGAAG  2160
480              K  K  E  E  N  E  I  R  S  D  D  A  K  V  E  E  A  R  E  E
                AATTTGAAAA TATAGCTTTA TGAAATATCA CAATTAACAT TCTACAACCA AAAAGAAATT  2220
500              F  E  N  I  A  L
                TGGACAATAT TTATTTCTAT TAAACTGTTC TAGGTAGGTT TCAACCTTAA GAAATTTATA  2280
                CAATATTATC ATATATATAT ATATATATAT ATAGCACAAT TCCCTTACGT TTACCAACCA  2340
                TGAACGTTCG TGGAAATCAA TGCATTATGT CTATAAGAGT ATTCCTAAAA GCAGGAGAAA  2400
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                AATATATACA AAACGGTTGG CCACTAAAAG ATGGAAATAA AAAAAGAAAA TAAATACTAC  2520
                TAGTAAACAT TTATAGAACG TCTTTTTGCA ACTATAATCA ATTCTGTTCA CCATATTATT  2580
                CTTTGCAAAT AGGGCTCAGT GGTCCAAGGA AATGAGACCT TGGAAAAGAT AACTAAAGTA  2640
                ACGACATTGA AGAGCATGAA CCATAAAAAT TCCATGAACA GTCTTTTCGT ACTGCTTATG  2700
                GTAATTGGGT TTTCGCCTTC TTTAGCAGGT TTTAAATCCT TTATCAAAGG TTCGCAG     2757
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FIG. 4. DNA sequence of the *SMI1* locus and predicted amino acid sequence of the encoded protein.

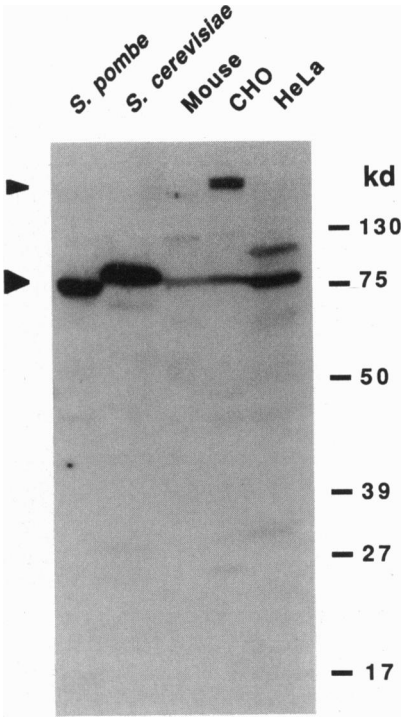

FIG. 5. Detection of the *SMI1* gene product and homologs by immunoblot analysis. Protein samples prepared from whole cells were separated on an SDS/polyacrylamide gel, and material was transferred to nitrocellulose for reaction with antipeptide antibodies and autoradiography. The mouse sample was from S194 plasmacytoma cells. The upper arrowhead represents a unique cross-reacting species. The lower arrowhead depicts a common cross-reacting band. Wild-type *S. cerevisiae* overexpressing SMI1 were used to increase its signal ≈30-fold.

DISCUSSION

Involvement of Calmodulin in MAR Sequence Attachment. Our genetic approach identifies genes involved at some level in putative matrix attachment, but how direct this involvement might be will require further analyses, such as for the unexpected connection between calmodulin and MAR repression (Fig. 2). Previous studies have revealed that the yeast *CMD1* gene is essential (25) and required for progression through mitosis (30). Although its cellular localization in yeast is most concentrated in growing buds (31), calmodulin along with at least seven calmodulin-binding proteins have been found in the nucleus of animal cells, and several of these

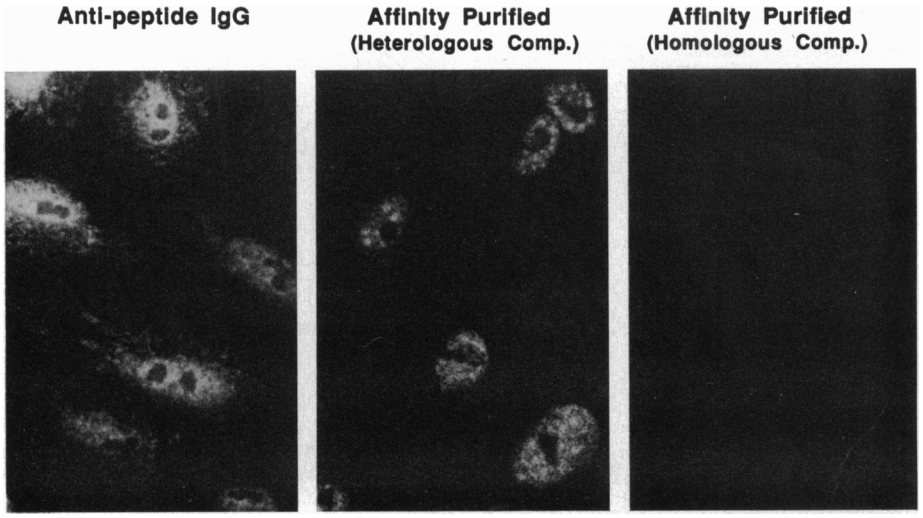

FIG. 6. Nuclear location of SMI1 homolog(s) in animal cells. CV-1 cells were subjected to immunofluorescent analysis by reaction with antipeptide antibodies before or after affinity purification, with homologous peptide competition (comp.), as indicated.

proteins exhibit enrichment in the nuclear matrix (32). Thus, calmodulin-dependent protein interactions could regulate MAR sequence attachment.

***SMI1*: Function and Conservation.** *SMI1* is required for both yeast cell growth and DNA replication at 37°C. The S-phase arrest is intriguing considering the similarities between MAR sequence motifs and the yeast ARS A domain consensus and the observation that certain heterologous MARs can serve as ARS elements in yeast (7) [but not the MAR studied here (unpublished results)]. SMI1 homologs are present in animal cells and are intranuclear (Figs. 5 and 6). Formosa and coworkers have independently cloned the *SMI1* gene from an expression library by using antibodies generated against yeast DNA-binding proteins (T. Formosa, personal communication). However, the protein is new in the data base and exhibits no major homology to known motifs; its function remains to be identified.

An Intriguing Complexity Underlies the Maintenance of Repression. The patterns of induction between different mutants and constructs are unexpectedly complex (Fig. 2). Perhaps this is not surprising because the colony-color assay monitors for maintenance of repression, which may, in turn, depend on combinatorial nuclear interactions. For example, we found that the *STE6*–reporter gene was most sensitive to derepression in a wide range of mutants studied. In addition to the α2 repressor, recent studies indicate the need for MCM1, SSN6, and TUP1 proteins for effective repression of *STE6* (23). Furthermore, each of these additional proteins has multiple functions. Moreover, *CEN3* is also a matrix-attachment sequence (5) but does not give induction in the *smi19* mutant (Fig. 2), indicating nonequivalence among matrix-binding sequences. In the future it will be interesting to characterize *smi69* because this mutant gave another distinctive induction pattern of MAR and *CEN* derepression, but not of *STE6* (Fig. 2).

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