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Functional Analysis of Met4, a Yeast Transcriptional Activator Responsive to *S*-Adenosylmethionine

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Transcription of the genes necessary for sulfur amino acid biosynthesis in *Saccharomyces cerevisiae* is dependent on Met4, a transcriptional activator that belongs to the basic region-leucine zipper protein family. In this report, we show that one mechanism permitting the repression of the sulfur network by *S*-adenosylmethionine (AdoMet) involves inhibition of the transcriptional activation function of Met4. Using a wide array of deleted LexA-Met4 fusion proteins as well as various Gal4-Met4 hybrids, we identify the functional domains of Met4 and characterize their relationship. Met4 appears to contain only one activation domain, located in its N-terminal part. We demonstrate that this activation domain functions in a constitutive manner and that AdoMet responsiveness requires a distinct region of Met4. Furthermore, we show that when fused to a heterologous activation domain, this inhibitory region confers inhibition by AdoMet. Met4 contains another distinct functional domain that appears to function as an antagonist of the inhibitory region when intracellular AdoMet is low. On the basis of the presented results, a model for intramolecular regulation of Met4 is proposed.

Sulfur metabolism of the yeast *Saccharomyces cerevisiae* involves a set of enzymes catalyzing the biosynthesis of cysteine, methionine, and *S*-adenosylmethionine (AdoMet). This metabolic pathway comprises more than 20 unlinked genes, the expression of which is subject to a specific regulation: in response to an increase of the intracellular AdoMet, transcription of the sulfur genes is turned off (32, 34).

To date, the products of two genes are known to be involved in the transcriptional control of this metabolic network. The first one, encoded by gene *CBF1* (also *CEP1* or *CPF1*), belongs to the basic region–helix-loop-helix protein family which recognizes the DNA motif RCACGTG (where R is a purine) (1, 3, 23). As demonstrated by mutational analysis of the promoter region of gene *MET25*, this motif acts in transcriptional activation of the sulfur network (32). It is present in either one or two copies upstream of almost all of the sulfur genes and also constitutes the CDE1 element of all the centromeres of *S. cerevisiae* (14). Accordingly, inactivation of gene *CBF1* results both in methionine auxotrophy and in a 9- to 25-fold increase in the rate of mitotic chromosome loss (1, 3). However, the DNA-bound Cbf1 may not act by itself as a transcriptional activator, since experiments with LexA-Cbf1 fusion protein indicated that Cbf1 lacks a transcription activation domain (33). Gene *MET4* encodes the other known product mediating transcriptional control of the sulfur network. Cloning of this gene revealed that Met4, which acts as a transcriptional activator, belongs to the basic region-leucine zipper (bZIP) protein family (33).

Numerous studies on transcriptional activators have shown that these proteins contain separate domains involved in DNA binding and in carrying out transcriptional activation (26). From these studies, several types of eukaryotic transcription activators were defined and classified as acidic, glutamine rich, or proline rich, according to the composition of their activation domains (5, 24). However, it has not been clearly established how activation domains function, and not much is known about how their activity is regulated. In particular, acidic domains were generally thought to be amphipathic α helices (12) or acid blobs (28) that recruit the transcription factors by virtue of their high density of negative charges. Although supported by numerous studies, this view was recently challenged by the work of Leuther et al. (20). By mutations, these authors diminished the charged residues without affecting dramatically the transcriptional activation function. Further studies on transcriptional activators thus appear to be required to determine how these molecules work.

Inspection of the Met4 amino acid sequence did not allow determination of whether Met4 belongs to one of the established classes of transcriptional activators. To address this question, we decided to perform a functional analysis of Met4. These experiments have allowed us to identify the activation domain of Met4. Furthermore, we show that the transcriptional activation function of Met4 is negatively controlled by the elevation of intracellular AdoMet, and we identify two distinct domains of Met4 that are implicated in this regulatory response. A model explaining the AdoMet-mediated control of the Met4 function is proposed.

MATERIALS AND METHODS

Strains. For functional analyses, pLexM4Δ*i* plasmids were transformed into strain C170 (*MAT***a** *ade2 his3 leu2 trp1 ura3 met4*::*TRP1*) bearing also a *GAL1-lexAop-lacZ* gene fusion integrated at the *ura3* locus. The *GAL1-lexAop-lacZ* fusion reporter is deleted of UAS_{GAL} and instead contains four LexA operators at position −167 (13). plexM4Δ*i* plasmids were transformed into strain CD106 (*MAT*α *ade2 his3 leu2 trp1 ura3 met4*::*TRP1*). Plasmids expressing Gal4-Met4 fusion proteins were transformed into strain Y526 (*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 gal4 gal80*), which contains an integrated *GAL1-lacZ* gene reporter activated by UAS_{GAL} (2). Plasmids expressing LexA-Gal4-Met4 fusion proteins were transformed into strain C170.

Standard yeast media were prepared as described by Cherest and Surdin-Kerjan (4). Yeast transformations were performed as described by Gietz et al. (9). Transformants were selected on solid YNB medium containing appropriate auxotrophic supplements (27).

Plasmids. (i) LexA-Met4 derivatives. To create internal deletions in Met4, we first generated sets of amino-terminal and carboxy-terminal fragments of Met4. To generate N-terminal fragments, plasmid pM4-4 (33) was first linearized by *Xba*I and treated with nuclease BAL 31. Aliquots were phenol extracted at regular time intervals. DNA samples were treated with Klenow fragment to blunt ends and ligated to the linker *olfus*, which provides *Kpn*I-compatible ends and also contains an *Xho*I site and a half *Sma*I site (see below). DNA samples were

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then restricted by *Eco*RI. Deleted fragments were purified by electrophoresis on agarose gels, ligated into M13mp18 digested by *Eco*RI and *Kpn*I, and dephosphorylated. An equivalent procedure was used to generate C-terminal fragments except that (i) *Not*I and *Bam*HI were used in place of *Xba*I and *Eco*RI, respectively, and (ii) the deleted fragments were ligated into M13mp19 digested by *Bam*HI and *Kpn*I and dephosphorylated.

After sequencing about 170 M13 derivatives, internal deletions were achieved through the ligation, into plasmid pSH2-1 (13), of two fragments, one encoding a deleted N-terminal part of Met4 and the other encoding a deleted C-terminal part of Met4. M13 derivatives bearing *MET4* N-terminal fragments were cut by *Xho*I (provided by the linker *olfus*), treated to blunt ends with Klenow fragment or mung bean nuclease when required, and then cut by *Eco*RI. For M13 derivatives bearing C-terminal fragments, the same procedure was used except that *Bam*HI was used in place of *Eco*RI. Pairs of fragments were combined, ligated into plasmid pSH2-1 digested by *Eco*RI and *Bam*HI, and dephosphorylated. Correct in-frame ligations were ascertained by restriction analyses. Indeed, because of the linker *olfus*,

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P-CCCTCGAGGTAC-OH
OH-GGGAGCTC-OH
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correct ligation events created a unique restriction site. The site thus created depends on the treatment of the *Xho*I-generated ends: combination of two fragments bearing *Xho*I ends and not treated generated an *Xho*I site, combination of two fragments bearing *Xho*I ends converted to blunt ends by the use of Klenow fragment generated a *Pvu*I site, and combination of two fragments bearing *Xho*I ends converted to blunt ends by the use of mung bean nuclease generated a *Sma*I site. For all LexA-Met4 derivatives, the amino-terminal boundary of the Met4 part is residue 16.

(ii) Met4 derivatives. To express modified Met4 proteins, we first cloned the *MET4* promoter region into plasmid pRS316 (29), an autonomously replicating sequence centromeric plasmid containing the *URA3* gene. This was done by transferring the *Stu*I-*Eco*RI fragment of pM4-1 (33) to plasmid pRS316 which had been digested by *Hin*dIII, treated with Klenow fragment to blunt ends, digested by *Eco*RI, and dephosphorylated. The *Stu*I-*Eco*RI fragment of pM4-I contains 600 bp of the 5′ upstream region of *MET4* and the region encoding the first 15 amino acids of Met4. The resulting plasmid was digested by *Eco*RI and *Bam*HI and ligated with *Eco*RI-*Bam*HI fragments from plasmids pM4-1, pLexM4Δ12, pLexM4Δ25, and pLexM4Δ30, yielding plasmids pM4-5, pM4Δ12, pM4Δ25, and pM4Δ30, respectively.

(iii) Gal4-Met4 derivatives. Plasmids expressing derivatives of Met4 fused to the DNA-binding domain of Gal4 were derived from plasmid pGBT9. Plasmid pGBT9 contains the region encoding the first 147 amino acids from Gal4 followed by a polylinker (2). To fuse portions of Met4 to the DNA-binding domain of Gal4, we used M13mp18 derivatives containing 3′ deletions of the *MET4* gene that were obtained during the construction of modified LexA-Met4 fusion proteins. We transferred *Eco*RI-*Bam*HI fragments from these M13mp18 derivatives into plasmid pGBT9, which had been digested by *Eco*RI and *Bam*HI. In addition to the Gal4 and Met4 residues, all of the resulting fusion proteins contain the same C-terminal extension of 20 amino acids encoded by the polylinker sequence.

(iv) LexA-Gal4-Met4 derivatives. To express LexA-Gal4-Met4 fusion proteins, we first constructed the plasmid pLexGAD1, which contains the activation domain of Gal4 (residues 768 to 881) fused to the DNA-binding domain of LexA (residues 1 to 87). This plasmid was generated by cloning the *Asp* 718-*Eco*RI fragment of pGAD424 (2) blunt ended by Klenow fragment treatment into plasmid pSH2-1 digested by *Eco*RI and treated with mung bean nuclease. Correct ligation events restored both the *Asp* 718 and *Eco*RI sites. In plasmid pLexGAD1, the region encoding the LexA-Gal4 fusion protein is thus followed by the same polylinker as that found on plasmid pSH2-1. We then used oligonucleotides to amplify fragments of plexM4-1, plexM4Δ16, and plexM4Δ30 that were cloned into pLexGAD1 digested by *Eco*RI and *Bam*HI, yielding pLexGADM4-1, pLexGADM4Δ16, and pLexGADM4Δ30, respectively. Amplifications were performed with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). The amplified fragments correspond to Met4 residues 145 to 666. All of the resulting fusion proteins contain the same C-terminal extension of seven amino acids resulting from the translation of the polylinker region.

Northern (RNA) blot analyses. Northern blotting was performed as described by Thomas (35), with total cellular RNA extracted from *S. cerevisiae* as described by Hoffman and Winston (16) and oligolabeled probes (15).

Immunoblot analyses. Total cellular extracts were prepared from exponentially growing cells. Cells were harvested by centrifugation at 4°C, washed once in 1 ml of ice-cold 20 mM Tris-HCl (pH 8.0)–50 mM $(NH_4)_2SO_4$–1 mM EDTA–25% glycerol and resuspended in 200 μl of the same buffer containing 1 mM phenylmethylsulfonyl fluoride and 7 mM β-mercaptoethanol. Cells were then disrupted by vortexing 10 times for 15 s each with an equivalent volume of 0.4 to 0.5 mm glass beads, and the extract was spun for 5 min at 4°C. Total protein from each extract (10 μg as quantitated by the Lowry method [21]) was separated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and electroblotted on a nitrocellulose membrane (BA85; Schleicher & Schüll, Dassel, Germany). LexA-Met4 fusion proteins were detected with a polyclonal antibody raised against LexA (kindly supplied by P. L. Moreau) used at a 1:1,500 dilution, followed by a 1:10,000 dilution of a donkey anti-rabbit immunoglobulin G-specific antibody conjugated to horseradish peroxidase. Peroxidase activity was revealed with an enhanced chemiluminescence kit (Amersham International, Amersham, England) as specified by the manufacturer.

Enzyme assay. β-Galactosidase assays were performed as follows. Ten milliliters of cells was grown in minimal medium to an optical density at 650 nm of between 1 and 1.4 (about 10^7 cells per ml). Cells were harvested by centrifugation at 4°C, and the supernatant was discarded. The cell pellet was immediately frozen in dry ice and stored at −80°C. For the assay, the cells were washed once in 1 ml of ice-cold 100 mM Tris-HCl (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, pelleted, and resuspended in 200 μl of the same buffer. Cells were then disrupted by vortexing 10 times for 15 s each with an equal volume of 0.45- to 0.50-mm glass beads. Following addition of 100 μl of 100 mM ice-cold Tris-HCl (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, the extract was spun for 5 min at 4°C. β-Galactosidase activity was assayed with 5, 10, 15, and 20 μl of each extract as described by Miller (25). The protein concentration of each extract was determined by the method of Lowry et al. (21), using 10, 20, 40, and 60 μl of the extract diluted fivefold in water. For each fusion protein, at least three independent transformants were assayed, and the standard error was less than 15%.

RESULTS

A previous study showed that a LexA-Met4 fusion protein is capable of efficiently activating transcription of a reporter gene placed downstream of LexA operators (33). Moreover, a pLexMet4 plasmid relieves the methionine auxotrophy of a *met4*-disrupted strain, provided that the expressed fusion protein contains the bZIP region of Met4. We show here that when cells transformed by plasmid pLexMet4-1 are grown in a medium containing a repressing amount of methionine, i.e., 1 mM L-methionine, expression of the reporter gene is decreased fourfold relative to that in nonrepressing growth conditions (Fig. 1A). This finding implies that the transcriptional activation function of Met4 is severely impaired by the increase of intracellular AdoMet. Indeed, it was demonstrated that the repressive effect of added methionine is actually mediated through the increase of intracellular AdoMet, owing to the efficient transformation of methionine into AdoMet by the two yeast AdoMet synthases (34).

To identify regions of Met4 capable of activating transcription as well as regions conferring AdoMet responsiveness, we constructed several series of internal deletions in Met4. Each derivative was expressed fused to the DNA-binding domain of the protein LexA and introduced on a plasmid into *met4*::*TRP1* cells. The cells also bore a *lexAop-lacZ* fusion gene in the chromosome, and β-galactosidase activity was used as a measure of the transcriptional function of each modified protein, since the DNA binding was achieved through the LexA moiety. In all cases, the bZIP domain of Met4 was conserved in order to preserve the possibility of complementing the methionine auxotrophy of the recipient strain.

Mapping of the functional regions of Met4. We first constructed two series of nested internal deletions, one removing amino-terminal parts of Met4 and the other removing carboxy-terminal parts. As shown in Fig. 1A, progressive deletions in the N-terminal region rapidly lead to variants without transcriptional activation capabilities: deletion Δ1 is fully active and inhibited by AdoMet, but the longer deletions, Δ2 to Δ5, virtually abolish the transcriptional activation function. In contrast, a considerable part of the C-terminal region of Met4, spanning residues 404 to 589 (deletions Δ6 to Δ8), can be deleted without drastically affecting either the transcriptional activation function or the AdoMet responsiveness. Deletion Δ9 reduces the transcriptional activity threefold under nonrepressive growth conditions. This deletion does not affect the transcriptional activity measured after growth in the presence of a repressing amount of methionine. In contrast, the longer deletion Δ10, which reduces the transcriptional activity only 1.6-fold under nonrepressive growth conditions, appears to be no

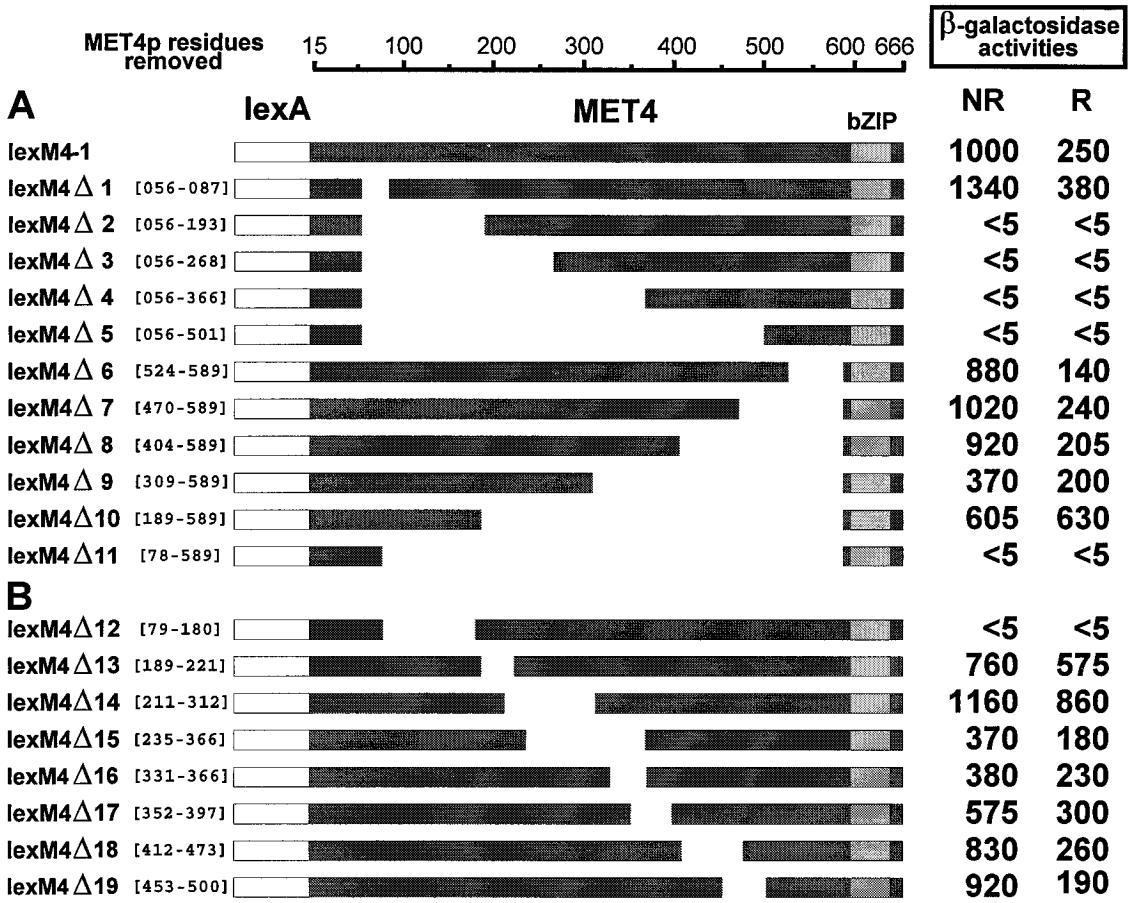

FIG. 1. Deletion analysis of the transcriptional activator Met4. (A) Nested internal deletions. (B) Small internal deletions. Schematic representations of the fusions between the DNA-binding domain of LexA (residues 1 to 87) and modified Met4 are shown. Plasmids encoding these fusion proteins were introduced into a *met4*::*TRP1* strain bearing a *lexAop-lacZ* fusion gene integrated in the chromosome. The exact amino and carboxy ends of the segments removed from Met4 are indicated on the left. The β-galactosidase activities were measured with cells grown in nonrepressive (NR; 0.05 mM L-methionine) and repressive (R; 1 mM L-methionine) growth conditions. The reported values represent averages of at least three assays performed with independent transformants and are expressed as arbitrary units (1,000 arbitrary units corresponds to 4,250 nmol of substrate transformed per min per mg of protein). Standard deviations were less than 15%.

longer inhibited by an increase of intracellular AdoMet. The longer deletion Δ11 is unable to activate the transcription of the reporter gene. These results suggest that the N-terminal part (residues 80 to 190) of Met4 comprises one activation domain and that the central part (around residue 300) of Met4 may contain domains required for AdoMet responsiveness as well as for high transcriptional activation function under nonrepressive growth conditions.

We next examined the effects, on both transcriptional activation function and AdoMet responsiveness, of a set of small deletions that covers the entire length of Met4 (Fig. 1B). Assays of β-galactosidase activity expressed from the reporter gene confirm that the Met4 activation domain is contained within a region encompassing residues 79 to 180 (region 79–180). Deletion of this region (Δ12) leads to an inactive LexA-Met4 derivative, whereas deletion Δ13, removing adjacent residues, leads to an active fusion protein. As shown in Fig. 1, region 79–180 appears to be the only region of Met4 whose deletion abolishes the transcriptional activation function of the LexA-Met4 fusion protein. The second set of deletions confirms the presence, around residue 220, of a domain required for AdoMet responsiveness. Derivative Δ14 is indeed a very potent activator that weakly responds to the increase of intracellular AdoMet. The addition of a repressing amount of methionine to the growth medium reduces its transcriptional activity only 1.3-fold, compared with the LexA–Met4-1 fusion protein, the activity of which in repressing growth conditions is 4-fold less than that in nonrepressing growth conditions. β-Galactosidase assays with transformants expressing derivatives Δ15, Δ16, and Δ17 suggest the presence, around residue 340, of a domain necessary for high activity of Met4 under nonrepressive growth conditions. In these growth conditions, derivatives Δ15 and Δ16 behave like derivative Δ9, having a transcriptional activity threefold lower than that of the LexA–Met4-1 fusion protein.

Taken together, these results present evidence that in addition to the bZIP region, Met4 comprises three regions of functional importance: an activation domain, a domain required for AdoMet responsiveness, and a domain required for high transcriptional activity under nonrepressive growth conditions. This finding prompted us to determine more accurately the boundaries of these different regions.

The transcriptional activation domain is contained within residues 95 to 144 of Met4. To define the activation domain of Met4, we prepared a set of small deletions that removed residues from the region spanning residues 79 to 180. As shown in Fig. 2, all deletions that remove amino acids between residues 95 and 144 lead to a decrease of the transcriptional activity of

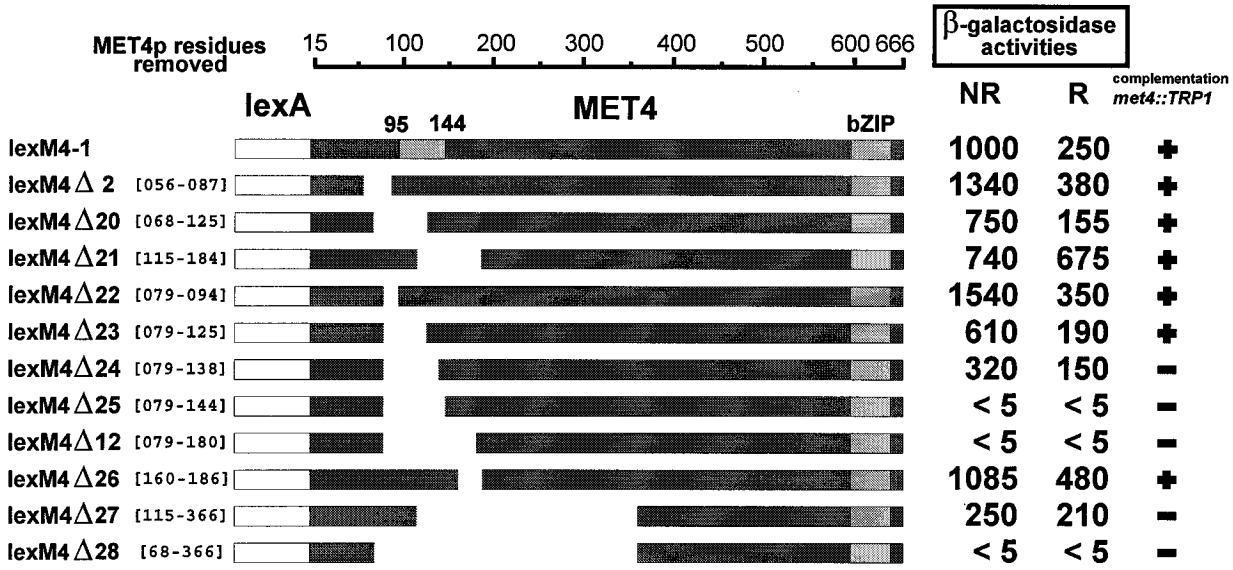

FIG. 2. The activation domain of Met4 is contained within residues 95 to 144. Schematic representations and assays are as for Fig. 1. On the right is indicated the ability of each plasmid expressing a LexA-Met4 fusion protein to relive the methionine auxotrophy induced by the inactivation of the chromosomal *met4* gene (1,000 arbitrary units of β-galactosidase activity corresponds to 4,250 nmol of substrate transformed per min per mg of protein).

the LexA-Met4 derivatives. When all of these amino acids are removed, the resulting fusion protein is completely unable to activate transcription. By contrast, derivatives Δ22 and Δ26, lacking adjacent residues, exhibit high transcriptional activation function. Furthermore, as an increasing number of residues of the region encompassing residues 95 to 144 are deleted, a gradual decrease of the activity is observed (for example, compare activities of deletions Δ23, Δ24, and Δ25). It is noteworthy that a similar observation was made during the functional analysis of the activation domain of Gcn4 (17). Since the fusion proteins have the bZIP domain of Met4, it was possible to test whether expression of these derivatives relieves the methionine auxotrophy induced by the inactivation of the chromosomal *MET4* gene. In all cases, lack of functional complementation is associated with deletions that severely affect the transcriptional activation function (Fig. 2). Expression of LexA-Met4 derivatives that are half or more than half as functional as the LexA–Met4-1 fusion protein relieves the methionine auxotrophy of strain C170, whereas expression of derivatives exhibiting 50% or less than 50% of the transcriptional activity of the LexA–Met4-1 fusion protein does not.

We performed additional experiments to ensure that the defect in the transcriptional activation function of modified LexA-Met4 fusion proteins indeed resulted from the removal of an activation domain. First, we measured the level of production and stability of the LexA-Met4 derivatives by immunoblot analysis. This was done with antibodies raised against LexA (a gift of P. L. Moreau). Cell extracts were prepared from cells expressing various modified LexA-Met4 fusion proteins, grown in either nonrepressive or repressive conditions. As shown in Fig. 3, equivalent amounts of LexA-Met4 fusion proteins are detected in each growth condition. Moreover, all of the derivatives tested are expressed at similar levels. Second, experiments which verified that deletions have the same effect in a nonchimeric Met4 context were performed. In this way, we also verified that lack of transcriptional activation function of LexA-Met4 derivatives did not result from an impairment of DNA binding by the LexA moiety. Deletions Δ12 and Δ25 were used to construct Met4 derivatives expressed from the *MET4* promoter on a centromeric plasmid. The resulting plasmids, pM4Δ12 and pM4Δ25, were introduced into a *met4*-disrupted strain, and the capability of the recombinant Met4 proteins to activate transcription of a sulfur gene was monitored by Northern blot analysis. Strain CD106 (*met4*::*TRP1*) transformed by either pM4-5 (wild type), pM4Δ12, or pM4Δ25 was first grown in a medium containing a repressing amount of methionine and then shifted to a medium without methionine. Total RNA was extracted at regular time intervals after the shift and analyzed with a probe specific to gene *MET25* (18). As shown in Fig. 4, in pM4-5-transformed cells, the transcription of gene *MET25* reaches the maximal level 80 min after the shift. By contrast, in pM4-Δ12-transformed cells, only a weak derepression is measured 140 min after the shift (the same result was obtained with the pM4Δ25 derivative; data not shown). These results thus confirm that removal of amino acids 94 to 144 abolishes the transcriptional activation function of Met4p.

Region 188–235 is necessary for AdoMet responsiveness. Analysis of the first set of deletions revealed that LexA-Met4 derivatives, lacking residues within Met4 region 190–320, appear to be less responsive to the increase of intracellular AdoMet than the original fusion protein. To determine which part of this region could be associated with the AdoMet inhibitory effect, we created a further series of small internal deletions that remove amino acids from the region spanning residues 190 to 320. As shown in Fig. 5, several deletions result in LexA-Met4 derivatives that are weakly inhibited by the increase of intracellular AdoMet. The results suggest that the major inhibitory effect can be ascribed specifically to the Met4 residues 188 to 235. Indeed, the transcriptional activity of derivatives deleted within this region is increased two- to fivefold under repressive growth conditions compared with the LexA–Met4-1 fusion protein. As these deletions weakly affect the transcriptional activities measured in nonrepressive growth conditions, the resulting ratios of AdoMet-mediated inhibition vary from 1.1 to 2, compared with the 4-fold inhibition measured with the fusion protein that contains the entire Met4. It is noteworthy that the more residues of Met4 region 188–235

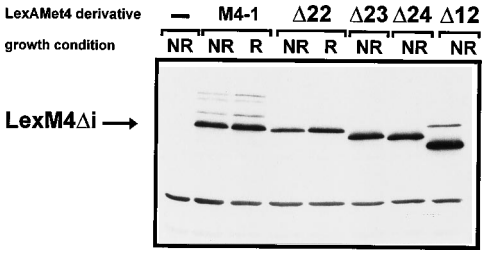

FIG. 3. Immunodetection of LexA-Met4 derivatives. Each lane contains 10 μg of total yeast protein that was separated on an SDS–10% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. LexA-Met4 fusion proteins were detected with a polyclonal antibody raised against LexA (see Materials and Methods). Extracts were prepared from strain C170 transformed by parental plasmid pSH2-1 (−) expressing the LexA(1–87) protein or plasmids expressing LexA-Met4 derivatives. LexA-Met4 fusion proteins were detected from cells grown in either nonrepressive (NR; 0.05 mM L-methionine) or repressive (R; 1 mM L-methionine) growth conditions. In addition to the LexA-Met4 derivatives, an unknown protein is recognized by the polyclonal antibody (lower band).

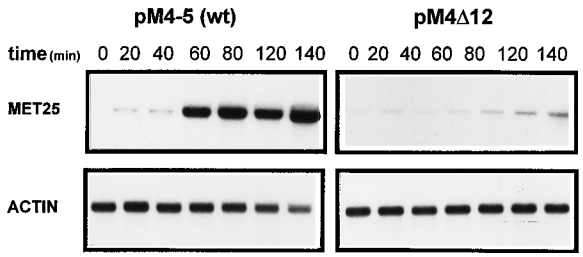

FIG. 4. Effect of a deletion within the activation domain of Met4 on the transcriptional derepression of a sulfur gene. Derepression kinetics of the transcription of gene *MET25* was monitored in cells expressing a wild-type Met4 or a modified Met4 lacking residues 79 to 180. The cells used were strain CD106 (*met4*::*TRP1*) transformed by either plasmid pM4-5 (wild type [wt]) or plasmid pM4Δ12. Cells were grown in B medium (4) in the presence of a repressing amount of methionine (1 mM L-methionine). When the cells reached a density of about 10^7/ml, they were harvested by filtration and washed. The cells were suspended in the same medium without methionine and shaken at 28°C. Samples were then withdrawn at different times, and total RNAs were extracted. For each time, 10 μg of total RNA was electrophoresed on a 1% agarose gel and transferred onto a nylon membrane. The transferred RNAs were hybridized to the radioactive *Xba*I-*Eco*RV fragment from the *MET25* gene (18). The actin probe was used as a control for the amounts of RNA loaded.

are removed, the less the resulting derivatives are inhibited by the increase of intracellular AdoMet (for example, compare derivatives Δ29, Δ30, and Δ32 in Fig. 5). Deletions of only half of the residues in region 188–235 result in an inhibition ratio of 1.5 to 2. This finding suggests that region 188–235 may contain redundant functional parts, although no sequence repetitions can be observed within this region. Immunoblot analyses were performed to verify that these LexA-Met4 derivatives were expressed at the same level (data not shown). We named the domain encompassing Met4 residues 188 to 235 the inhibitory region. To determine to which extent the deletion of the inhibitory region affects the AdoMet-mediated repression of a natural *MET* promoter, we used deletion Δ30 to construct a Met4 derivative lacking the inhibitory region that was expressed from the *MET4* promoter on a centromeric plasmid. Strain CD106 (*met4*::*TRP1*) transformed by either pM4-5 (wild type) or pM4Δ30 was first grown in a medium lacking methionine and then shifted to a medium containing a repressing amount of methionine. Total RNA was extracted at regular time intervals after the shift and analyzed with a probe specific to gene *MET25* (18). As shown in Fig. 6, for both the pM4-5- and pM4Δ30-transformed cells, addition of a repressing amount of methionine to the medium results in the same rapid repression of the transcription of the *MET25* gene. However, at 40 and 50 min, a low residual transcription of the *MET25* gene is measured in pM4Δ30-transformed cells. Repression of *MET25* gene expression thus appears to occur through another mechanism in addition to the inhibition of the Met4 activation function. However, it remains to be determined whether the same observation could be generalized for all of the sulfur genes that are controlled by Met4. This point is currently under investigation in our laboratory.

A domain encompassing residues 312 to 375 is required for high transcriptional activity under nonrepressive growth conditions. The first series of deletions also revealed a third functional domain in Met4. This domain seems to be necessary for high transcriptional activation function under nonrepressive growth conditions. This third region was tentatively located within residues 300 to 400. To define more accurately this domain, we created another set of internal deletions. As shown in Fig. 7, this functional domain is comprised within Met4 residues 312 to 375. Indeed, under nonrepressive growth conditions, most of the deletions that remove parts of this portion of Met4 reduce the transcriptional activation function threefold relative to that of the LexA–Met4-1 fusion protein. In contrast, adjacent deletions Δ34 and Δ38, which remove Met4 residues 235 to 312 and 375 to 430, respectively, lead to LexA-Met4 derivatives that, under nonrepressive growth conditions, activate transcription as efficiently as the LexA–Met4-1 fusion protein. In contrast to what we observed for the activation domain, all of the deletions within Met4 region 312–375 lower to the same extent the transcriptional activation function, irrespective of the number of residues removed. As a control, we verified by immunoblot analyses that the modified LexA-Met4 derivatives from this series are expressed at the same level (data not shown). The region encompassing Met4 residues 312 to 375 was named the auxiliary domain. As shown in Fig. 7, the expression of LexA-Met4 fusion proteins lacking the auxiliary domain cannot relieve the methionine auxotrophy resulting from the inactivation of the chromosomal *met4* gene. The LexA-Met4Δ38 derivative seems to be one exception in our deletion series, as this derivative does not complement the *met4*::*TRP1* strain although it has a strong transcriptional potential. To date, we have no explanation for this result.

Intramolecular regulation and AdoMet responsiveness. To further establish that we have identified autonomously functioning domains and to investigate more precisely their functional relationships, we fused different parts of Met4 to the DNA-binding domain of another yeast transcriptional activator. In this way, we also address the question of whether the regions described by the deletion series are sufficient to confer transcriptional activity or regulation. We used the DNA-binding domain of Gal4, and the transcriptional activities of the resulting fusion proteins, as well as their regulation by AdoMet, were monitored by using a *GAL1-lacZ* fusion gene integrated in the chromosome.

We first show that the region encompassing Met4 residues 95 to 144, when directly fused to the DNA-binding domain of Gal4, is indeed capable of efficiently activating the transcription of the reporter gene (Fig. 8). In contrast, Gal4-Met4 fusion proteins containing either the auxiliary domain or both the inhibitory region and the auxiliary domain lack transcriptional activation function. These findings, together with the results reported above, allow us to conclude that the full transcriptional activity potential of Met4 is indeed contained within residues 95 to 144.

We next examined the role of the inhibitory region and the auxiliary domains by constructing Gal4-Met4 fusion proteins

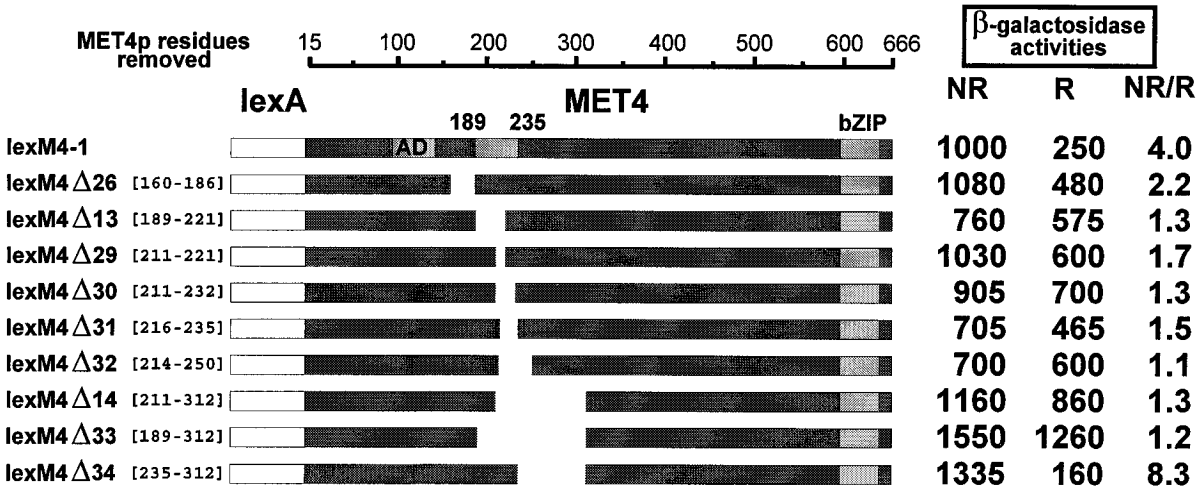

FIG. 5. AdoMet inhibition of Met4 is mediated through a distinct region located within residues 189 to 235. Schematic representations and assays are as for Fig. 1. On the right is indicated the ratio of activities measured after growth in nonrepressive and repressive growth conditions (1,000 arbitrary units of β-galactosidase activity corresponds to 4,250 nmol of substrate transformed per min per mg of protein).

having these domains in addition to the Met4 activation domain. The presence of the inhibitory region alone reduces 3.5-fold the transcriptional activity in nonrepressive growth conditions (Fig. 8). We therefore conclude that the inhibitory region can inhibit the Met4 activation domain even when the intracellular AdoMet level is low. This inhibition could be prevented by the presence of the auxiliary domain. Addition of the auxiliary domain indeed results in a twofold increase of the transcriptional activity of the Gal4-Met4 fusion protein. However, in the Gal4-Met4 context, addition of the auxiliary domain restores only half of the transcriptional activity potential of the activation domain of Met4.

The transcriptional activity of the Gal4-Met4 fusion proteins was also measured in repressive growth conditions. As expected from the results presented above, the Gal4-Met4 derivative containing only the activation domain of Met4 does not respond to the increase of intracellular AdoMet (Fig. 8). AdoMet responsiveness requires the presence of the inhibitory region. The transcriptional activation function of Gal4-Met4 fusion proteins that contain the entire Met4 inhibitory region is inhibited eightfold by the increase of intracellular AdoMet.

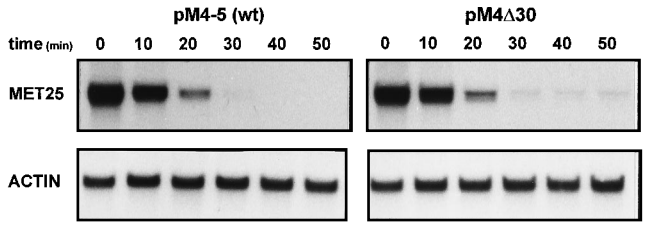

FIG. 6. Effect of a deletion within the inhibitory region of Met4 on the transcriptional repression of a sulfur gene. Repression kinetics of the transcription of gene *MET25* was monitored in cells expressing a wild-type Met4 or a modified Met4 lacking residues 211 to 232. The cells used were strain CD106 (*met4*::*TRP1*) transformed by either plasmid pM4-5 (wild type [wt]) or plasmid pM4Δ30. They were grown in B medium (4) in the presence of 0.1 mM sulfate as the sulfur source. When the cell concentration reached about 10^7/ml, L-methionine was added to the medium at a final concentration of 1 mM. Samples were then withdrawn at different times, and total RNAs were extracted. For each time, 10 μg of total RNA was electrophoresed on a 1% agarose gel and transferred onto a nylon membrane. The transferred RNAs were hybridized to the radioactive *Xba*I-*Eco*RV fragment from the *MET25* gene (18). The half-life of *MET25* mRNA was previously shown to be about 8 min (18). The actin probe was used to monitor the amounts of RNA loaded.

The Gal4 activation domain can be regulated by the Met4 inhibitory region. To provide more decisive evidence that the Met4 inhibitory region consists of a functionally distinct domain responding to the increase of intracellular AdoMet, we determined whether a different activation domain from another transcription factor could be regulated by the Met4 inhibitory region. To test this possibility, we constructed LexA-Gal4-Met4 fusion proteins that contain the DNA-binding domain of LexA, the activation domain of Gal4, and different parts of Met4 (see Materials and Methods). The activation domain of Gal4 used in these constructions comprises residues 768 to 881 of Gal4 (22). These new derivatives were expressed under the control of the promoter region of the *ADH1* gene and introduced on a plasmid into *met4*::*TRP1* cells which also bore a *lexAop-lacZ* fusion gene in the chromosome. As shown in Fig. 9, expression of a LexA-Gal4-Met4 fusion protein having the Met4 inhibitory region leads to expression of the reporter gene under repressive growth conditions that is 2.7-fold lower than that in nonrepressive growth conditions. This result indicates that Met4 residues 146 to 666 confer AdoMet regulation on the Gal4 activation domain. This result was further substantiated by demonstrating that in the absence of the Met4 inhibitory region, the transcriptional activation function of the LexA-Gal4-Met4 hybrid is weakly sensitive to the increase of intracellular AdoMet. Indeed, the ratio of AdoMet inhibition of the transcriptional activity of derivative LexGADM4Δ30 is 1.4. This value equals the ratio of AdoMet inhibition of the LexA-Met4Δ30 fusion protein, in which the same residues within the inhibitory region were deleted (see Fig. 5). In contrast to what we observed with the Met4 activation domain, the Met4 auxiliary domain is not required for preventing the inhibition of the transcriptional activity of the Gal4 activation domain in nonrepressive growth conditions.

DISCUSSION

The transcriptional activator Met4 is a rather large protein comprising 666 amino acid residues. The results presented here demonstrate that Met4 contains, in addition to its bZIP domain, three separable domains of functional importance.

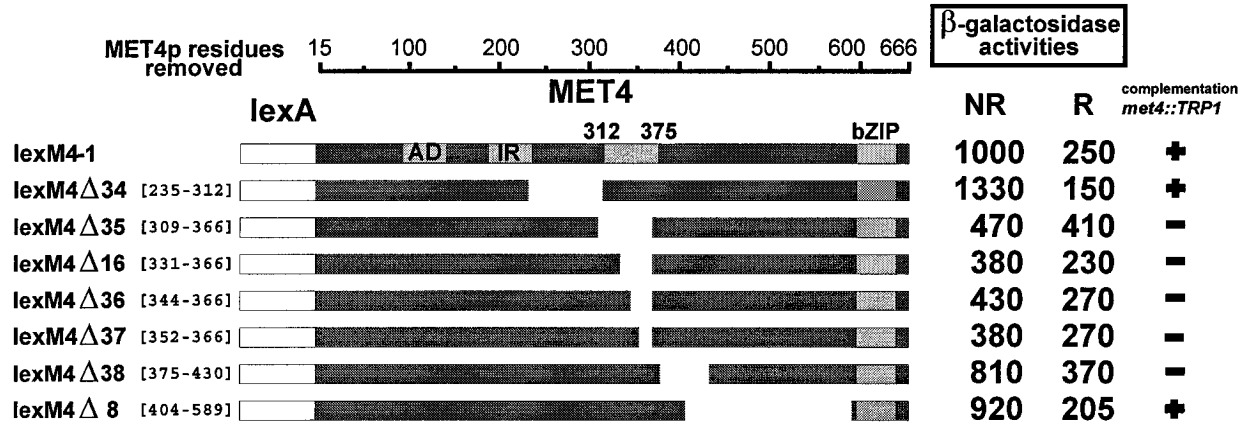

FIG. 7. A domain contained within residues 312 to 375 is required for high transcriptional activity of Met4 under nonrepressive growth conditions. Schematic representations and assays are as for Fig. 1. On the right is indicated the ability of each plasmid expressing a LexA-Met4 fusion protein to relieve the methionine auxotrophy induced by the inactivation of the chromosomal *met4* gene (1,000 arbitrary units of β-galactosidase activity corresponds to 4,250 nmol of substrate transformed per min per mg of protein).

These domains are responsible for the Met4 transcriptional activation function and its regulation.

Our analysis reveals that Met4 contains only one activation domain, which is located in its N-terminal part between residues 95 and 144. This identification was established by constructing nested as well as small internal deletions in Met4 and by showing that LexA-Met4 derivatives from which region 95–144 was deleted lack transcriptional activation function. Furthermore, we show that when directly fused to the DNA-binding domain of Gal4, region 95–144 of Met4 is capable of efficiently activating the transcription of a reporter gene placed downstream of Gal4 binding sites. Examination of the amino acid composition of region 95–144 from Met4 reveals that this activation domain is not rich in proline or glutamine, unlike those of some activators from other organisms (5, 8, 19). In contrast, this domain is particularly rich in asparagine residues, which account for 21% of the residues of this region. The Met4 activation domain is also an acidic region, containing eight acidic residues and no basic residue, and therefore appears to belong to the class of acidic activation domains. Deletion analysis of the Gcn4 activation domain as well as mutagenesis analysis of the Gal4 activation domain showed a correlation between the strength of activation and the acidity (11, 17). Our analysis also reveals a relationship between negative charge and activation. For example, comparison of transcriptional activities of LexA-Met4 derivatives Δ23 and Δ24 shows that deleting a seven-amino-acid segment containing three acidic residues drastically lowers the transcriptional activity (Fig. 2). However, it must be noted that neither the asparagine-rich property nor the acidity are features specific to region 95–144 in Met4. Indeed, asparagine is the most represented amino acid (13.5%) in Met4, which is also substantially hydrophilic, with a high percentage (14%) of negatively charged amino acids. Thus, several amino acid segments rich in both aspara-

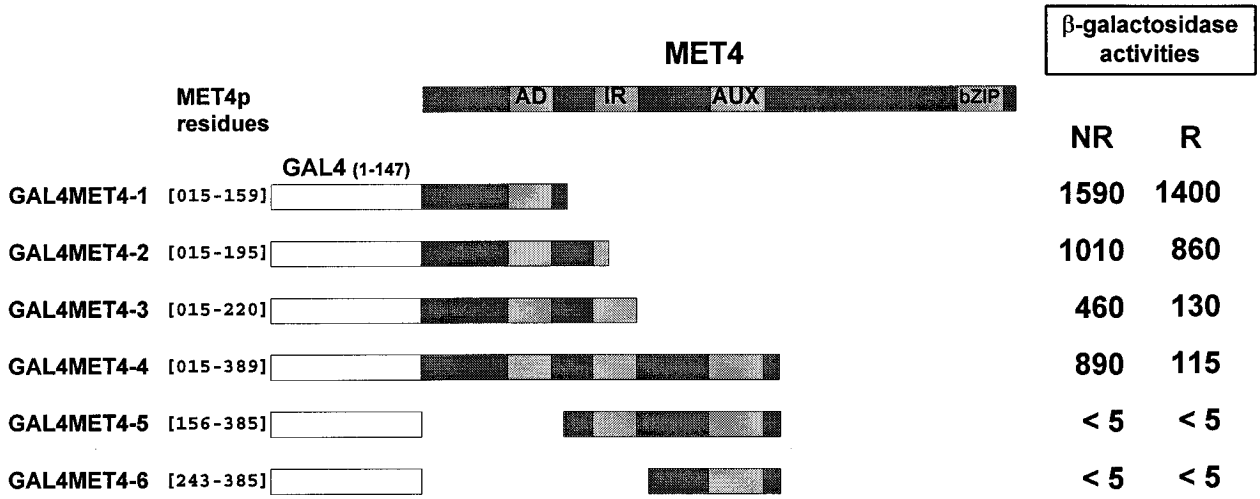

FIG. 8. Transcriptional activation by Gal4-Met4 fusion proteins. Schematic representations of the fusions between the DNA-binding domain of Gal4 (residues 1 to 147) and various fragments of Met4 are shown. Plasmids expressing Gal4-Met4 fusion proteins were transformed into strain Y526 containing an integrated *GAL1-lacZ* reporter gene activated by UAS_{GAL}. The exact amino and carboxy ends of the fused fragments of Met4 are indicated on the left. The β-galactosidase activities were measured with cells grown in nonrepressive (NR; 0.05 mM L-methionine) and repressive (R; 1 mM L-methionine) growth conditions. The reported values represent averages of at least three assays performed with independent transformants and are expressed as nanomoles of substrate transformed per minute per milligram of protein. Standard deviations were less than 15%. AD, activation domain; IR, inhibitory region; AUX, auxiliary domain.

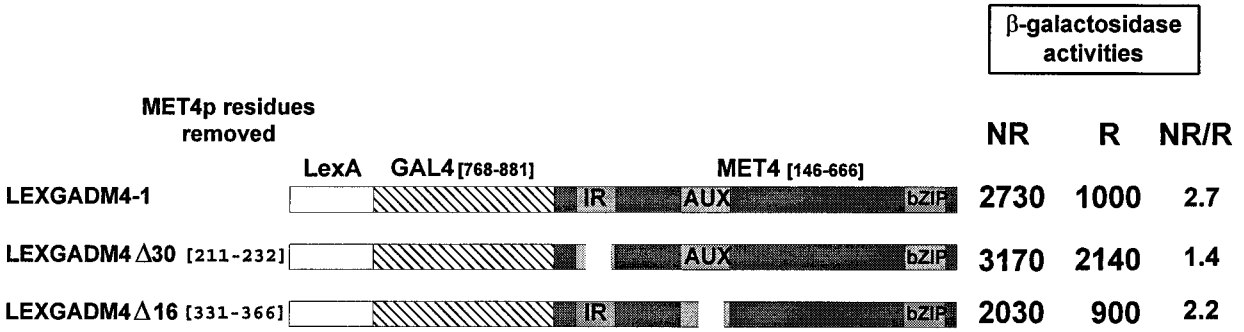

FIG. 9. AdoMet inhibition of LexA-Gal4-Met4 fusion proteins. Schematic representations of the fusions between the DNA-binding domain of LexA (residues 1 to 87), the activation domain of Gal4 (residues 768 to 881), and wild-type or deleted 146–666 region of Met4 are shown. Plasmids expressing LexA-Gal4-Met4 fusion proteins were transformed into strain C170 containing an integrated *lexAop-lacZ* reporter gene. The exact amino and carboxy ends of the deleted region of Met4 are indicated on the left. The β-galactosidase activities were measured with cells grown in nonrepressive (NR; 0.05 mM L-methionine) and repressive (R; 1 mM L-methionine) growth conditions. The reported values represent averages of at least three assays performed with independent transformants and are expressed as nanomoles of substrate transformed per minute per milligram of protein. Standard deviations were less than 15%. On the right are indicated the ratio of the activities measured after growth in nonrepressive and repressive growth conditions. IR, inhibitory region; AUX, auxiliary domain.

gine and acidic residues are found in Met4. Leuther et al. (20) recently postulated that acidic residues are important in activation, not because they interact with general transcription factors but because their interspersal within activation domains tends to increase the accessibility of the residues that interact with the factors. Other mutagenesis studies of activation domains also suggested that the most important residues for activation are not necessarily the predominant amino acid (6, 10). According to these results, it seems likely that a particular structure, rather than a compositional trait, should specify the activation domain of Met4. More genetical and biochemical data are thus clearly required to determine what structural features distinguish region 95–144 from the rest of Met4 and allow it to function as an activation domain.

Our study also reveals that the transcriptional activation function of Met4 is subject to a negative regulation independent of its DNA-binding capacity. Assays performed with LexA-Met4 derivatives demonstrate that the Met4 activation function is fourfold inhibited when intracellular AdoMet increases. Negative transcriptional regulation of the sulfur network therefore consists of a mechanism preventing the Met4 activation function and, superimposed on this regulation, of a repression mechanism acting through the *MET30* gene product (31). A similar combination of regulatory mechanisms is known to control the expression of the genes required for galactose metabolism in *S. cerevisiae* (7). Results presented here show that inhibition of the Met4 activation function requires a distinct region, located between residues 188 and 235. Deletions in this region eliminate most of the AdoMet responsiveness of the LexA-Met4 derivatives. Furthermore, when fused alone to the DNA-binding domain of Gal4, the activation domain of Met4 functions in a constitutive manner, unresponsive to the increase of intracellular AdoMet. AdoMet responsiveness is restored by the addition of the inhibitory region to the fusion proteins. The function of the inhibitory region is not specific to the activation domain of Met4; experiments using LexA-Gal4-Met4 fusion proteins indeed show that it can inhibit the transcriptional activation function of the Gal4 activation domain. Thus, unrelated heterologous activation domains can be negatively controlled by AdoMet through the inhibitory region of Met4.

The existence of another distinct domain is evident within Met4p residues 312 to 375. The deletion of this region, called the auxiliary domain, reduces the transcriptional activation function of Met4 threefold under nonrepressive growth conditions. Deletion analyses as well as experiments with Gal4-Met4 fusion proteins prove that this domain is devoid of intrinsic transcriptional activation capability. The role of this domain was elucidated by experiments with Gal4-Met4 fusion proteins which demonstrate that in the absence of the auxiliary domain, the inhibitory region prevents to a large extent the operation of the Met4 activation domain even when the intracellular AdoMet level is low. Addition of the auxiliary domain relieves this negative effect. The auxiliary domain thus appears to function as an antagonist of the inhibitory region under nonrepressive growth conditions.

Considering all of our results, we propose the following model to explain the intramolecular regulation of the transcriptional activator Met4. Because the inhibitory region is distinct from the activation domain and functions with heterologous activation domains, we believe that under high intracellular AdoMet concentrations, this region governs the interaction with a regulatory protein which would prevent the activation domain from contacting components of the basal transcription machinery. Under low intracellular AdoMet concentrations, the reversal of the inhibition of the activation domain would be facilitated by the auxiliary domain. A related model was recently proposed by Stone and Sadowski (30) to explain how the Gal4 activation function is inhibited by glucose. In this case, glucose inhibition is mediated through a repeat of three inhibition domains. Moreover, the function of these inhibition domains appears to be negatively controlled by another region of Gal4, called the glucose-responsive domain. Our experiments suggest that the auxiliary domain of Met4 could be the functional equivalent of the glucose-responsive domain of Gal4. However, because the auxiliary domain seems, within the limits of our experiments, to function specifically with the activation domain of Met4, it is difficult to assess whether this domain may actually be an AdoMet-responsive domain. Further insights into the AdoMet-mediated regulation of Met4 should emerge from the identification of the regulatory protein predicted to interact with the Met4 inhibitory region. Experiments are currently under way in our laboratory to identify this factor.

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