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The yeast putative transcriptional repressor RGM1 is a proline-rich zinc finger protein

Francisco Estruch
Departament de Bioquímica i Biologia Molecular, Facultats de Ciències, Universitat de València, Spain

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ABSTRACT

I have cloned a yeast gene, *RGM1*, which encodes a proline-rich zinc, finger protein. *rgm1* mutants do not show any obvious phenotype but overexpression of *RGM1* gene greatly impairs cell growth. The proline-rich region of RGM1 attached to a heterologous DNA binding domain is able to repress the expression of the target gene. RGM1 shares similar zinc finger motifs with the mammalian Egr (early growth response) proteins as well as proline-rich sequences with a high serine and threonine content, suggesting that RGM1 and Egr proteins could have functional similarities.

INTRODUCTION

Regulation of transcription plays a major role in the control of gene expression in eukaryotic cells. Transcriptional regulation involves an interplay between regulatory DNA sequences and site-specific DNA binding proteins. In recent years much effort has been focused on identifying such sequence-specific DNA binding proteins and how their binding results in increased or decreased transcription of the associated genes.

A comparison of different transcriptional factors indicates the existence of several common motifs that allow the binding of the protein to DNA in a sequence specific manner. These motifs include the helix-turn-helix, the helix-loop-helix, the leucine zipper and the zinc finger (1). The zinc fingers were first described in TFIIIA, a transcriptional factor required for the transcription of *Xenopus laevis* 5S RNA genes (2). In this protein the zinc finger consensus sequence $C\text{-}N_2\text{-}C\text{-}N_{12}\text{-}H\text{-}N_3\text{-}H$ is repeated nine times and the conserved cysteines and histidines of each repeat have been shown to chelate one zinc atom (3). In the yeast *S. cerevisiae* the transcriptional activators ADR1 (4), SWI5 (5) and ACE2 (6) and the repressor MIG1 (7) contain C_2H_2 zinc finger sequences.

The ability of a transcriptional factor to bind an upstream element is not sufficient to elicit a change in the transcriptional activity. It should also contain also a regulatory domain to interact with the transcriptional apparatus. There are also several classes of transcriptional activation domains. The first class described is characterized by a high content of negatively charged amino acid residues that may be present in an amphipatic α-helical structure (8, 9). In mammals, glutamine-rich and proline-rich domains have also been shown to activate transcription (10, 11). The proline-rich domain of CTF/NF-1 consists of nearly 25% proline residues and the content of serine and threonine is also high. Proline-rich regions have been found in other proteins involved in the control of gene expression, including the *Drosophila* Fushi tarazu and Krüppel proteins (12, 13), the steroid receptor for progesterone and estrogen (14), and the proteins encoded by the immediate early growth response (*Egr*) genes, which are rapidly activated following mitogenic stimulation (15, 16). Much less is known about transcriptional repression. Different, non excluding, models have been proposed (17) and examples where a single protein can serve either as a transcriptional activator or repressor depending on the promoter have been described (18). Recently, an alanine-rich region with transcriptional repression activity has been identified in the *Drosophila* zinc finger protein Krüppel (19). However, except for the acidic domains, the assignation of a general role for all these regions in transcriptional regulation will require the identification of a larger number of transcriptional factors.

I report here the cloning and sequencing of the yeast *RGM1* gene. This gene encodes a protein with two C_2H_2 zinc fingers suggesting that RGM1 could be a DNA binding regulatory protein. Expression of the *RGM1* gene under the control of the inducible *GAL10* promoter reduces cell growth. The reduction in the activity of the target gene observed when the proline-rich region of RGM was attached to a DNA binding domain of known specificity suggests a possible role of this sequence in transcriptional repression.

MATERIALS AND METHODS

Strains, media and genetic methods

The *Saccharomyces cerevisiae* strains are listed in Table 1. Yeast media were prepared according to Sherman *et al* (20). Glucose, galactose and sucrose were used at 2%. SETOH media contain 2% ethanol, 0.1% glucose and 0.7% yeast nitrogen base supplemented with required amino acids. Standard methods were used for genetic analysis (20), yeast (21) and *E. coli* transformation (22).

Cloning of the RGM1 gene

The *Eco*RI- *Eco*RI fragment that includes the region encoding the zinc fingers of *MSN2*, a gene that in multicopy restores invertase expression in *snf1-ts* mutants (F. E. and M. Carlson, unpublished results), was used as a probe to screen a yeast

genomic library (23) at low stringency. About 20,000 colonies were screened by hybridization, and plasmid DNA was isolated from the positive clones. The plasmids containing the *MSN2* gene were identified by restriction analysis and Southern blotting by using the same probe. Two additional positive clones were obtained. The first one contained a gene with homology to *MSN2* in other regions besides the zinc finger domain (F. E. and M. Carlson, unpublished results). The second one included the *RGM1* gene that is the subject of this report.

Disruption of the *RGM1* gene

Standard methods were used for manipulation and analysis of DNA (22). pEB73-Δ1::URA3 was constructed by first subcloning the *Eco*RI-*Bam*HI fragment of pEL73 into pUC19 (24). In the resulting plasmid, pEB73, the *Eco*RV-*Eco*RV fragment was replaced with the *URA3* gene in a 1.1 kb *Hin*dIII fragment (see Fig 1). The *Eco*RI-*Bam*HI fragment from pEB73-Δ1::URA3 was used to replace (25) the wild type sequence on one chromosome of the diploid MCY1093×MCY1094. The diploid was sporulated and subjected to tetrad analysis. Four spore clones of one tetrad were analyzed by genomic blot hybridization to confirm that the deletion-insertion was present (not shown).

Plasmid constructions

The entire open reading frame of *RGM1* gene was fused from the *Mnl*I site (position −2) to the inducible yeast *GAL10* promoter in the vector YEp52 (26), which contains the yeast 2 mm origin of replication and the *LEU2* gene as selecting marker. In the resulting plasmid, pEGAL73, the first ATG following the *GAL10* promoter is the first ATG of the *RGM1* gene. A modified version of this plasmid, pEGAL73fs, was made by inserting the polylinker sequence of pUC19 from the *Hin*dII site to the *Hin*dIII site between the *Mnl*I site of *RGMI* and the *Hin*dIII site of YEp52. This introduces an additional ATG codon not in frame with *RGM1*.

To construct the fusion of the proline-rich region of RGM1 with the DNA binding motif of MIG1, the *Acc*I-*Eco*RI fragment from pEB73 was blunted and inserted in the *Xho*I site of pMIG1 (7) previously filled in. This produces a plasmid, pEMR1, which contains a gene fusion between the first 96 codons of *MIG1* and the sequence between codons 83 to the end of *RGM1*. In plasmid pEMR1inv the fragment *Acc*I-*Eco*RI was inserted in the inverted orientation. pMIG1fs was constructed by digesting pMIG1 with *Xho*I, filling the ends with Klenow fragment and religating with T4 ligase. This creates a +1 frame shift that introduces a stop codon at position 99.

Nucleotide sequence analysis

Restriction fragments were subcloned into M13mp18 and M13mp19 (27) and sequenced by the method of Sanger *et al* (28) using the T7 sequencing™ kit (Pharmacia) with the 17-nucleotides universal primer. The sequence of both strands was determined.

Table 1. List of *Saccharomyces cerevisiae* strains.

Strain	Genotype
MCY1093	*MATa his4-539 ura3-52 lys2-801 SUC2*
MCY1094	*MATa ura3-52 ade2-101 SUC2*
MCY1389	*MATa ura3-52 leu2::HIS3 SUC2*
PEY10	*MATa his4-539 lys2-801 ade2-101 rgm1-Δ1::URA3 SUC2*

Other methods

Preparation of glucose-repressed and derepressed cultures and assays of secreted invertase activity were as described (29).

RESULTS

Cloning and localization of the *RGM1* gene

The *RGM1* gene was cloned by searching for homologues of the *MSN2* gene. *MSN2* encodes a zinc finger protein that in multicopy restores invertase expression in *snf1-ts* mutants (F. E. and M. Carlson, unpublished). From this screening, a clone with weak but significant hybridization signal was isolated. The plasmid carried by this clone, pEL73, contained a 10-kb insert of yeast genomic DNA. In this insert the region of homology was localized by restriction analysis and Southern blotting (see Materials and Methods). A 1.3-kb *Bam*HI-*Eco*RI containing the homologous gene was identified (see Fig 1).

Sequence of *RGM1*

Figure 2 shows the complete nucleotide sequence of the *RGM1* gene and its noncoding flanking regions. A single open reading frame of 211 codons was observed. The gene is predicted to encode a protein of 23,868 daltons. The analysis of this sequence shows the presence of two C_2H_2 zinc finger motifs in the N-terminal part of the protein. Figure 3 shows an alignment of these fingers with the Krüppel consensus and other zinc fingers. The position of the highly conserved amino acids is strictly maintained, except that the first repeat contains five amino acids between the two cysteine residues and that the second zinc finger contains four residues between the two histidines. The so-called H/C link (30) located between the zinc fingers is also highly conserved.

A computer search was performed with the sequences in GenBank (release 67) by using the program TFASTA (31). The best homology was found between the zinc fingers of RGM1 and those encoded by the human gene deleted in Wilm½s tumour cells (32), the yeast *ADR1* gene (4) and the mammalian early growth response genes *Egr-1* or *zif/268* (15) and *Egr-2* or *Krox-20* (16) (see Fig. 3).

The second main feature of the RGM1 primary sequence is the high content (15%) of proline residues. The C-terminus of RGM is particularly rich with 24% of prolines (from position 95 to the end). Interestingly, both Egr proteins have in common

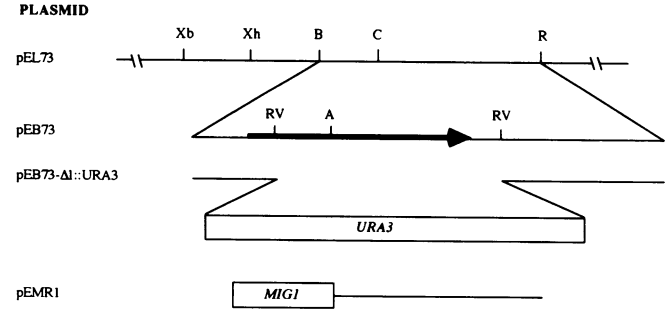

Figure 1. Restriction maps of *RGM1* gene and plasmids. Plasmids are described in the text, and only the yeast DNA segments are shown. Solid bar indicates the *RGM1* coding region, and arrowhead indicates the 5′ to 3′ direction of ORF. Restriction sites; A, *Acc*I; B, *Bam*HI; C, *Cla*I; R, *Eco*RI; RV, *Eco*RV; Xb, *Xba*I; Xh, *Xho*I. Only relevant sites are shown.

the presence of regions with an elevated content of proline (23% between positions 109 and 205 in *Krox-20* and 24% between positions 164 and 239 in *zif/268*), although they share little sequence similarity in other regions besides the zinc fingers. The proline-rich domains of RGM1 and Egr proteins have also in common a high content of serine and threonine.

The sequence Pro-Lys-Arg-Asn-Lys-Asp in position 6 is similar to the previously identified nuclear localization determinants (29).

Disruption of the chromosomal *RGM1* locus

A deletion/substitution mutation was constructed in the plasmid pEB73-Δ1::URA3 (Fig 1). This mutation was introduced into the genome of the diploid strain MCY1093×MCY1094. Diploids were sporulated and subjected to tetrad analysis (see Materials and Methods). Tetrads yielded four viable spores indicating that *RGM1* is not an essential gene.

Since *RGM1* was isolated by its homology with *MSN2*, a gene apparently involved in the regulation of *SUC2* expression (F. E. and M. Carlson unpublished results), I tested the ability of *rgm1* mutants to grow in different carbon sources. No defect in growth on raffinose, galactose or glycerol was noted. The possibility of constitutive expression of *SUC2* in the mutants was excluded since mutants were unable to grow on raffinose in the presence of 2-deoxyglucose. *rgm1* mutants were also able to grow in minimal media without any additional requirement (not shown).

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                                TTTTCTTTCATTGTATCTTCTTGATTTCAACTATTGTTTGTGTGTA  -121
TATGAACAGACGCCTCCTTCTCTTTCTTTCGTGTATTCTTGATTCAATTGCGACTTCATA  -60
GCACTATTCCTCCCCACTCACGTCTATAGTCCAGGACAATTAGCATGACACTGGTTAAAC  -1

ATGAGAGGAAAACAACCGAAAAGGAACAAAGACAACGCTTCTGTCAAGAGAAATTATAGA   60
 1  M  R  G  K  Q  P  K  R  N  K  D  N  A  S  V  K  R  N  Y  R

TGCGTGGGATATCCTGATTGTAACATGAGTTTCAATAGGACCGAACATTTGGCTAGACAT  120
21  C  V  G  Y  P  D  C  N  M  S  F  N  R  T  E  H  L  A  R  H

ATCAGGAAACACACAGGGGAGAAACCATTCCAATGCAATATCTGCCTTAAATTCTTCAGT  180
41  I  R  K  H  T  G  E  K  P  F  Q  C  N  I  C  L  K  F  F  S

AGAATCGATAACTTAAGACAACATCAATCTTCTGTTCATTCGGACGTTGATTTGATGTCC  240
61  R  I  D  N  L  R  Q  H  Q  S  S  V  H  S  D  V  D  L  M  S

TTACGTAGACTGCAGCAGTCCGCAAATAGCACTGCGAACGACCCTAACGCAACAAGAATG  300
81  L  R  R  L  Q  Q  S  A  N  S  T  A  N  D  P  N  A  T  R  M

TTCCCGCAATTACGCCCATATGGAATTGTTGTCCAGCCAGTGCCTGTTCCATACAATCTG  360
101  F  P  Q  L  R  P  Y  G  I  V  V  Q  P  V  P  V  P  Y  N  L

CCCATATCAACTCCTGCTAGCCCACAAGACACTATATCTCTTTACGCACCACCATACTTC  420
121  P  I  S  T  P  A  S  P  Q  D  T  I  S  L  Y  A  P  P  Y  F

CCTCATCCTATGCCATCTGCTCCCATACCATTGCCGCATCAGCCTCCTCCACTACCAATA  480
141  P  H  P  M  P  S  A  P  I  P  L  P  H  Q  P  P  P  L  P  I

TACAGCTATATGCAACCACTCTTCCTGAACCACACTCCCATACAAAACCACAACATTGTA  540
161  Y  S  Y  M  Q  P  L  F  L  N  H  T  P  I  Q  N  H  N  I  V

GAGCTCCCTCCAGATAGCAGTGACACTCCAGCATCGCCGTCTAAGGTGCAAAGTTTTGAC  600
181  E  L  P  P  D  S  S  D  T  P  A  S  P  S  K  V  Q  S  F  D

CAGGCCAAGGACGCTTCACCAAACGCAAAAAAATAAGCTCTACCGCACCCACTCCTCCAT  660
201  Q  A  K  D  A  S  P  N  A  K  K  *

TTCACAAGCCGCATCATTGCTGCTATTGTACCCTCTTGGCCATTCGCGTGCGCGTTGATT  720
GCCGTTATTATGCAGATATTTTGATATCGCTTGCCGTTTGGCTCATCTCCGCGCCCTTAA  780
AATATATCCGCTTCTGTTGGGAAATGCCGAGCATAAAAATGCCGAAAATATAAAAAGAAC  840
AAGAACAATACCCTTCGAGGGAAACGCTATTTTCCT                           876
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Figure 2. Nucleotide sequence of *RGM1* gene and deduced amino acid sequence. Nucleotides are numbered on the right, and amino acids are numbered on the left. The two zinc finger motifs are underlined. The sequence has been transmitted to the EMBL Data Library (accesion number X59861)

Overexpression of RGM1 reduces cell growth

To test the effect of the overproduction of RGM1, the gene was cloned under the control of the inducible *GAL10* promoter in the plasmid pEGAL73. The expression of *RGM1* is now regulated by galactose. The plasmid was introduced into a wild type strain and growth was tested. Transformants were able to grow on both glucose and galactose, although when grown on galactose, the transformants carrying the plasmid pEGAL73 produced considerably smaller colonies than those carrying the vector plasmid. Transformants carrying the *RGM1* gene fused with the *GAL10* promoter in the inverted orientation or carrying the plasmid pEGAL73fs, where an additional ATG codon out of frame was introduced between the *GAL10* promoter and the *RGM1* coding sequence (see Materials and Methods), showed wild type growth (results not shown). To measure the inhibitory effect of RGM1 overexpression, growth curves were made both in glucose and galactose. As it can be observed in Figure 4, overexpression of *RGM1* under the control of *GAL10* promoter greatly impairs cell growth in a galactose-specific manner. These results suggest that RGM1 could be involved in the repression of a process necessary for normal cell growth in yeast.

A MIG1-RGM1 fusion protein inhibits carbohydrate catabolism

The phenotype caused by the overexpression of *RGM1* suggests that it could act by repressing the transcription of its target gene(s). Since this gene or genes have not yet been identified, this possibility was tested by replacing the RGM1 zinc fingers by the zinc fingers of the MIG1 protein. MIG1 is a repressor that binds to two sites in the upstream region of the *SUC2* gene and whose overproduction inhibits *SUC2* and *GAL* genes expression (7). The fusion gene in the multicopy plasmid pEMR1 was introduced in the wild type strain MCY1389 and its effect was tested by the capability to grow on glucose, sucrose or galactose as carbon sources and by the secreted invertase activity of the transformants. Figure 5 shows that overexpression of the fusion protein inhibits growth on sucrose and galactose to an extent similar to that produced by the overexpression of MIG1 (Fig 5 b, c). Growth on glucose was unaffected (Fig 5 a). In transformants carrying the fusion protein, derepressed levels of secreted invertase at low glucose concentration (29) were reduced

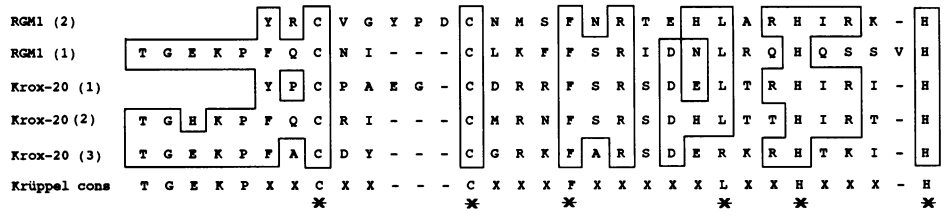

Figure 3. Alignment of RGM1 zinc fingers with fingers of Krox-20 and the consensus sequence of the Krüppel repeating unit. Identities to any of RGM1 zinc fingers are boxed. The amino acids strictly conserved in the Krüppel finger motif are labelled with asterisk.

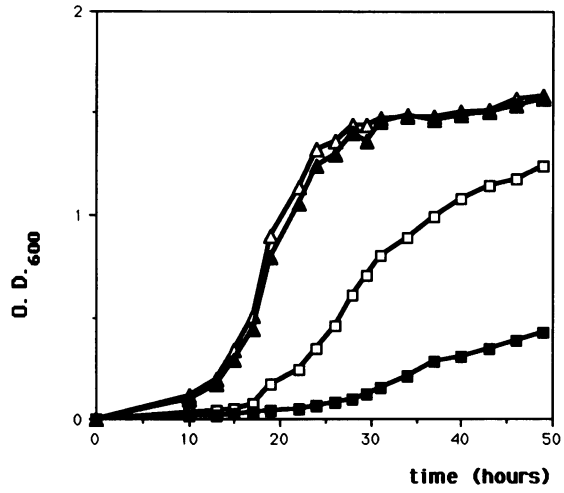

Figure 4. Effect of RGM1 overproduction on cell growth. Wild type strain MCY1389 carrying the control vector YEp52 (open symbols) or pEGAL73 (closed symbols) were grown in minimal media with glucose (triangles) or galactose (squares) as carbon source. Cells were inoculated from a freshly grown culture in SETOH. Cell growth was measured by optical density at 600 nm.

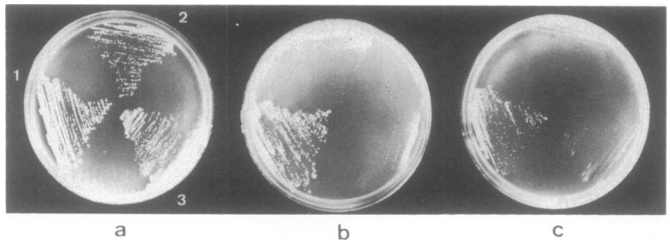

Figure 5. Effect on cell growth of MIG1-RGM1 fusion. Wild type strain MCY1389 was transformed with plasmid pEMR1inv (control) (1); pEMR1 (MIG1-RGM1 fusion protein) (2) or pMIG1 (3). Transformants were streaked on solid minimal medium containing 2% glucose (a), 2% sucrose (b) or 2% galactose (c) and the plates were incubated at 30°C for 3 days.

9–10 fold relative to the wild type strain carrying the vector plasmid pHR81 (33), although the invertase activity of pMIG transformants was still lower (Table 2). This reduced invertase activity should be responsible for the growth defect on sucrose of pEMR1 transformants. The truncated MIG1 protein containing only the zinc finger domain produced when the fragment containing the *RGM1* gene was cloned in the inverted orientation does not cause either any growth defect (Fig. 5) or significant invertase activity reduction (Table 2). Transformants carrying the truncated MIG1 produced by frame-shift in the *Xho*I site (see Materials and Methods) were able to grow both in sucrose and galactose, although colony size was slighly smaller than that of transformants carrying the vector plasmid (not shown)

DISCUSSION

I report here the isolation and partial characterization of *RGM1*, a gene that when overexpressed reduces cell growth. The amino acid sequence of RGM1 protein reveals the existence of two C_2H_2 zinc finger motifs similar to those found in the *Xenopus laevis* transcription factor IIIA (TFIIIA) and typified by the

Table 2. Effect of the overproduction of MIG1-RGM1 fusion protein.

Strain (plasmid)	Invertase activity	
	Repressed	Derepressed
MCY1389 (pHR81)	5	296
MCY1389 (pMIG1)	3	13
MCY1389 (pEMR1)	5	32
MCY1389 (pEMR1inv)	8	201

Invertase activity is expressed as mg of glucose released/min/100 mg (dry weight) of cells. Values represent the average of assays of two transformants

Drosophila gap gene Krüppel. Both zinc fingers have conserved all the residues that are generally invariant in these domains (Figure 3). The presence of this DNA binding motif suggests that the function of RGM1 should be at a transcriptional level. However, the absence of any obvious phenotype in *rgm1* mutant does not bring any clue to the identity of its target gene. Although *RGM1* was cloned by its homology with *MSN2*, the facts that the similarity is restricted to the zinc finger region and that *RGM1* does not share any of the phenotypical features of *MSN2* suggest that there is no direct functional relationship between these two genes.

An indication about the nature of RGM1 function was obtained when this protein was overproduced under the control of the *GAL10* promoter. RGM1 overproduction substantially reduces cell growth, raising the possibility that RGM1 could repress a gene or a set of genes whose products are necessary for normal mitotic growth. Nevertheless, from this experiment one cannot exclude the possibility that RGM1 is acting as a transcriptional activator of a gene encoding a protein whose excess is toxic for the cell. To distinguish between these two possibilities, the proline-rich region of RGM1 was attached to the heterologous DNA binding domain of MIG1. The fusion protein was able to repress the expression of *SUC2*, the known target of MIG1, suggesting a direct role of RGM1 in transcriptional repression through its proline-rich region. Alternative mechanisms by which the fusion protein could repress transcription (17) seem unlikely. The lack of any noticeable effect on *SUC2* expression of the truncated protein containing only the DNA binding motif of MIG1 argues against an indirect mechanism by which the repressor competes with the activator for binding to the regulatory sites in the upstream region of the target gene. The reported inhibitory effect of the overexpression of activatory domains by 'squelching' of general transcription factors (34) can be excluded because the growth defect is restricted to sucrose and galactose as carbon sources. Therefore, the most likely possibility is that RGM1 interacts directly with some general components of the transcriptional apparatus or other ancillary factors in a way that reduces the rate of formation of a functionally active initiation complex.

The proline-rich domain is not an unusual motif among transcriptional factors. Since transcriptional regulators contain distinct domains for DNA binding and for regulation of the transcription (35), the small size of RGM1 suggests that this proline-rich domain is the region of the protein that interacts with the transcriptional machinery and accounts for the repressor function of RGM1. This interpretation is supported by the inhibitory effect exerted by this domain when it is attached to a heterologous DNA binding domain.

Interestingly, the Egr proteins whose zinc fingers are very similar to those in RGM1 have also proline-rich domains. *Egr-1* and *Egr-2* genes are rapidly and transiently activated after

stimulation of murine fibroblasts with serum. Both proteins have almost identical zinc fingers but no other similarity except for the presence of proline-rich regions (15, 16). It has been suggested that a possible role of Egr proteins could be the repression of the immediate early response genes (15), although Krox-20 could act also as activator (36).

It is conceivable that a group of proline-rich transcriptional regulatory proteins is involved in the rapid response to external stimuli and that RGM1 is related to this class of factors. To establish this I have started the identification of the gene(s) regulated by RGM1

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