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Dissection of the Bifunctional ARGRII Protein Involved in the Regulation of Arginine Anabolic and Catabolic Pathways

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ARGRII is a regulatory protein which regulates the arginine anabolic and catabolic pathways in combination with ARGRI and ARGRIII. We have investigated, by deletion analysis and fusion to LexA protein, the different domains of ARGRII protein. In contrast to other yeast regulatory proteins, 92% of ARGRII is necessary for its anabolic repression function and 80% is necessary for its catabolic activator function. We can define three domains in this protein: a putative DNA-binding domain containing a zinc finger motif, a region more involved in the repression activity located around the RNase-like sequence, and a large activation domain.

ARGRII is one of the three proteins involved in the regulation of arginine metabolism in *Saccharomyces cerevisiae*. In combination with ARGRI and ARGRIII, it represses the synthesis of five anabolic enzymes and induces that of two catabolic enzymes (2, 14). Analysis of the control regions of these anabolic and catabolic coregulated genes allowed the definition of the primary target of the ARGR proteins upstream of the transcription initiation site (8, 11, 12), but for the *ARG5,6* gene, part of the control region is transcribed (30). Measurement of steady-state mRNA levels by Northern blot analysis shows that the regulation by arginine seems to operate at a posttranscriptional level (10, 28). However, recent data obtained by measuring the levels of *ARG1* and *ARG3* mRNA by pulse-labeling showed a repression of these mRNAs when the cells are grown in the presence of arginine (9). Although the question is still open, we have proposed a model in which the ARGR proteins could bind specifically to DNA and, in the presence of arginine, also interact with the nascent mRNA, thus somehow preventing its translation (29). The functional analysis of ARGR proteins could provide some useful information concerning the mechanism of regulation of arginine metabolism. ARGRII is a protein of 880 amino acids with features common to other DNA-binding proteins: a zinc finger motif in its N-terminal region and two helix-turn-helix motifs (31). In contrast to other yeast regulatory proteins, ARGRII also possesses a region of identity with various retroviral and pancreatic RNases (29). This region could be responsible for its interaction with mRNA. Thus, the role of the ARGRII regulatory protein seems to be more complex than that of other regulatory proteins which activate or repress gene transcription.

Functional analysis of several transcriptional factors such as GAL4, GCN4, HAP1, and ADR1 has shown that two rather small domains are sufficient for their functions (DNA binding and activation domains) (6, 20–22, 25, 37, 42). These results were obtained by systematic deletions of various portions of these proteins and also by fusion of different parts to the DNA-binding domain of the lexA protein.

A similar approach was used to define the domains of the ARGRII protein necessary to repress and induce the expression of arginine anabolic and catabolic genes. Our results show that in contrast to the other regulatory proteins described above, 80 to 90% of ARGRII is indispensable for its functions.

MATERIALS AND METHODS

Strains and media. Yeast strain 10R34d-II (*ura3 argRII::CAR1*) (insertion of a 0.7-kb *Bgl*II-*Bgl*II fragment from the *CAR1* gene at the *Bgl*II site of ARGRII) was used as a recipient strain for all plasmids containing the *ARGRII* deletions. This strain clearly has an argRII phenotype (see Table 2), but the altered ARGRII protein, when overexpressed, has retained a reduced DNA-binding capacity (data not shown). Strain 1C2123a (*ura3 leu2 argRI argRIII*) was used for transformation with all *lexA-ARGRII* fusion plasmids (multicopy) and reporter plasmid p1840 (24). All plasmids constructed in this work were amplified in *Escherichia coli* XL1-B [*endA1 hsdR17*(r_k m_k) *supE44 thi-1* λ *recA1 gyr96 relA1* Δ (*lac*) (F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r) (Stratagene). The plasmid-bearing yeast strains were grown in a minimal medium with 0.02 M $(NH_4)_2SO_4$ as the nitrogen source (this medium is designated M.am). M.am contains 3% glucose, vitamins, and mineral traces (27). L-Arginine (1 mg/ml) was added as indicated to M.am. M. ornithine was the same medium with the ammonium being replaced by 1 mg of ornithine per ml as the nitrogen source. Some strains were also cultivated on a medium with 2% galactose (see Fig. 5 legend and Table 2).

Construction of the *ARGRII* deletions. The deletions of *ARGRII* were produced either by exonuclease III-mung bean nuclease treatment or by using convenient restriction sites, some of which were created by oligonucleotide-directed in vitro mutagenesis with the Amersham kit.

The amino-terminal deletions were constructed by exonuclease III-mung bean nuclease treatment of a Bluescript plasmid (+SK) containing the *ARGRII* gene on a 4-kb *Pvu*II-*Bam*HI DNA fragment. This plasmid was digested with *Kpn*I and *Sal*I and treated with exonuclease III for the appropriate time and then with mung bean nuclease and finally ligated in the presence of excess *Bam*HI linker. The resultant plasmids, BS43, BS34, and BS39, were sequenced to find the position of the insertion of the *Bam*HI linker. They have a *Bam*HI restriction site at positions −143, +12, and +60, respectively (position +1 being the translational start codon), the last two leading to N-terminus-truncated

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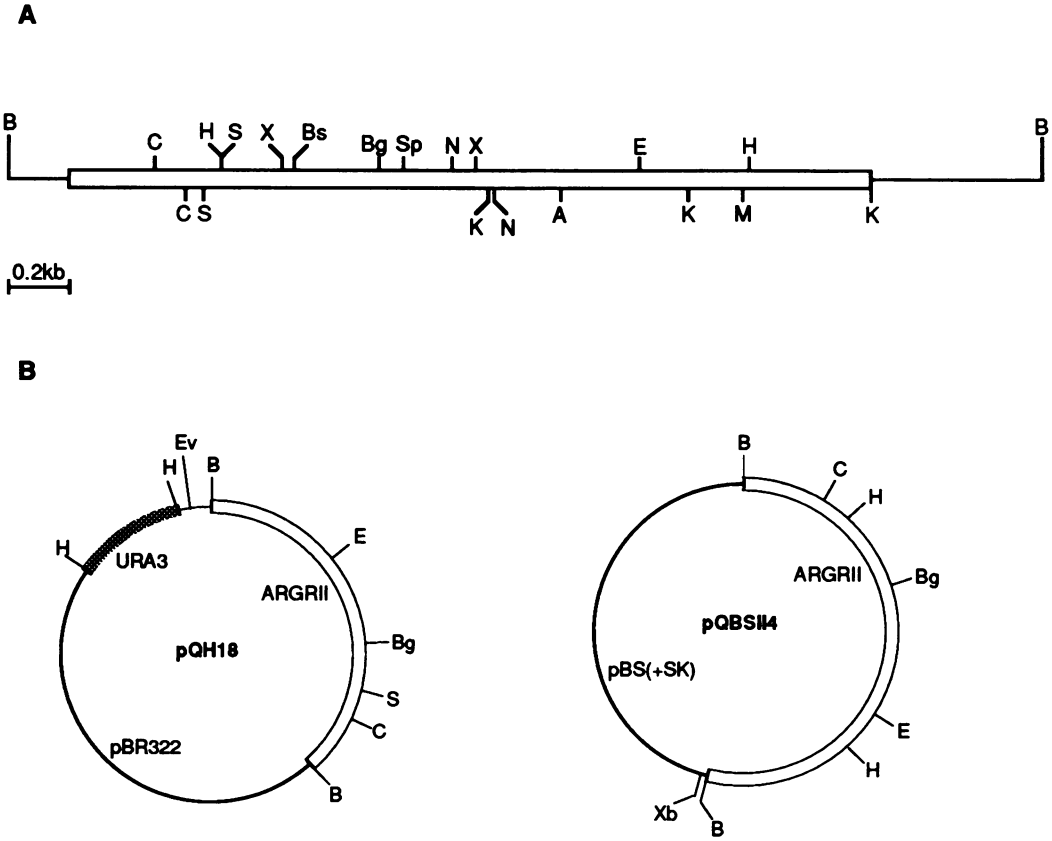

FIG. 1. Restriction map of the 3.4-kb *ARGRII* gene (A) and of two *ARGRII* plasmids (B). (A) The open box represents the *ARGRII* open reading frame. The restriction sites over the open box are naturally existing sites, and those below the box were created by in vitro oligonucleotide-directed mutagenesis. (B) pQH18 contains the *ARGRII* gene, *URA3* gene, and pBR322 whose *Cla*I site has been destroyed. pQBSII4 contains the *ARGRII* gene and the phagemid pBS(+SK) whose sites from *Kpn*I to *Hin*dIII in the polylinker have been removed to make the deletion procedure convenient. Enzyme abbreviations: A, *Aat*II; B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Nco*I; S, *Sac*I; Sp, *Sph*I; X, *Xho*I; Xb, *Xba*I. Symbols: □, *ARGRII*; ▩, *URA3*; ——, vector.

proteins devoid of an *ARGRII* promoter. To express these deleted proteins, the *Bam*HI fragments from BS34, BS39, and BS43 were inserted into the *Bam*HI site of YEP52 plasmid (pBR322 *LEU2* 2 μm, a gift from F. Lacroute) containing the galactose-inducible *GAL10* promoter. These constructions led to the synthesis of ARGRII proteins beginning either at Met-1 (YEP43), Met-12 (YEP34), or Met-61 (YEP39).

For the internal deletions, the 3.4-kb *Bam*HI-*Bam*HI fragment of *ARGRII* from plasmid BS43 was inserted into the Bluescript vector (+SK) on which the restriction sites from *Kpn*I to *Hin*dIII in the polylinker had been previously destroyed, producing plasmid pQBSII4 (Fig. 1B), and in pBR322 with the *Cla*I site destroyed and containing *URA3*, producing plasmid pQH18 (Fig. 1B). For the internal deletions, the *ARGRII* plasmids, pQH18, pQBSII4, and their derivatives which were produced after creating new restriction sites by in vitro mutagenesis, were digested with appropriate restriction endonucleases (Table 1; Fig. 1A). To produce in-frame junctions, the resultant vector fragments were either directly ligated to produce Δ97–127, Δ145–165 (pQΔRII23), Δ233–442, and Δ419–464 or treated with Klenow enzyme or T4 DNA polymerase to create blunt ends and then ligated to produce Δ96–166, Δ166–243 (pQΔRII3), Δ461–534, Δ533–625, Δ628–677, and Δ745–880, or, after treatment with Klenow enzyme or T4 DNA polymerase, ligated in the presence of an excess amount of octameric *Bgl*II linker to produce Δ96–243, Δ245–338, Δ166–338, Δ340–416, Δ339–625, Δ419–625, and Δ676–743. The deletion mutant (Δ839–845) with a deletion of only 7 amino acids was created directly by in vitro mutagenesis. To be sure that all internal deletions were in frame, the junctions in all deleted mutants were determined by DNA sequencing. Finally, to express these ARGRII deletions in yeast cells, *Bam*HI-*Bam*HI fragments of wild-type (pQH7) or deleted *ARGRII* genes were inserted into a low-copy-number plasmid pFL38 (pUC19 *URA3 CEN6 ARS*, a gift from F. Lacroute), leading to all the different pQH strains listed in Table 2. To test the binding capacity of the deleted ARGRII proteins, *Bam*HI-*Bam*HI fragments were also inserted into a high-copy-number plasmid pFL44 (pUC19 *URA3* 2 μm, a gift from F. Lacroute).

Construction of the *lexA-ARGRII* fusions. To construct *lexA-ARGRII* fusions, plasmids pQH46 and pQH51 (Fig. 2) had to be constructed to allow correct phasing for all fusions. The *Bam*HI-*Bam*HI fragment from plasmid pRB500 (a gift from R. Brent [5]) was inserted into pBS(+SK) to yield pQH46. From pQH46, a 1.8-kb *Sma*I-*Xmn*I fragment, which contains the promoter of the *ADHI* gene (P_{ADHI}) and a portion of the *lexA* gene encoding 87 amino-terminal residues of lexA protein, was inserted into pBS(+SK) at the *Kpn*I site, which had been blunt ended by T4 DNA polymer-

TABLE 1. Enzymes used in construction of internal deletions of ARGRII and the new created junctions

Derivatives of deleted ARGRII	Enzymes	Amino acid sequence in junctions[a]
Δ97–127	*Cla*I	96 126 NID NKT
Δ145–165	*Sac*I	144 166 TDK SSA
Δ96–167	*Cla*I, *Sac*I, T4 DNA pol.[b]	95 168 NIA ASI
Δ166–243	*Sac*I, *Bst*EII, T4 DNA pol.	165 244 LE VTL
Δ96–243	*Cla*I, *Bst*EII, Klenow, 8-mer linker	95 244 I adl VTL
Δ246–339	*Bst*EII, *Bgl*II, Klenow, 8-mer linker	245 340 VT rsg SL
Δ166–339	*Bst*EII, *Bgl*II, Klenow, 8-mer linker	165 340 E vtrsg SL
Δ341–417	*Bgl*II, *Nco*I, Klenow, 8-mer linker	340 418 RS rsa WK
Δ419–464	*Nco*I	418 465 NPW NYK
Δ233–442	*Xho*I	232 443 IS RNA
Δ341–625	*Bgl*II, *Eco*RI, Klenow, 8-mer linker	340 627 RS rs EFV
Δ418–625	*Nco*I, *Eco*RI, Klenow, 8-mer linker	417 626 P cr SEFV
Δ461–534	*Kpn*I, *Aat*II, T4 DNA pol.	460 535 S s V
Δ533–625	*Aat*II, *Eco*RI, T4 DNA pol.	532 626 E EFV
Δ628–677	*Eco*RI, *Kpn*I, T4 DNA pol.	627 678 EF DSL
Δ676–743	*Kpn*I, *Mlu*I, T4 DNA pol.	675 744 I adl RVI
Δ745–880	*Mlu*I, *Kpn*I, T4 DNA pol.	744 GKR a stop

[a] Numbers indicate the positions of amino acids in the ARGRII protein; lowercase letters represent amino acids read from the linkers or from codons created by ligating two fragments. The one-letter code for amino acids is used.
[b] pol., Polymerase.

ase treatment, to produce plasmid pQH51 (Fig. 2A). To construct pLAII96, pQBSII4 was digested with *Sma*I and *Cla*I and the resulting vector fragment was blunt ended with Klenow enzyme and ligated to the *Sma*I-*Xmn*I fragment from pQH46. Figure 2B describes the sequence of the *LexA*-vector junction allowing construction of in-frame *LexA-X* gene fusions. pLAII163 was constructed in two steps. In the first, the 1.7-kb *ARGRII Hin*dIII-*Hin*dIII fragment from pQBSII4 was blunt ended with Klenow enzyme and inserted at the *Sma*I site of pQH51. Second, the *Bst*EII-*Xba*I fragment from this plasmid was replaced by the *Bst*EII-*Xba*I fragment of *ARGRII* from pQBSII4 which now contains the entire C-terminal sequence of *ARGRII*. pLAIIΔ23 was constructed directly by inserting the *Sma*I-*Xmn*I fragment from pQH46 into pQΔRII23 at the *Cla*I site. To construct pLAIIΔ3, the *Cla*I-*Eco*RV fragment was isolated from pQΔRII3, filled in with Klenow enzyme, and inserted at the filled-in *Xho*I site of pQH51. For all *lexA-ARGRII* fusions described above, the sequence of the junction between *lexA* and *ARGRII* genes was determined by DNA sequencing to be sure that the fusions were in frame. All of the other *lexA-ARGRII* fusions in which the portions of *ARGRII* contain various deletions were constructed by replacing the *Bst*EII-*Xba*I fragment of pLAII96 with those of various deleted *ARGRII* plasmids. The *Bam*HI-*Bam*HI fragment of pAAH5, a yeast expression vector made by G. Ammerer (1), was replaced by the *Bam*HI-*Bam*HI fragments containing P_{ADHI} and various *lexA-ARGRII* fusions.

Yeast transformation. Plasmids were introduced into yeast cells by using the method of Hinnen et al. (18) with some modifications. Glucuronidase/arylsulfatase (from Boehringer, Mannheim, Federal Republic of Germany) was used instead of glusulase to generate spheroplasts, and regeneration medium was the selective medium containing 3% agar and 1 M sorbitol.

Enzyme assay. Ornithine carbamoyltransferase (EC 2.1.3.3) (OTCase) and arginase (EC 3.5.3.1) activities were measured as described previously (32). To measure the activities of various *LexA-ARGRII* fusions, yeast cells were first transformed with *LexA-ARGRII* plasmids and reporter plasmid p1840 (24) that contained the *GAL1-LacZ* fusion gene and a *LexA* operator. The various activities of *LexA-ARGRII* fusions were determined by their ability to activate β-galactosidase (EC 3.2.1.23) production. β-Galactosidase activity was assayed by the method described by Miller (34).

DNA manipulation and DNA sequencing. Restriction reactions were performed as recommended by the supplier. Filling in by Klenow enzyme and creation of blunt ends by T4 DNA polymerase were done by the methods of Maniatis et al. (26). DNA fragments were isolated from agarose gels by electroelution. Plasmid DNA was prepared by the alkaline lysis method (4) or rapid-boiling lysis (19).

Both single-strand DNA and denaturated double-strand DNA were used as templates for DNA sequencing. For preparation of single-strand DNA template, *E. coli* XL1-B harboring phagemid pBS(+SK)-*ARGRII* recombinant plasmids was grown overnight. Cells were diluted (1:20) into fresh medium and grown again until the optical density at 660 nm reached 0.4. Helper phage R408 was added to the mixture, and cells were grown for another 6 to 7 h. Single-strand DNA templates were prepared as described by Messing (33). For double-strand DNA sequencing, pBR322-*ARGRII* recombinant plasmids were denatured with alkalin (7). DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (40) with home-made oligomers as primers.

Gel retardation assay. (i) Extract preparation. The *argRII* strain 10R34d-II was transformed with different *ARGRII* deletions inserted into the multicopy plasmid pFL44 (pUC19 *URA3* 2 μm). We used the French pressure cell to make extracts from 1 liter of culture grown overnight on M.am plus 1 mg of arginine per ml to a cell concentration of 4×10^6 cells per ml. Proteins were extracted in extraction buffer by the method of Pfeifer et al. (36) and precipitated with $(NH_4)_2SO_4$ to 100% saturation. The protein pellet was resuspended in heparin-buffer A (10 mM Tris · HCl [pH 8.0], 50 mM $(NH_4)_2SO_4$, 1 mM zinc EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol), and applied to a 10-ml heparin-Sepharose column equilibrated with the same buffer. The column was washed with 40 ml of buffer A. Nucleic acid-binding proteins were collected by washing the column with 40 ml of buffer B [buffer A with 800 mM $(NH_4)_2SO_4$]. Proteins were then reprecipitated with solid $(NH_4)_2SO_4$ to 100% saturation. The pellet was resuspended in buffer C (buffer A, but with 20 mM Tris · HCl and 25% glycerol) and dialyzed thoroughly. The protein concentrations were determined by using the Folin assay. Proteins were stored at −80°C.

(ii) DNA probe. The DNA used in these assays is a 160-bp *Alu*I-*Alu*I fragment containing the control region of the *ARG5,6* gene (30). This fragment was end labeled with [γ-^{32}P]ATP (Amersham) by using polynucleotide kinase by the standard method (26).

(iii) Binding assay. The binding reaction took place in a 20-μl volume containing 4 mM Tris · HCl (pH 8.0), 40 mM NaCl, 4 mM $MgCl_2$, 5% glycerol, 1 μg of carrier DNA (calf

TABLE 2. Specific activities of OTCase and arginase in strains harboring various argRII deletion plasmids

Strains	Plasmids[a]	Deletions[b]	OTCase sp act[c]		Arginase sp act[c]	
			M.am	M.am + arginine	M.am	M.am + arginine
BJ210 (*argRII-10*)			62	58	3	5
10R34d-II (*argRII*::*CARI*)			60	61	4	4
10R34d-II (*argRII*::*CARI*)	pFLI *argRII*::*CARI*		58	55	4	5
10R34d-II (*argRII*::*CARI*)	pQH7	no Δ	23	10	26	77
	YEP43	no Δ	44	19	38	181
	YEP34	Δ1–12	46	20	17	143
	YEP39	Δ1–60	58	53	4	6
	pQΔRII20	Δ97–127	66	55	4	5
	pQΔRII23	Δ145–165	68	64	27	55
	pQΔRII2	Δ96–166	62	64	4	5
	pQΔRII3	Δ166–243	8	2	12	16
	pQΔRII7	Δ96–243	61	58	3	3
	pQΔRII8	Δ245–338	10	3	33	87
	pQΔRII16	Δ166–338	15	8	18	25
	pQΔRII9	Δ340–416	62	61	3	3
	pQΔRII13	Δ419–464	65	63	3	3
	pQΔRII6	Δ233–442	62	57	3	3
	pQΔRII5	Δ339–625	61	69	3	3
	pQΔRII10	Δ419–625	74	62	3	3
	pQΔRII19	Δ461–534	71	63	4	3
	PQΔRII15	Δ533–625	65	59	2	3
	pQΔRII17	Δ628–677	26	7	18	74
	pQΔRII18	Δ676–743	56	53	5	3
	pQΔRII22	Δ745–880	63	57	5	3
	pQΔRII21	Δ839–845	38	15	45	138

[a] All pQ plasmids are low-copy-number plasmids; YEP34, YEP39, and YEP43 plasmids are multicopy plasmids in which the *ARGRII* gene is directed by the *GAL10* promoter. The strains transformed with the YEP plasmids were grown on galactose (2%) instead of glucose as the carbon source.

[b] The numbers correspond to the positions of the first and last amino acids which are deleted.

[c] OTCase and arginase specific activities are expressed in micromoles of citrulline and urea, respectively, per hour per milligram of protein produced at 30°C. These activities are summarized in Fig. 3.

thymus DNA), 10 μg of purified proteins, 5 mM L-arginine, and 1 ng of labeled DNA probe. This reaction mix was incubated for 20 min at 25°C and loaded immediately into a 5% polyacrylamide gel (acrylamide to bis-acrylamide [wt/wt] in TEB buffer). Electrophoresis was carried out for 4 h at 120 V. The gel was dried and autoradiographed.

Western blot analysis. Western immunoblot analysis was performed by the method of Neal-Burnette (35). Crude protein extracts from exponentially growing cells were prepared in buffer C (see above). Total protein (100 μg) was subjected to electrophoresis in a 10% polyacrylamide gel containing sodium dodecyl sulfate as described by Laemmli (23). Protein was transferred to a nitrocellulose membrane (Sartorius) by electrotransfer at room temperature for 1.5 h at 80 mA in a buffer containing Tris · HCl (pH 8.3), 192 mM glycine, and 20% (vol/vol) methanol. The nitrocellulose filter was then processed to detect protein by enzyme-linked immunodetection after Western blot analysis (Protoblot A.P system; Promega). The primary antibodies were rabbit anti-LexA (a gift from R. Brent).

RESULTS

Deletions created in *ARGRII*. By using amino acid sequence analysis, we defined several regions in ARGRII. The N-terminal portion from amino acids 21 to 48 contains six cysteine residues similar to zinc finger motifs present in several DNA-binding proteins (31). According to previous results, this region overlaps nuclear targeting sequences of ARGRII (3). Two other potential DNA-binding regions (helix-turn-helix motif comparable to the bacterial DNA-binding motif but not to homeo-domains) are located between amino acids 426 and 444 and between amino acids 839 and 845. The transcriptional activation domain of several regulatory proteins appears to be rich in acidic amino acids (17, 21, 25, 37). In ARGRII, three acidic regions are found, between amino acids 101 and 113, 564 and 630, and 859 and 874. Two other more specific regions present in ARGRII have been identified: an RNase-like region (amino acids 133 to 203) and a region of homology to the *E. coli* arginine repressor ARGR (amino acids 454 to 551) (29). To test the implication of these different regions in ARGRII function, we constructed a set of deletions, some of which required the creation of restriction sites by in vitro mutagenesis (see Materials and Methods). The low-copy-number plasmid pFL38 (pUC19 *URA3 CEN6 ARS*) with different *ARGRII* deletions was used to transform an *argRII* strain (genomic disruption of part of the *ARGRII* gene). The ability of mutant derivatives to repress the anabolic gene *ARG3* and to induce the catabolic gene *CAR1* were checked by enzymatic assays of OTCase (the product of the *ARG3* gene) and arginase (the product of the *CAR1* gene) in the presence or absence of arginine in the growth medium. The reference levels of OTCase and arginase in *argRII* mutant strains are listed at the top of Table 2 (BJ210 [*argRII-10*] or 10R34d-II [*argRII*::*CAR1*]). The recipient strain used for transformation clearly has an argRII phenotype, and the disrupted *argRII* gene is incapable of restoring repression of OTCase and induction of arginase in the presence of arginine, even on a multicopy plasmid. In these experiments, some deleted *ARGRII* genes are expressed from the *GAL10* promotor (YEP34, YEP39, YEP43), whereas the others are under the control of the *ARGRII* promoter. Deletions in ARGRII lead to three classes of phenotypes (Table 2; Fig. 3). Only 3

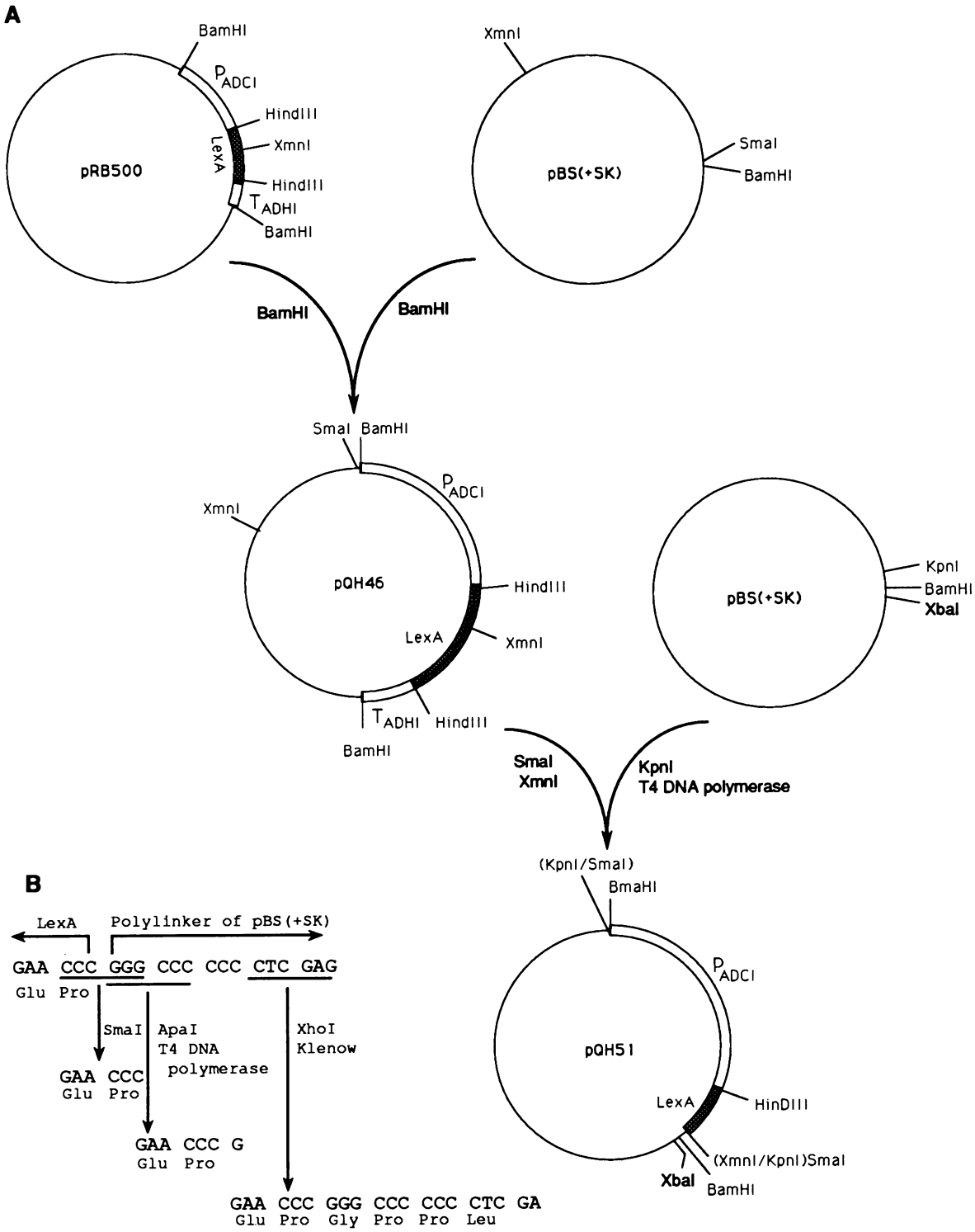

FIG. 2. Construction of plasmids pQH46 and pQU51. (A) Symbols: □, insert; ——, vector; ▩, portion of LexA. The restriction sites that have been destroyed in pQH51 are in parentheses. (B) Junction sequences between LexA and the polylinker of the Bluescript vector in pQH51. *Sma*I, *Apa*I, or *Xho*I sites can be used to produce in-frame LexA fusions.

deletions out of 20 have no effect on ARGRII function: Δ1–12 (YEP34), Δ628–677 (pQΔRII 17), and Δ839–845 (pQΔRII 21). When the *ARGRII* gene is overexpressed, the first 12 amino acids can be removed without any effect on ARGRII function in vivo (compare OTCase and arginase levels in YEP34 with those in YEP43 containing the wild-type gene, both under the control of the *GAL10* promotor). However, the higher levels of arginase in YEP34 and YEP43

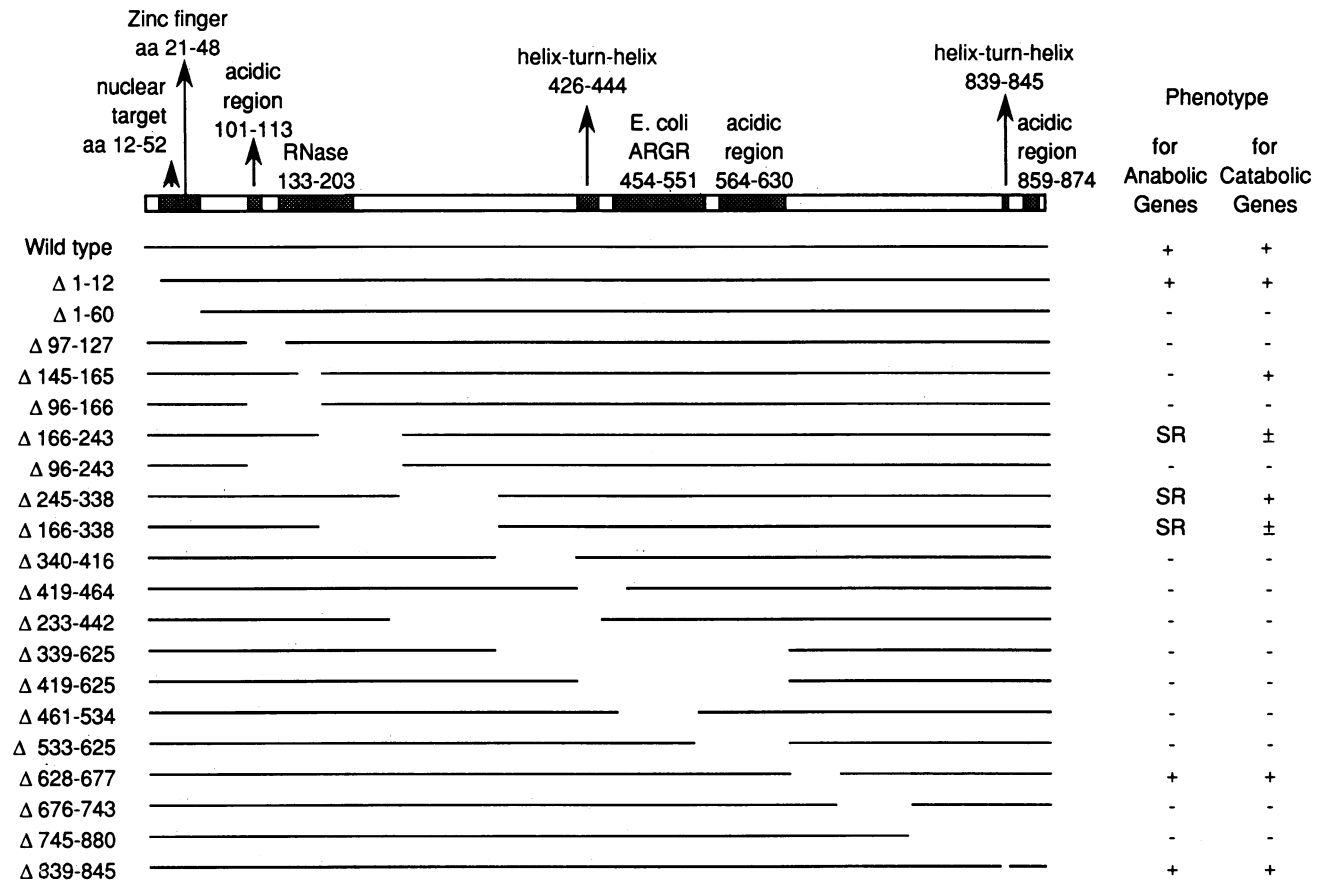

FIG. 3. Effect of deletions created in the *ARGRII* gene on repression and induction of arginine anabolic and catabolic enzymes. The organization of the ARGRII protein on the basis of amino acid sequence analysis is shown at the top of the figure. The numbers correspond to the position of the amino acids starting and ending the different regions. This figure also shows the different amino acid sequences that were deleted in ARGRII. Deletions were constructed as described in Materials and Methods and Table 1. The bars indicate which part of the protein is present. The numbers indicate the endpoints of each deletion. Plasmids containing *ARGRII* wild-type or deletion mutants were introduced into an *argRII* strain (10R34d-II). OTCase and arginase activities were assayed from cells grown in M.am with or without arginine (1 mg/ml). These activities are summarized in this figure: +, wild-type repression for an anabolic gene and wild-type induction for a catabolic gene; −, absence of repression or induction; ±, difference from wild-type values; these differences are discussed in the text. SR means super repression.

strains are probably due to an increase in the copy number of these plasmids as if ARGRII protein was limiting for induction, whereas repression is reduced when ARGRII is overexpressed, as reported by Dubois and Messenguy (15). The difference between the arginase activities of YEP34 and YEP43 strains on both media is not significant, as the level of variability in these measurements is about 20%. The deletion of the C-terminal helix-turn-helix motif (pQΔRII 21) also has no effect on ARGRII function. Thirteen deletions lead to a typical argRII phenotype: absence of repression of OTCase (derepressed level around 60 to 70 units) and absence of induction of arginase (basal level around 3 to 5 units) if one compares the arginase and OTCase levels in strains transformed with these plasmids and with the wild-type plasmid (pQH7), which is used as the wild-type control. Among these, Δ1–60 (YEP39), which removes both the cysteine-rich region and the nuclear target signal (3, 28), has lost its in vivo activity. Deletion of a segment containing the internal helix-turn-helix motif (pQΔRII13) also leads to a mutant phenotype. It is striking that most of the deletions simultaneously impair the regulation of anabolism and catabolism. Only three deletions affect these two regulations differently. The deletion Δ145–165 (pQΔRII23) leads to an argR phenotype for anabolic regulation, while induction is rather normal (compared with the basal arginase level of any *argR* mutant grown on M.am plus arginine). This deletion is located in the region of highest homology with retroviral RNases. The deletion Δ245–338 (pQΔRII8) also affects the regulation of anabolism but leads to a stronger repression of OTCase synthesis. This enzyme level is already significantly reduced when cells are grown on M.am, probably because of a better repression by the arginine endogenous pool. The deletions Δ166–243 (pQΔRII3) and Δ166–338 (pQΔRII16) have two effects: an absence of induction of catabolism but significant and reproducible higher arginase basal levels and a stronger repression of anabolism (5R in Fig. 3). The lower OTCase level in these mutants than in the strain transformed with pQH7 (even when grown on M.am) could be due to a better repression by the arginine endogenous pool. From the data presented in Fig. 3 and Table 2, we conclude that most of the deletions lead to simultaneous loss of anabolic and catabolic regulation. At least 80 to 90% of this protein is indispensable for its function. However, the stability of the ARGRII-deleted proteins could be impaired, leading to a loss of

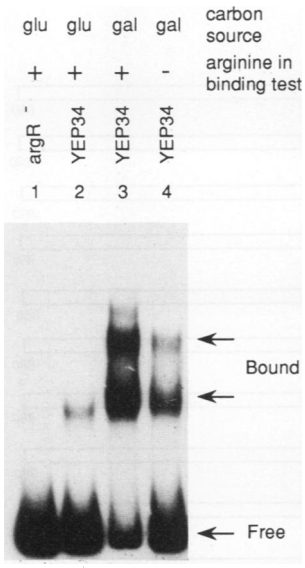

FIG. 4. Detection of ARGRII-dependent protein complexes to *ARG5,6* control region. The end-labeled 160-bp *Alu*I-*Alu*I DNA fragment (about 1 ng) containing the control region of *ARG5,6* was incubated with yeast extracts (10 μg) (see Materials and Methods) prepared from strain 5716c grown on M.am-glucose-arginine (lane 1) or strain 02431a transformed with p*GAL10 ARGRII* (YEP34) grown on M.am-arginine-uracil (20 μg/ml) (lane 2) or M.am-galactose-arginine-uracil (lanes 3 and 4). In all in vitro assays, 5 mM L-arginine was added, except in lane 4.

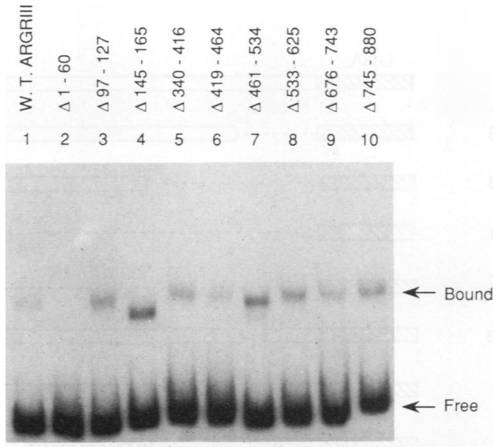

FIG. 5. Effect of deletions in ARGRII on binding activity. Extracts from strain 10R34d-II transformed with wild-type (W.T.) pARGRII on a multicopy plasmid (lane 1), pARGRIIΔ1–60 (galactose as the carbon source) (lane 2), pARGRIIΔ97–127 (lane 3), pARGRIIΔ145–165 (lane 4), pARGRIIΔ340–416 (lane 5), pARGRIIΔ419–464 (lane 6), pARGRIIΔ461–534 (lane 7), pARGRIIΔ533–625 (lane 8), pARGRIIΔ676–743 (lane 9), and pARGRIIΔ745–880 (lane 10) were incubated with the 160-bp *Alu*I-*Alu*I DNA fragment containing the control region of the *ARG5,6* gene, in the presence of 5 mM L-arginine, according to Materials and Methods.

regulatory function although the deleted sequences could be dispensable. This question is addressed in the following section.

In vitro binding of *ARGRII* mutants to the *ARG5,6* control region. We have developed an in vitro binding assay to show the specific binding of the ARGR proteins to the control region of different arginine coregulated genes (see Materials and Methods). An arginine-stimulated binding to the *ARG5,6* promoter requires the overexpression of the *ARGRII* gene (Fig. 4). No DNA-protein complexes are observed with extract from a triple *argRI argRII argRIII* strain (lane 1). Binding is also absent when the test is performed with extracts from the *argRII::CAR1* recipient strain (see Fig. 1, lane 13, in the accompanying paper [15a]). Therefore, it is unlikely that the mutated protein of this strain would interfere in the assay with the binding of the overproduced deleted ARGRII proteins tested. A weak binding is observed when *ARGRII* gene is poorly expressed (YEP34 grown on glucose; Fig. 4, lane 2). The binding is strongly enhanced when the protein extract contains overproduced ARGRII protein (YEP34 grown on galactose [lane 3]). A weaker binding is observed when the assay is performed in the absence of arginine in the binding assay (lane 4). The integrity of ARGRI and ARGRIII proteins is also required for observation of the formation of DNA-ARGR complexes (15a, 16). To test the DNA-binding capacity of the deleted ARGRII proteins, strain 10R34d-II was transformed with the deleted genes inserted into the multicopy number plasmid pFL44. In this experiment, expression of the different ARGRII deleted genes is under the control of the ARGRII promoter. Therefore, as described in the accompanying paper (15a), only one protein-DNA complex is observed. Strains transformed with these plasmids behave as the strains described in Table 2. As already mentioned, the altered ARGRII protein produced in the recipient strain should not interfere in our in vitro binding assays, as it is not overexpressed. Only deletion of the first 60 amino acids (zinc finger) leads to the loss of binding of the ARGR complex to target DNA (Fig. 5, lane 2). As we have no antibody against ARGRII protein, it is impossible to show whether the protein devoid of the zinc finger motif is stable. The binding that we observed with the other deleted proteins is still stimulated by arginine (data not shown), indicating that none of the deletions impair the interaction with the effector. However, it is not known whether the ARGRII protein is the target of arginine. The mobility of the ARGR-DNA complex is not significantly affected by the presence of the different deletions in ARGRII protein except for Δ145–165 (see Discussion). In this case, the slight difference in mobility is reproducible only if 5 μg of DNA carrier is added to the binding assay instead of 1 μg, as described in Materials and Methods. Therefore, most of the deletions created in ARGRII do not affect the binding of ARGR proteins to the *ARG5,6* promoter under our in vitro conditions, with overexpressed ARGRII and partly purified proteins. This may not represent the in vivo situation.

As the deleted ARGRII proteins can still participate in the formation of a DNA-protein complex, this indicates that they are stable and suggests that the loss of ARGRII function observed in vivo is not the result of protein instability. Thus, most *ARGRII* deletions seem to affect ARGRII function.

Analysis of the lexA-ARGRII proteins. Brent and Ptashne (6) have shown that the hybrid protein lexA-GAL4 (in which the GAL4 DNA-binding domain has been replaced by the lexA DNA-binding domain) is capable of activating the transcription of a gene located downstream of the LexA operator region. This technique was used to define transcription activating regions present in several regulatory proteins (20, 38). As ARGRII is implicated in the induction of expression of arginine catabolic genes, a similar approach was used to define the part of the protein responsible for the

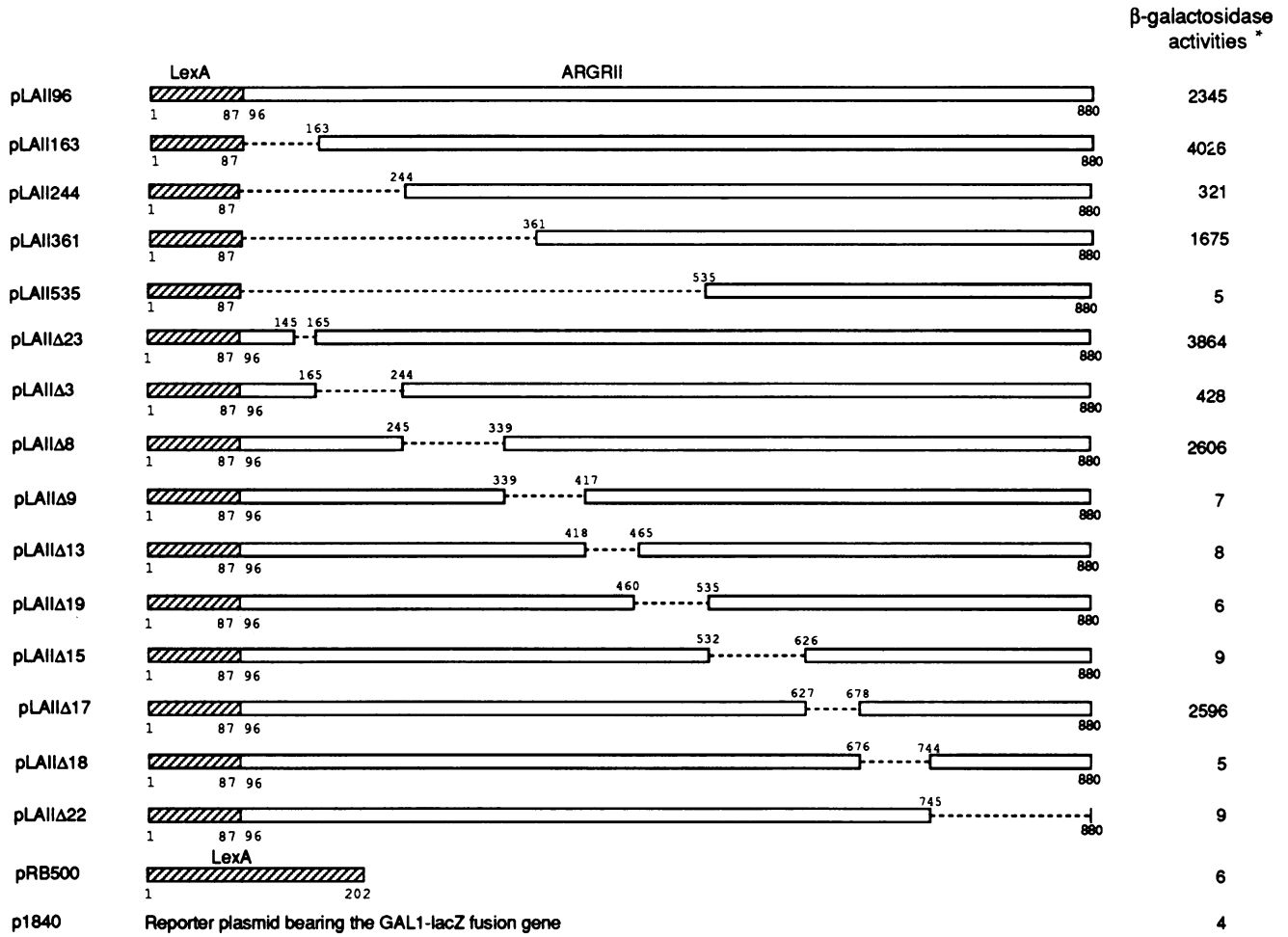

FIG. 6. Determination of β-galactosidase activities in a *lexA-ARGRII* fusion and its deleted derivatives. The different constructions are described in detail in Materials and Methods. Symbols: ▨, lexA part (amino acids 1 to 87); □, ARGRII part (amino acids 96 to 880). The numbers around the dashed lines indicate the exact endpoints of each deletion. Strain 1C2123a was cotransformed with the reporter plasmid p1840 and the various plasmids in the list on the left-hand side of the figure. Specific β-galactosidase activities in transformed cells grown on minimal medium were determined and are presented on the right-hand side of the figure. β-Galactosidase specific activity is expressed in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein. *, Identical β-galactosidase activities are obtained if the cells are grown on M.am plus arginine and if the recipient strain is *ARGRI ARGRIII* instead of being *argRI argrIII* as in strain 1C2123a.

function. We constructed a fusion of the 87 amino-terminal residues of lexA protein to the 785 carboxy-terminal amino acids of ARGRII. The fusion gene was inserted into a plasmid that directs the synthesis of lexA-ARGRII under the control of the *ADH1* promoter (described in Materials and Methods). We cotransformed *S. cerevisiae* 1C2123a (*ura3 leu2 argRI argRIII*) with this plasmid and another one (p1840) bearing part of the *GAL1* gene fused to *lacZ* and under the control of the lexA operator.

To determine the amount of transcription of the *GAL1-lacZ* fusion gene, we measured β-galactosidase activity in these doubly transformed cells grown with and without arginine. The 785 carboxy-terminal amino acids of ARGRII are capable of activating transcription of the gene adjacent to the lexA operator (pLAII96). Indeed, the β-galactosidase activity in this strain is strongly increased compared with the activity in a strain transformed with either pRB500 (p*ADH1 LexA* 2 μm *LEU2*) or p1840 (p*oplexA GAL1 lacZ* 2 μm *URA3*) (5).

In GAL4 and GCN4 activator proteins (6, 20, 25), a short acidic region fused to the lexA DNA-binding region is sufficient to activate transcription. To define more precisely the part of the protein involved in transcription activation, we constructed 14 other *lexA-ARGRII* fusions. Deletions up to amino acid 361 do not destroy the activation capacity of lexA-ARGRII protein, although a reduction is observed for two of them (pLAII244 and pLAIIΔ3). However, all deleted proteins from amino acids 339 to 880 totally lose activation function, except Δ627–678 (pLAIIΔ17), which is a very short deletion. Although the region between amino acids 339 and 880 contains two acidic stretches, these are not sufficient to ensure activation of transcription, as deletions outside of acidic regions lead to the loss of activation.

This activation is independent of the presence of arginine in the growth medium (Fig. 6) and, unlike the arginine system, does not require functional ARGRI and ARGRIII proteins, as the recipient strain is *argRI argRIII*. Indeed, β-galactosidase activities are not significantly different if the recipient strain is *ARGRI* and *ARGRIII* (Fig. 6).

To ensure that the lack of activation capacity of some

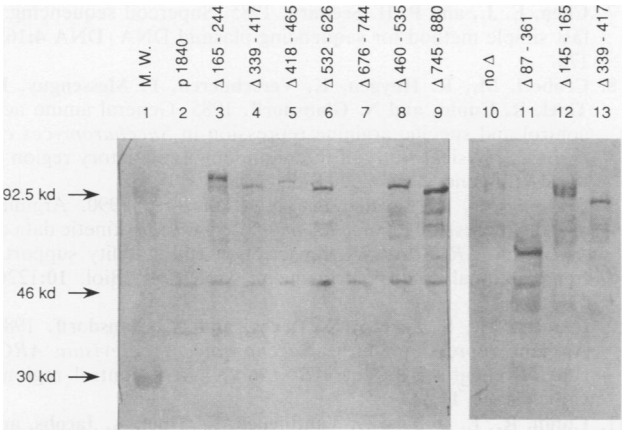

FIG. 7. Detection of lexA-ARGRII fused proteins by Western blot. Each lane contains 100 μg of total yeast protein. After overnight electrophoresis at 40 V on a 10% polyacrylamide gel containing sodium dodecyl sulfate, proteins were electrotransferred to a nitrocellulose membrane as described in Materials and Methods. The lexA-ARGRII protein was visualized by immunodetection with lexA antibodies and anti-rabbit antiserum conjugated with alkaline phosphatase. Extracts were prepared from strain 1c2123a (grown on M.am) transformed with the reporter plasmid (p1840) alone (lane 2); with pLAIIΔ3 plus p1840 (lane 3); with pLAIIΔ9 plus p1840 (lane 4); with pLAIIΔ13 plus p1840 (lane 5); with pLAIIΔ15 plus p1840 (lane 6); with pLAIIΔ18 plus p1840 (lane 7): with pLAIIΔ19 plus p1840 (lane 8); with pLAIIΔ22 plus p1840 (lane 9); with pLAII96 plus p1840 (lane 10); with pLAII361 plus p1840 (lane 11); with pLAIIΔ23 plus p1840 (lane 12) and with pLAIIΔ9 plus p1840 (lane 13). Lane 1 contains standard protein molecular mass markers in kilodaltons (kd). Lanes 1 to 9 and lanes 10 to 13 were obtained independently.

chimeric lexA-ARGRII proteins is not due to their instability in vivo, we have measured their presence by Western blot with antibodies raised against lexA (Fig. 7). In all extracts, except in the one from strain pLAIIΔ18 (Δ676–744) we can detect the presence of the chimeric proteins. However, these proteins seem to be less stable in vitro, because degradation products were observed in most of our experiments. The different sizes of the deleted proteins are in agreement with the lengths of the deletions.

DISCUSSION

Functional analysis of eukaryotic regulatory proteins has revealed that they contain multiple domains that are separable structurally and functionally. Our analysis of the ARGRII protein was aimed at understanding the mechanism by which, in cooperation with ARGRI and ARGRIII, ARGRII controls the expression of arginine anabolic and catabolic genes. These latter genes are also regulated by three other gene products: CARGRI, CARGRII, and CARGRIII (13, 14).

Deletion analysis of the *ARGRII* gene shows that in contrast to other regulatory proteins, 80 to 92% of this protein is required for its functions. The binding domain of ARGRII seems to be localized in its N-terminal portion containing a zinc finger motif. Deletion of this part of the protein leads to an argR phenotype, for anabolic as well as for catabolic genes, and the deleted ARGRII protein has lost the ability to bind its cognate DNA sequence in vitro. However, we have no proof that this protein is stable. All other *ARGRII* deletions tested do not impair in vitro DNA binding, and the mobility of the protein-DNA complex is only slightly affected in Δ145–165. As the ARGR system contains several proteins (15a), the mobility of the protein-DNA complex is probably not directly related to the size of each protein.

Two other putative DNA-binding motifs (helix-turn-helix with no homology with homeo-domains) were positioned between amino acids 426 and 444 and between amino acids 839 and 845. The C-terminal helix-turn-helix is dispensable for ARGRII function, whereas a segment containing the internal motif is necessary for ARGRII activity in vivo but does not affect DNA binding in our in vitro analysis. This result does not rule out the possibility of its participation in an in vivo binding of the ARGR protein to DNA. Only one other deletion (Δ628–677) has no effect on ARGRII function. Because we have shown that most of the deleted proteins are present in our in vitro binding assay, we can conclude that 80 to 92% of the proteins are indispensable for ARGRII activity. Of these, 13 deletions are *argR* for anabolism and catabolism. Only deletion Δ145–165 clearly dissociates both regulations, as this mutant is totally impaired in repression of anabolic genes but induction of catabolism is rather normal. These 21 amino acids are localized in the region presenting the best identity with some RNases. As postulated in our model, this region could be implicated in the posttranscriptional control of arginine anabolic genes (29). Results obtained in vivo and in vitro localize the arginine control region of anabolic genes between the TATA box and the initiation of transcription for *ARG3* (8) and partially in the transcribed region for *ARG5,6* (30). The ARGR complex could bind to these DNA regions, and the RNase-like region of ARGRII could interact with the nascent mRNA in the presence of arginine and prevent, for example, its capping. This model does not imply that ARGRII has an RNase activity, but rather a RNA-binding site.

Two other deletions adjacent to Δ145–165, Δ166–243 and Δ245–338 (or Δ166–338), lead to a particular phenotype. We observe a lower OTCase level in these mutants even when grown on M.am, which could be the result of a better repression by the arginine endogenous pool and this modified ARGRII protein, as if the region between amino acids 166 and 338 had a negative effect on the posttranscriptional function of ARGRII.

Our analysis of the different deletions does not allow us to position the arginine-binding site on ARGRII, as was first postulated on the basis of a certain degree of identity between ARGRII and the *E. coli* ARGR repressor. The binding of the deleted ARGRII proteins to the *ARG5,6* promoter is still stimulated by arginine. This question is still open.

As ARGRII possesses three acidic regions, we also tested the activation capability of these regions by using the lexA system. In this system the chimeric protein lexA (1 to 87)-ARGRII (96 to 880) indeed has a transcriptional activator capacity. This activation capacity is independent of the presence of arginine in the growth medium and of ARGRI and ARGRIII gene products. There is a parallel between the activation capacity of ARGRII in the lexA fusions and induction of the catabolic *CAR1* gene product (arginase) except for the deletion Δ96–244, leading to a total loss of arginase induction and only in a drop of activation capacity. Similar experiments carried out with the *lexA-ARGRI* and *lexA-ARGRIII* fusion genes do not show any activation capacities of these two proteins (39). These results lead us to propose a revised model for regulation of the catabolic pathway by ARGR and CARGR gene products.

From measurements of the steady-state mRNA levels in arginine anabolic and catabolic genes, we had proposed a model in which the arginine-specific regulation occurs at a posttranscriptional level. Our results are still in agreement with this proposal for the anabolic pathway. The mRNA values obtained for *CAR1* did not show the variations expected on basis of arginase activity, measured after growth on M.am, M.am plus arginine, or arginine as the sole nitrogen source. In contrast, Sumrada and Cooper (41) proved a good correlation between *CAR1* mRNA and arginase levels after growth on M. proline and M. arginine. These observations, together with the role of ARGRII as a transcriptional activator for arginine metabolism, lead us to propose a new model with a cascade of transcriptional and posttranscriptional regulation. In this new model the arginine upstream activation sequence located upstream of the *CAR1* TATA box (11, 29) is the target of the ARGR activator regulatory complex. This binding competes with that of CARGRI protein, acting as a repressor. Indeed, the target of CARGRI overlaps the arginine upstream activation sequence (11, 30). The expression of the *CAR1* mRNA flux produced in the presence of arginine could then be modulated by the action of CARGRII and CARGRIII when ammonia is still present. The posttranscriptional role of CARGRII and CARGRIII is supported by the fact that the function of these two repressors requires the presence of the coding part or 3′ end of *CAR1* and *CAR2* (11, 12).

In conclusion, 92% of the ARGRII protein is necessary for its anabolic function and 80% is necessary for its catabolic function. We can, however, define three domains in this protein: a putative DNA-binding domain localized in the N-terminal portion, a region more involved in the repressor activity and situated around the RNase-like domain, and a large activation domain that contains two acidic regions. This domain is also necessary for proper repression. As ARGRII has a double regulatory function and has to interact with other proteins to bind to DNA, it is not surprising that almost the entire protein is required for efficient regulation.

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