

Activation of gastrin gene transcription in islet cells by a RAP1-like *cis*-acting promoter element

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Abstract Gastrin transcription in islet cells is activated by a *cis*-regulatory sequence containing a binding site for the yeast transcription factor RAP1. The DNA–protein interactions between RAP1 protein and the gastrin DNA element determined by methylation interference assays are identical to those of RAP1 and yeast genes. Point mutations in the gastrin RAP1 binding site, which abolished RAP1 binding, decreased transcriptional activation by this sequence. Islet cells revealed a DNA binding protein with RAP1-like binding specificity. These findings support the conclusion that gastrin transcription is activated in mammalian cells by a RAP1-like transcription factor.

Key words: Transcription factor; Gastrin promoter; Islet cell; RAP1

1. Introduction

Gene transcription is highly conserved between yeast and mammalian cells. Not only are general RNA polymerase II transcription factor components, such as TFIID, highly conserved [1] but so are gene-specific transcription factors. The similarity between yeast HAP2/3 transcription factor and the mammalian CCAAT box binding protein, CBP1, is perhaps the most striking example of homology between yeast and mammalian transcription factors with a 75% amino acid homology in their DNA binding domains [2,3]. It is likely that mammalian homologs remain to be identified related to other classes of yeast transcription factors. One candidate is the yeast DNA binding protein RAP1. RAP1, also known as GRF1 or TURF [4,5] is a highly abundant multifunctional DNA binding protein which effects both transcriptional activation and silencing in yeast. The RAP1 binding site at the HMR silent mating-type locus is required for full repression of transcription [6–10]. RAP1 binding sites are also found upstream of a large number of genes including ribosomal and glycolytic enzyme genes activating gene transcription [11–14]. RAP1 also binds to ARSs (autonomously replicating sequences) and telomeres of yeast chromosomes to regulate cell division [10,15]. Since RAP1 regulates many biological processes common to yeast and mammalian cells, homologs of RAP1 with conserved DNA binding domains could regulate gene transcription in mammalian cells. One approach to identifying these factors is to demonstrate that mammalian genes contain RAP1 binding sites in DNA sequences controlling transcription. The gastrin gene is transiently expressed in fetal pancreatic islet cells and the encoded peptide is suggested to be involved in islet cell neogenesis [16,17]. This paper reports that both, the rat and human gastrin promoter contain a RAP1 binding site within a *cis*-regulatory domain controlling gastrin gene transcription in islet cells. Point mutations in this gastrin RAP1 binding site, which abolished RAP1 binding, also decreased transcriptional activation

by this sequence. Furthermore, extracts of islet cells contained a DNA binding protein with RAP1-like binding specificity.

2. Materials and methods

2.1. Cell lines and DNA transfection

The rat insulinoma (RIN 38A) cell line [18], hamster insulinoma cell line (HIT) and rat pituitary cell line GH4 [19] were cultured as described. For transient transfections, subconfluent HIT and GH4 cells were transfected in quadruplicates with gastrin–CAT constructs and gastrin reporter gene activity measured by assaying CAT enzyme activity [20].

2.2. Gastrin reporter gene construction

Synthetic oligonucleotides containing the RAP1 consensus site, single or multiple point mutations in the RAP1 consensus sequence or the nucleotide sequences flanking the RAP1 binding site were synthesized (Fig. 5) ligated into the *Nde*I site of the 82 GASCAT reporter gene construct [20]. All constructs were confirmed by restriction analysis and DNA sequencing.

2.3. Production of RAP1 protein and nuclear protein extracts

The RAP1 protein was cloned from a lambda gt11 expression library (unpublished results) using the ligand binding technique [21]. Sequencing revealed identity with the RAP1 sequence reported [22]. The RAP1 cDNA fragment containing the RAP1 coding sequence was subcloned into the vector pTRA-1, a derivative of pGEM-3Z, which fuses a human α -globin ATG and Kozak sequence in frame with the RAP1 sequence. The tailored RAP1 clone designated pTRA-RAP1 contained the ATG positioned immediately to the SP6 promoter. pTRA-RAP1 template linearized with *Xba*I (nucleotide 3670) was transcribed using SP6 polymerase under standard conditions (Promega Biotec) in the presence of the cap analog m7G5'ppp5'G (Pharmacia, Inc.) and translated using the rabbit reticulocyte lysate system (Promega Biotec). Mock translation reactions were identical but not primed with RAP1 RNA. Nuclear protein extracts were prepared as described [23] and protein concentration determined by the method of Bradford [24].

2.4. Gel retardation assays

Oligonucleotides used in this study were 5'-GATCCC-CACCCCATTCCTCTCGCCTGGACT-3' (hGRD), 5'-GGGATCC-ACCCCATTCCTCTCGCTGTGGGAGTCTGGCCT-3' (rGRD WT, wild type), 5'-GGGATCCACACACATTCCTCTCGCTGTGGGAGTCTGGCCT-3' (rGRD mt, mutant), 5'-GGGATCCTTATATTG-CAAAAACCCATCAACCTTGAAAA-3' (HMRE WT, wild type), 5'-GGGATCCTTATATTGCAAAAACACATCAACCTTGAAAA-3' (HMRE mt, mutant), (mutant oligonucleotides are in lower-case type, RAP1 consensus sequences are underlined, RAP1 recognition sites in

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the noncoding strands are displayed in *italics*). Single-stranded oligonucleotides were labeled with [32 P]ATP using T4 DNA kinase, hybridized with the complementary strands, purified by nondenaturing polyacrylamide gel electrophoresis and recovered on DEAE membranes in 1% agarose gels. Binding reactions using nuclear cell extracts were performed as described [20]. Binding reactions with in vitro produced protein were performed using 5 μ l of in vitro translated RAP1 protein or mock lysate.

2.5. Methylation interference analyses

Sense and antisense strands of a 80 base pair fragment containing the rat gastrin promoter sequence 5'-CACACCCATTCTCTCGC-TGTGGGAGTC-3' (nucleotides -95 to -69) were separately labelled using [32 P]dATP (3,000 Ci/mmol; Amersham) and Klenow polymerase (Bethesda Research Laboratories, Inc.) and treated with 0.25% dimethyl sulfate in 50 mM sodium cacodylate, 1 mM EDTA (pH 8.0) for 5 min at 22°C to partially methylate the DNA. The fragment was recovered by several ethanol precipitations and washed twice with 80% ethanol before using in standard binding reaction. The binding reaction was electrophoresed through a 2% agarose gel in 0.5 \times TBE, fragments present in complexed and unbound DNA bands recovered into DEAE membranes and phenol extracted before piperidine cleavage. The cleavage products from each fragment were separated on a 10% polyacrylamide sequencing gel and detected by autoradiography [25]. The G and G + A sequencing reactions were performed as described [26].

3. Results

3.1. The gastrin promoter contains in the islet regulatory domain a RAP1 binding sequence

Comparison of the rat (rGRD, unpublished results) and human (hGRD, [20]) gastrin promoter sequences revealed a high sequence conservation in the islet regulatory domains (Fig. 1a). Furthermore, a good match to the consensus recognition sequence for the yeast DNA binding protein RAP1, A(A/C)ACCCANN(C/A)(C/A)Y(C/A), [10,22,27] was observed in this conserved promoter region of the gastrin gene (Fig. 1b).

3.2. Binding of in vitro produced RAP1 protein to the rat gastrin promoter sequence

To test whether RAP1 binds to the rat gastrin islet regulatory domain, yeast RAP1 was translated in vitro using reticulocyte lysate programmed by a SP6 RNA polymerase transcript of the RAP1 cDNA. Gel mobility shift assays were performed to test binding of in vitro translated yeast RAP1 protein to a labelled DNA oligonucleotide (rGRD) containing the RAP1-like element in the gastrin promoter (Fig. 2). Lysates programmed with RAP1 SP6 transcript formed a slowly migrating specific

(a)	hGRD	5' CCCACCCATTCTCTCGCCT	GGACTC	3'
	rGRD	5' CCACACCCATTCTCTCGCTGTGGGAGTCTGGCCTC		3'
(b)	RNR2	5' ACACCCAGACCTC	3'	
	PGK	5' AAACCCAGACACG	3'	
	rGRD	5' ACACCCATTCTC	3'	
	hGRD	5' CACCCATTCTC	3'	
	HMRL	5' AAACCCATCAACC	3'	
	HMRE	5' AAACCCATCAACC	3'	
	RAP1 Consensus	5' A A A C C C A N N C A T T	3'	
		GC	CC	
		* * *		

Fig. 1. (a) Sequence comparison of the human gastrin islet regulatory element (hGRE, nucleotides -108 to -83) and the corresponding element in the rat gastrin promoter (rGRE; nucleotides -96 to -62). Conserved nucleotides are displayed in bold. (b) Sequence alignment between RAP1 binding sites of RNR2 [38], PGK [13], HMRE, HMLE [22], hGRD [20], rGRD. The consensus RAP1 binding site is given in bold. Conserved residues are indicated with asterisk.

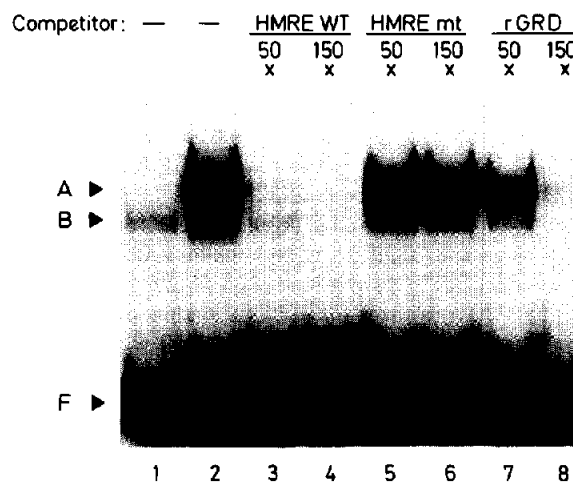


Fig. 2. Demonstration of RAP1 binding to the gastrin islet regulatory domain (rGRD). Gel retardation assays were performed using mock (lane 1) and RAP1 (lanes 2–8) programmed reticulocyte lysates and the rGRD sequence as DNA binding probe (lanes 1–8). Competitor oligonucleotides included HMRE WT, HMRE mt and rGRD sequences in 50- or 150-fold molar excess. A and B indicate DNA-protein complexes; F, free migrating DNA binding probe.

protein-DNA complex with the rGRD gastrin DNA probe (Fig. 2, lane 2) that was not seen with mock translated lysate (Fig. 2, lane 1). RAP1 binding was competed by a 50- and 150-fold molar excess of unlabelled rGRD gastrin sequence (Fig. 2, lanes 7 and 8). Binding was also specifically displayed with a 50-fold molar excess of an unlabelled oligonucleotide containing the wild type yeast HMRE sequence, a well characterized RAP1 binding site (Fig. 2, lane 3). However, DNA binding was not competed by a 150-fold molar excess of the mutant HMRE sequence oligonucleotide (HMRE mt) which differs from the wild type sequence by a single base mutation ACCCA to ACaCA (Fig. 2a, lane 6). This mutation has been shown to prevent RAP1 binding in yeast promoters [8,10].

The nucleotide residues in the rGRD DNA element necessary for RAP1 binding were determined by methylation interference analysis. Both, the coding and noncoding strands of the rat gastrin regulatory domain were endlabelled to detect contacts at the G residues opposite to the C residues in the RAP1 consensus sequence (ACCCA). RAP1 makes specific contacts with the highly conserved G-C base pairs in the RAP1 consensus binding site of the noncoding strand of the rat gastrin regulatory domain (Fig. 3) similar to that seen in the RAP1 binding site in the yeast PGK promoter (Fig. 1c) [13]. Analysis of the coding strand detected no additional DNA/protein contacts between the rGRD sequence and the recombinant yeast transcription factor (Fig. 3).

3.3. Islet nuclear extracts contain a protein with RAP1-like DNA binding activity

Islet nuclear extracts were examined for RAP1-like DNA binding activity by gel mobility shift assays using the rGRD probe containing the RAP1 recognition site (rGRD WT, Fig. 1). Binding was also analysed with a mutant gastrin probe (rGRD mt, Fig. 1) containing an identical sequence save for the single C-A transversion disrupting RAP1 binding. Three complexes (C1 to C3) were observed using the wild type rat gastrin

sequence as probe (Fig. 4, lane 1). Competition with a 50-fold excess of unlabelled wild type gastrin sequence rGRD WT abolished the upper and middle complexes C1 and C2, while the lower complex C3 was only partially displaced (Fig. 4, lane 2). However, competition with a 50-fold excess of unlabelled mutant gastrin sequence, rGRD mt, displaced the middle complex C2 but not the upper complex C1 (Fig. 4, lane 3). Furthermore, only complexes C2 and C3 could be resolved when using the mutant gastrin sequence rGRD mt as probe, when the upper complex C1 could not be observed (Fig. 4, lane 4). These findings support the hypothesis that a DNA binding protein in islet cells binds specifically dependent on the RAP1 recognition site in the gastrin promoter.

3.4. The RAP1 element in the gastrin promoter mediates activation of gastrin gene transcription in islet cells

To determine whether the RAP1 binding sequence functioned as a transcriptional regulatory element, islet and pituitary tumour cell lines were transfected with gastrin reporter gene constructs (RAP-GASCAT). The constructs comprised synthetic oligonucleotides of the sequence from –75 to –107 of the gastrin gene containing the RAP1 binding site ligated 5' to a truncated gastrin reporter gene 82GASCAT [20]. The synthetic oligonucleotides comprised the wild type sequence or

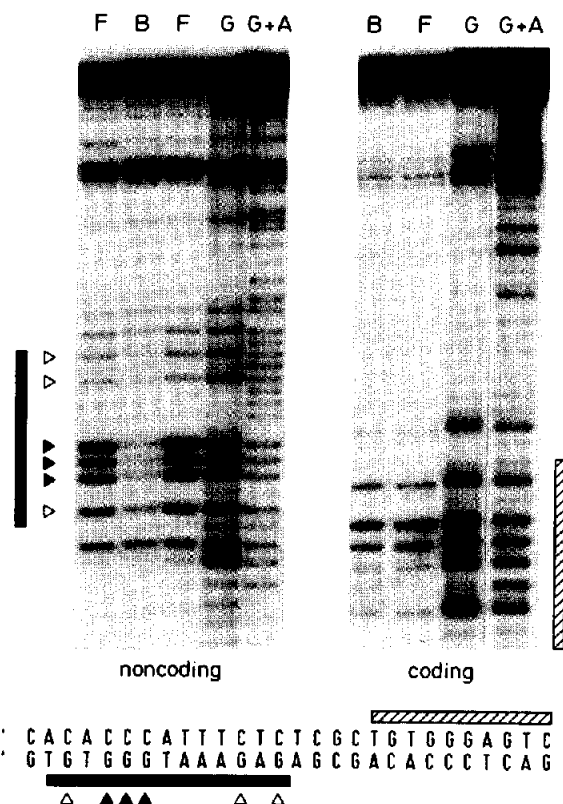


Fig. 3. Methylation interference of the rGRD-RAP1 complex A (Fig. 2, lane 2). Coding and noncoding strands are displayed. F, free fragment. B, fragment bound to RAP1. G and G + A refer to Maxam and Gilbert sequencing reactions. The positions of the RAP1 binding elements in the rat gastrin regulatory domain (rGRD) relative to the autoradiograph are shown on the left and right side. G residues at which methylation interfered with RAP1 binding to the rGRD are indicated with triangles. Solid triangles represent a greater degree of interference than open triangles.

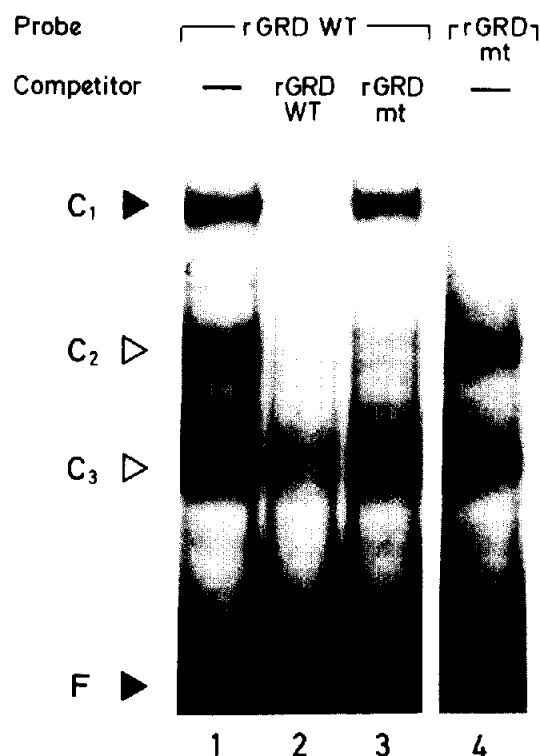


Fig. 4. Demonstration of a RAP1-like DNA binding activity in islet cell extract. Gel retardation assays using RIN 38A cell extracts and the rGRD WT sequence (lanes 1–3) or rGRD mt sequence (lane 4) as labelled probes. Unlabelled rGRD WT sequence and rGRD mt sequence were used as competitors in lanes 2 and 3, respectively. C1, C2 and C3 indicate DNA/protein complexes. F, free migrating probe.

mutations in the RAP1 binding site or in residues flanking the RAP1 sequence. These constructs were transiently transfected into GH4 and HIT cell lines and transcriptional activity measured by assaying CAT activity (Fig. 5). Mutations within the RAP1 binding site decreased reporter gene expression in both cell lines in contrast to mutations outside the RAP1 binding site which did not decrease reporter gene expression. Furthermore, there was a strong quantitative correlation between the strength of RAP1 binding and transcriptional activity amongst the different reporter gene mutants. The single C–A point mutation in the ACCCA motif, which was the site of strongest methylation interference of RAP1 binding (Fig. 3) markedly decreased reporter gene activity. Mutations in nucleotides which showed weak methylation interference of RAP1 binding (Fig. 3) only weakly decreased reporter gene activity. Point mutations in sequences downstream of the RAP1 site in the gastrin promoter had only little effect on promoter activity. These data suggest that the rat gastrin gene contains a positive *cis*-regulatory element which binds a transcriptional activator protein with sequence specificity similar to the yeast transcription factor RAP1.

4. Discussion

Many studies have shown that yeast transcription factors share both, structural and functional homology with mammalian transcription factors [28–30]. One feature of this conservation is that homologous mammalian and yeast transcription

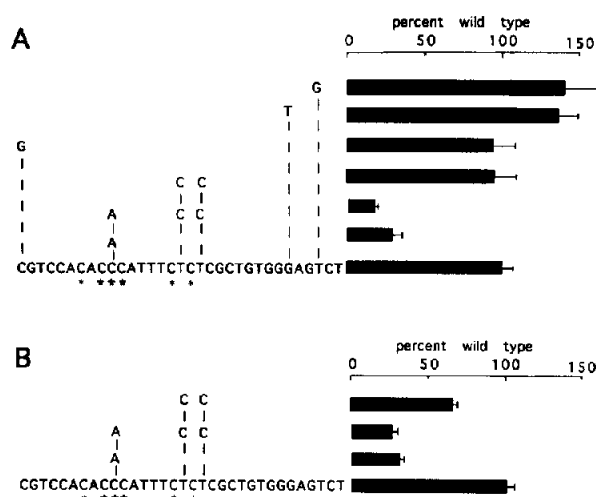


Fig. 5. The RAP1 binding site in the rat gastrin promoter is a positive *cis*-regulatory element. CAT receptor gene expression in HIT and GH4 cells transiently transfected with CAT reporter gene constructs with the wild type or mutated rat RAP1 binding sites. Reporter gene activity for each construct is shown as a percentage of the wild type construct activity (mean \pm S.E.M., $n = 4$). The wild type sequence is shown at the bottom where dark or light asterisks indicate sites of strong or weak methylation interference of RAP1 binding. The nucleotide substitution in the mutant constructs are shown above.

factors recognize identical DNA sequences. Thus, the presence of a recognition motif for a yeast transcription factor within a *cis*-regulatory element of a mammalian promoter may indicate that this site is bound by a mammalian homologue of that transcription factor. This has been verified by many examples, including the TGACTGCA, CAAT and CAnnTG motifs binding leucine zipper and helix loop helix proteins [2,31–34]. Although RAP1 is an abundant multifunctional DNA binding protein which regulates highly conserved fundamental eukaryotic functions, no homologue protein has been identified in mammalian cells. Recently, however, Pollice et al. [35] reported the presence of a RAP1 binding site in the polyoma virus enhancer which activated transcriptional activity in yeast cells. Similar RAP1 binding sites are also found in the SV40 and BPV enhancers. The functional significance of this RAP1 site in the polyoma enhancer was not clear since the RAP1 site was not associated with binding sites for other transcription factors as would be anticipated for functional *cis*-regulatory elements in promoters and enhancer elements. Furthermore, detection of a RAP1-like binding protein in mammalian cells has not been reported nor has the RAP1 site been shown to be a *cis*-regulatory element controlling transcription in mammalian cells.

The present study has identified a RAP1 binding site within a *cis*-regulatory sequence (GRD) which controls gastrin gene transcription in islet cells [20]. The RAP1 binding site in the gastrin promoter is adjacent to *cis*-regulatory elements which bind transcriptional repressors (ATTCCTCT) or activators (CATATGG). This is consistent with previous studies in yeast suggesting that the regulatory function of RAP1 is determined by the context of its binding site and, presumably its interaction with other factors [36,37]. Gel mobility shift assays and methylation interference studies also demonstrated sequence specific DNA–protein interactions between the gastrin DNA element

and recombinant RAP1 protein identical to those reported for RAP1 binding sites in yeast promoters. Point mutations within the GRD which abolish RAP1 binding greatly decreased transcriptional activity of reporter genes transfected into mammalian cells, suggesting that mammalian cells express a transcriptional activator which binds to RAP1 binding sites. Gel mobility shift assays revealed a specific DNA–protein complex formed between GRD DNA sequences containing the RAP1 gastrin site and a protein in islet cell nuclear extracts. Although further characterization of this factor is required to confirm RAP1-like properties of this DNA binding protein, these findings support the hypothesis that mammalian gene transcription is activated by homologs of the yeast transcription factor RAP1.

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