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The glioma-amplified sequence 41 gene (*GAS41*) is a direct Myb target gene

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ABSTRACT

The retroviral oncogene v-*myb* encodes a transcription factor (v-Myb) which transforms myelomonocytic cells *in vivo* and *in vitro*. It is thought that v-Myb exerts its biological effects by deregulating the expression of specific target genes, most of which are still unknown. The chicken glioma-amplified sequence 41 gene (*GAS41*) is located immediately downstream of the *lysozyme* gene, a known Myb-regulated gene. The *GAS41* promoter colocalizes with a CpG island which also functions as an origin of replication. Since the *GAS41* promoter contains several potential Myb-binding sites (MBSs) we have investigated whether *GAS41* is a v-Myb target gene. Our results show that the *GAS41* gene is directly activated by a v-Myb/estrogen receptor fusion protein. Furthermore, our studies reveal that the *GAS41* promoter is stimulated by v-Myb in co-transfection experiments and that the DNA-binding activity of v-Myb is crucial for transactivation of the promoter. Electrophoretic mobility-shift assays (EMSA) indicate that several Myb-binding sites, residing ~250 bp upstream of the transcriptional start site, are bound by Myb *in vitro*. Furthermore, chromatin immunoprecipitation assays demonstrate that v-Myb is bound to the *GAS41* promoter *in vivo*. Taken together these findings identify the *GAS41* gene as a novel v-Myb target gene. We have also analysed the *GAS41* replication origin in myelomonocytic cells and have failed to observe significant differences in origin activity in cells expressing or not expressing v-Myb.

INTRODUCTION

The glioma-amplified sequence 41 gene (*GAS41*) was originally isolated from a glioma cell line (1) and was shown to be frequently amplified in glial tumours (2) which account for 40% of the central nervous system tumours (3,4). The GAS41 protein is highly conserved among humans (U61384), mice (AA529582), chickens (AJ396415), *Drosophila melanogaster* (AAF52462) and *Caenorhabditis elegans* (CAB1234) (5). It lies immediately downstream of the well-studied *lysozyme* gene which is known to be flanked by two matrix attachment regions (MARs) (6). The location of *GAS41* with respect to the *lysozyme* gene is conserved among human (12q13–q15), mouse (chromosome 10D2) and chicken (accession no. AF410481). The GAS41 protein is related to the human AF-9 and ENL transcription factors (2) and contains a potential α-helical acidic activation domain, as is found in many eukaryotic transcription factors (7). GAS41 was recently found to be an interaction partner of the human MLL-AF10 fusion protein which is detected in ~5–10% of human acute leukaemias (8) thus linking *GAS41* overexpression to human malignant transformation. In addition, Debernardi *et al*. (8) have shown that GAS41 interacts with INI1 (Integrase Interactor 1) which is the human homologue of the yeast SNF5 protein. SNF5 is a component of the SWI/SNF complex which in turn is known for its ATP-dependent chromatin remodelling activity (9). The GAS41 protein is located mainly in the interphase nucleus, but not in the nucleolus, and is uniformly distributed in the cell during mitosis (10). Harborth *et al*. (10) have also reported that GAS41 interacts with NuMA (nuclear mitotic apparatus protein) which is a component of the nuclear matrix in interphase cells. The *GAS41* gene is ubiquitously expressed in humans and chickens. It has been shown that GAS41 is essential for cell viability, as a homozygous *GAS41*-deficient cell line could not be obtained by homologous recombination. However, both copies of the gene could be disrupted in the presence of a tetracycline-regulated *GAS41* cDNA. When tetracycline is withdrawn from these cells, total RNA synthesis is drastically decreased leading to cell death (11).

The retroviral oncogene v-*myb* encodes a transcription factor that transforms myelomonocytic cells and causes leukaemia in chickens [reviewed in (12)]. The protooncogene c-*myb*, from which v-*myb* is derived, is highly expressed in most haematopoietic progenitor cells and is essential for the development of the haematopoietic system. It has been shown that mice lacking a functional c-*myb* gene die during embryonic development because of multiple defects of the haematopoietic system (13). The proteins encoded by v-*myb* and c-*myb* bind to the sequence motif PyAA$^{C}/_{T}$G (14) and activate promoters containing such sites (15–18). It is generally believed that cell transformation by v-*myb* is caused by the activation of specific target genes. Until now, a number of genes have been identified as bona fide Myb target genes, including *mim*-1 (16), the *lysozyme* gene (19), *bcl*-2 (20,21), *tom*-1 (22) and *GBX*2 (23) among others. Some of these genes have been studied in detail; however, it has not yet been established whether any of the known Myb target genes play a crucial role in cell transformation by v-Myb.

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MATERIALS AND METHODS

Cell culture

The chicken cell lines BM2 (AMV-transformed myeloblasts) and HD11 (MC29-transformed macrophages) have been described previously (24,25). 10.4 is a derivative of the HD11 cell line expressing an E26 v-Myb/ER fusion protein (26). To investigate the direct stimulation of the c*GAS41* gene 10.4 cells were treated with 50 μg/ml cycloheximide for 15 min and then incubated in the presence or absence of 2 μM β-estradiol for a further 5 h.

Northern blotting

Preparation of polyadenylated RNA and northern blotting were performed as described in (24). As an internal control for mRNA quantities, a specific probe for the ribosomal *S17* gene was used.

Reporter genes and transfections

The luciferase reporter genes were constructed by cloning a 1.3 or a 0.4 kb GAS41-promoter fragment into the luciferase vector pGL3-Basic (Promega). Co-transfection experiments with different Myb-expressing vectors were performed using either an E26 v-Myb (pCDE26 v-myb) or an AMV Myb expression vector (pCDAMV v-myb). As a control an empty expression vector (pcDNA3) was co-transfected. Alternatively, an expression vector for an AMV v-Myb mutant lacking the DNA binding domain (pVM 130) or the corresponding empty expression vector (pVM 111) was used. All vectors have been described previously (15,27). The β-galactosidase reporter gene pCMVβ was obtained from Clontech. Transfection experiments were performed by calcium phosphate coprecipitation as described in (24).

Electrophoretic mobility-shift assay (EMSA)

The following pairs of single-stranded oligonucleotides were annealed and used for EMSAs: Myb1+2_sense, 5′-GCTACCCGCTTGGCAGTTTTAAACGCATCCCTCAAT-3′; Myb1+2_anti, 5′-AATGAGGGATGCGTTTAAAACTGCCAAGCGGGTAG-3′; Myb3_sense, 5′-TCCCTCATTAAAACGACTTATACG-3′; Myb3_anti, 5′-GCGTATAGTCGTTTTAATGAGGGA-3′; Myb4_sense, 5′-ACTATACGCAAACGCCTTCCCGT-3′; and Myb4_anti, 5′-GACGGGAAGGCGTTTGCGTAT AGT-3′. After annealing, oligonucleotides were radiolabelled by filling-in the ends using [α-^{32}P]dCTP. Preparation of bacterial full-length v-Myb protein and binding experiments were performed as described previously (28).

Chromatin immunoprecipitation (ChIP)

Approximately 10^8 BM2 or HD11 cells were incubated in 1/10 crosslinking solution containing 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris–HCl, pH 8.0, and 11% formaldehyde for 1 h and quenched for 5 min in 125 mM glycine. After washing in ice-cold phosphate-buffered saline, the cells were treated for 20 min each with washing solutions A (0.25% Triton X-100, 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5 mM EGTA) and B (200 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA). Nuclei were sonicated on ice (4 times for 20 s in 2 min intervals) in egg lysis buffer (ELB) (120 mM NaCl, 50 mM Tris–HCl, pH 7.5, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM sodiumpyrophosphate, 1 mM phenylmethylsulfonyl fluoride and 0.1% Nonidet P-40). DNA–protein complexes were incubated with two different Myb-specific antibodies, normal rabbit serum or no antibody. After precipitating with protein-A–Sepharose, the samples were washed several times in ELB buffer. Following elution with 0.5% SDS the DNA was recovered by reverse-crosslinking for 6 h at 37 and 65°C, respectively, in a buffer containing 0.5% SDS; 10 mM DTT and 100 μg proteinase K. The immunoprecipitated DNA was then purified by phenol–chloroform extraction and ethanol precipitation. PCR amplification was performed with primers specific for the chicken *GAS41* promoter region, forward 5′-GTGTTTCCCGCTTCCTTTCTTAA-3′ and reverse 5′-GCGTTCATGCCGTTTCCAT-3′. Alternatively, *Lysozyme* intron-2-specific primers, forward 5′-GGTAGTAAAAGCTTGACCCTTGCA-3′ and reverse 5′-GTTCACCTCTCCTCCCCTTCA-3′ were used. PCR products were resolved on a 10% polyacrylamide gel and stained with ethidium bromide.

Nascent strand abundance assay

Analysis of replication origin activity was performed according to Giacca *et al*. (29). Total genomic DNA was isolated from ~10^8 exponentially growing HD11 or BM2 cells with Nucleobond CB kit (Macherey&Nagel) following the manufacturer's instructions. DNA was resuspended in TNE buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) was heat-denatured for 8 min at 95°C followed by rapid cooling on ice. Denatured DNA was layered on a linear neutral sucrose gradient (5–30% in TNE) and size fractionated by centrifugation in a Beckman SW28 rotor at 26 000 r.p.m. for 20 h at 20°C. Nascent strand DNA fractions with an average DNA size of 1–1.5 kb were precipitated with ethanol and resuspended in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). The purified nascent DNA strands were analysed by quantitative real-time PCR using the following primer pairs: Lys_−8.1 kb_for 5′-AAGCTTCAGCAGGTCATTGT CTT-3′; Lys_−8.1 kb_rev 5′-GCTTCGACATCAGTTCCACTCT-3′; Lys-In2(+1.9 kb)_for 5′-GGTAGTAAAAGCTTGACCCTTGCA-3′; Lys-In2(+1.9 kb)_rev 5′-GTTCACCTCTC CTCCCCTTCA-3′; GAS-prom(+3.3 kb)_for 5′-GTGTTTCCCGCTTCCTTTCTTAA-3′; GAS-prom(+3.3 kb)_rev 5′-GCGTTCATGCCGTTTCCAT-3′; Lys_+4.7 kb_for 5′-GGAGCTGACTGAGCTCCCTTTC-3′; Lys_+4.7 kb_rev 5′-AGCCAAATGCAGCCAAAGC-3′; Lys_+8.8 kb_for 5′-ATCGTGTGATTTTCGGATGGAA-3′; Lys_+8.8 kb_rev 5′-ATCTCG GCTTTCAATAGGCTTTTC-3′.

Real-time PCR quantification

Q-PCR was carried out on a GeneAmp 5700 SDS (Applied Biosystems) with the qPCR Core kit for SYBR I (Eurogentec) following the manufacturer's instructions. Primers were chosen with PrimerExpress 2.0 Software (Applied Biosystems). A 5-fold serial dilution of sheared genomic DNA with a known concentration was used to create a standard curve. Genomic equivalents (1 genomic equivalent = 1.25×10^{-12} g) of the samples were extrapolated and relative abundance was calculated. The specificity of the PCR products was checked by melting curve analysis and agarose gel electrophoresis (data not shown).

RESULTS

Expression of *GAS41* mRNA is induced by v-Myb

Recently, the *GAS41* gene has attracted our attention as a potential Myb-regulated gene. It was shown that the *GAS41* gene resides very close to a known Myb target gene, the chicken *lysozyme* gene (6). The *lysozyme* gene is embedded in a 24 kb domain of general DNaseI sensitivity that is flanked by two boundary elements that coincide with MARs [(30); for review see (31)]. It has been shown that a set of DNaseI-hypersensitive sites (DHSs) are necessary for correct spatial and temporal expression of the gene (32). The *GAS41* gene is situated immediately downstream of the chicken *lysozyme* gene (Figure 1) and, thus, lies within its transcriptional domain

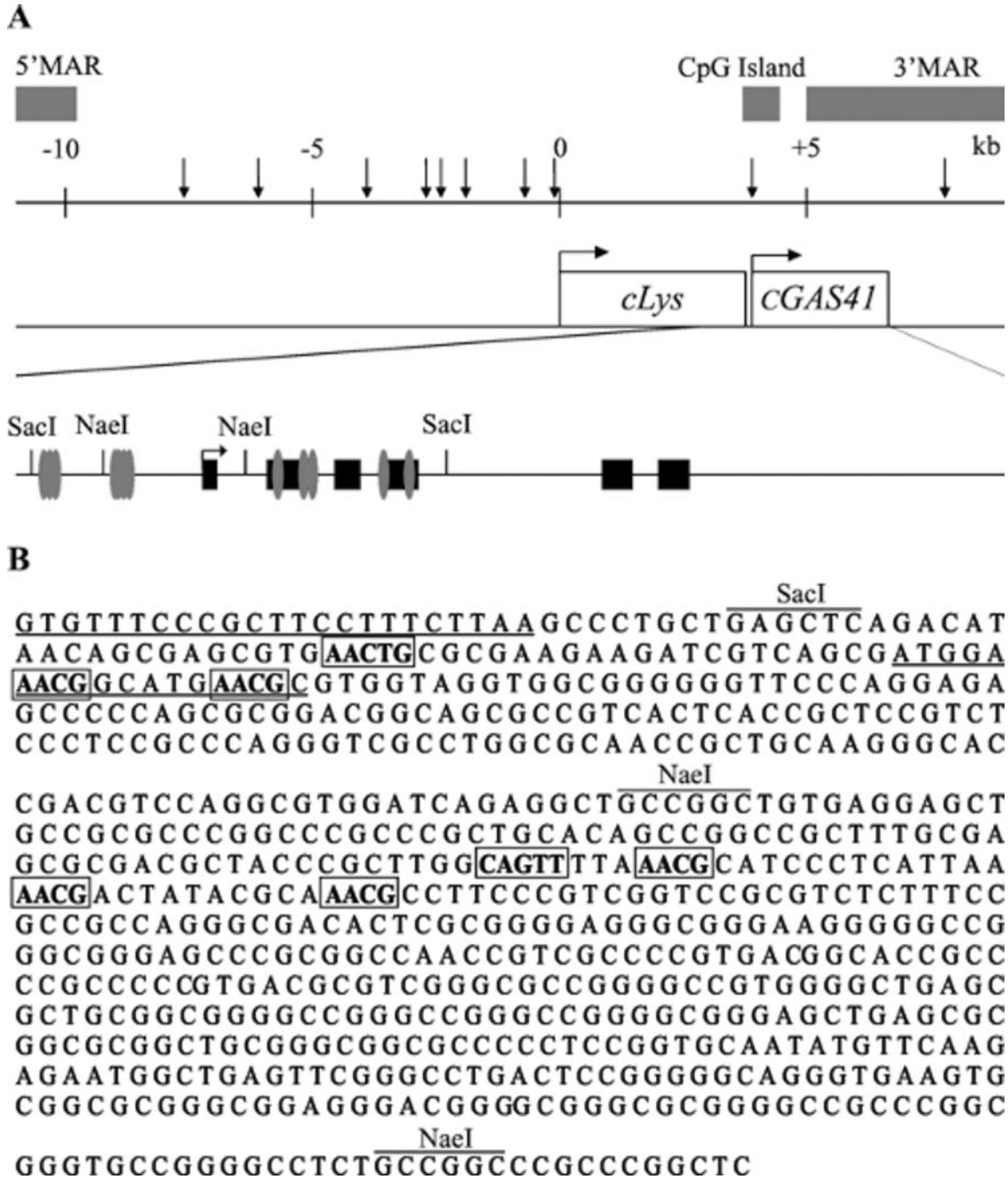

Figure 1. Transcriptional domain of the *lysozyme* (*cLys*) and *cGAS41* gene. (**A**) Grey boxes mark the boundaries of the general DNaseI-sensitive region and coincide with MARs. The location of a CpG-island which contains the *GAS41* promoter is also indicated as a grey box. DHSs within this domain are shown as vertical arrows. The orientation of the *lysozyme* and the *GAS41* genes and their transcriptional start sites are visible as open boxes and arrows. At the bottom the region of the *GAS41* gene is expanded. Potential MBSs are displayed as grey ovals and exons as dark boxes. For better orientation, SacI and NaeI restriction sites are shown. (**B**) Nucleotide sequence of the promoter region of the *GAS41* gene. Annealing sites for primers used in ChIP experiments are shown underlined, potential MBSs are marked with boxes. The transcriptional start site is indicated by an arrow. For better orientation one SacI and both NaeI restriction sites are shown.

(6). The promoter of the *GAS41* gene coincides with a CpG island. In addition, Phi-van and coworkers (33,34) showed that an origin of bidirectional replication (OBR) is located within the CpG island of the *GAS41* promoter (Figure 1A). Interestingly, the cell-type-specific expression patterns of the *lysozyme* and *GAS41* genes are very different. While the expression of the *lysozyme* gene is restricted to certain cell types, such as myelomonocytic cells, *GAS41* appears to be ubiquitously expressed. Since the chicken *lysozyme* gene is a direct Myb target gene and since the *GAS41* promoter region contains several potential Myb binding sites (Figure 1B), we were interested to know whether *GAS41* is also an Myb target gene.

To determine whether *GAS41* expression is regulated by Myb we performed northern blot analyses with polyadenylated RNA of 10.4 cells. 10.4 is a subclone of the chicken macrophage cell line HD11 stably expressing a v-Myb/estrogen receptor fusion protein (26). Upon β-estradiol treatment the v-Myb/ER fusion protein is activated and upregulates the expression of a number of Myb target genes (22,26,35,36). 10.4 cells were cultivated for 24 h in the presence or absence of 2 μM β-estradiol. Polyadenylated RNA from these cells was then isolated and analysed by northern blotting with probes specific for *GAS41*, the *lysozyme* gene and, as an internal control, the ribosomal *S17* gene (Figure 2A). As shown in Figure 2A, *GAS41* mRNA level was higher in estrogen-treated 10.4 cells compared to untreated cells, suggesting that chicken *GAS41* is indeed an Myb target gene. The effect of v-Myb on the expression of *GAS41* was lower than its effect on the expression of the *lysozyme* gene, indicating that both genes are regulated independently by Myb.

v-Myb directly increases *GAS41* mRNA expression

We next wished to know if *GAS41* is directly activated by v-Myb/ER. It had been shown that treatment of 10.4 cells with β-estradiol does not alter v-Myb/ER expression but solely activates the fusion protein independently of *de novo* protein synthesis (22,26). The v-Myb/ER system, therefore, allows to investigate if a particular gene is directly induced by v-Myb by analysing the effect of β-estradiol on this gene in the presence or absence of a protein synthesis inhibitor, such as cycloheximide (22,26). To determine if the stimulation of *GAS41* expression by v-Myb is direct, 10.4 cells were cultivated in the presence or absence of 50 μg/ml cycloheximide for 15 min, prior to addition of β-estradiol, and further incubated for 5 h. We treated the cells for only 5 h with β-estradiol (as opposed to 24 h in the experiment shown in Figure 2A) to minimize the toxic effects of cycloheximide. Polyadenylated RNA was prepared from these cells and analysed by northern blotting. A representative experiment is illustrated in Figure 2B. As shown in this figure activation of *GAS41* by v-Myb was observed in the absence as well as in the presence of cycloheximide. Since the induction of Myb target gene expression by v-Myb/ER increases with time after addition of β-estradiol (26,36), the overall induction by v-Myb/ER is lower in Figure 2B (5 h treatment with β-estradiol) than in Figure 2A (24 h treatment). To rule out that this effect is restricted to *GAS41*, we also analysed the expression of *lyzozyme* (Figure 2B) which is known to be highly Myb-inducible (Figure 2A). Under these conditions lysozyme expression is drastically lowered. From the data shown in Figure 2B we concluded that v-Myb directly induces *GAS41* expression.

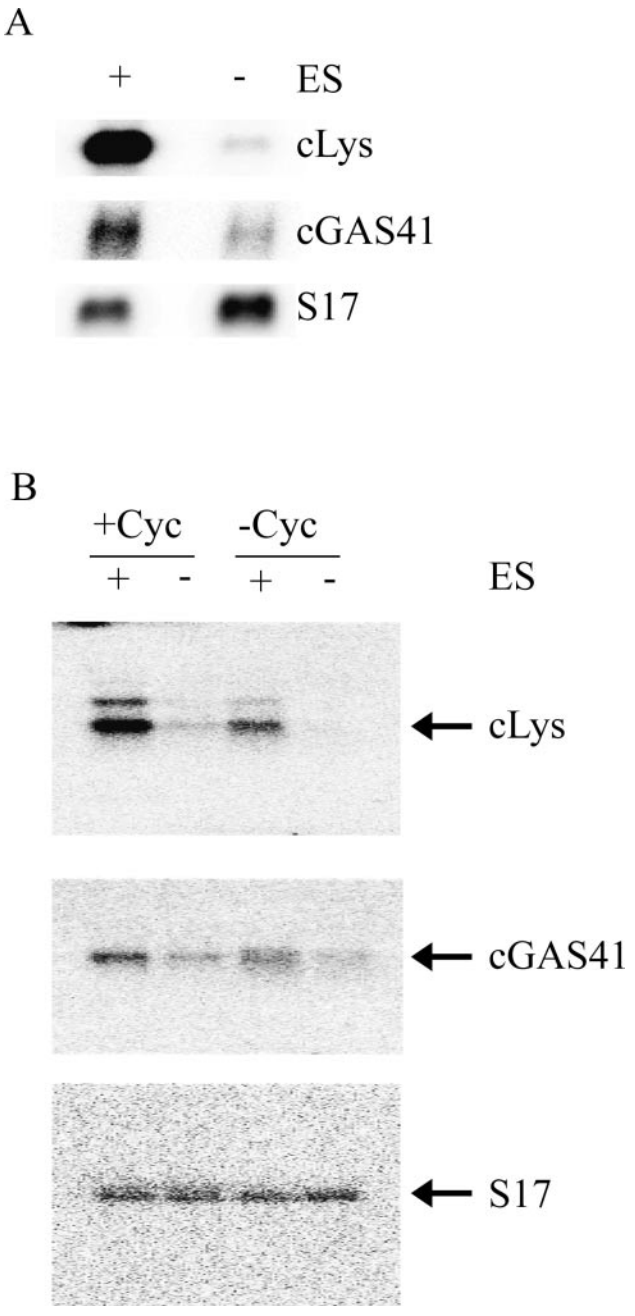

Figure 2. Induction of *GAS41* expression by a v-Myb/ER fusion protein. (**A**) Polyadenylated RNA from 10.4 cells grown for 24 h in the presence (+ ES) or absence (− ES) of 2 μM β-estradiol was analysed by a northern blot experiment using a *GAS41*-specific probe (middle). For comparison, induction of *lysozyme* (*cLys*) expression is shown at the top. Expression of the chicken *S17* gene was analysed as a control (bottom). (**B**) Stimulation of *GAS41* expression in the presence of cycloheximide. 10.4 cells were grown in the presence (+ Cyc) or absence (− Cyc) of 50 μg/ml cycloheximide either with (+ ES) or without (− ES) 2 μM β-estradiol. Cycloheximide was added to the cultures 15 min prior to hormone treatment. Cells were cultivated for 5 h and polyadenylated RNA was analysed by a northern blot experiment using a *lysozyme*- (top) or a *GAS41*-specific probe (middle). A chicken *S17* probe was used as control (bottom).

The *GAS41* promoter is stimulated by binding of v-Myb

To provide further evidence for the idea that *GAS41* is a direct Myb target gene, we performed transient transactivation studies with two different *GAS41* promoter constructs.

A schematic illustration of these reporter genes is shown in Figure 3. Potential *Myb*-binding sites are indicated as grey ovals. 10.4 cells expressing the v-Myb/ER fusion protein were transfected with the reporter genes and cultivated for 24 h in the presence or absence of β-estradiol before reporter gene activity was determined (Figure 3A). In case of both constructs, the luciferase expression levels were Myb inducible, supporting the idea that the *GAS41* promoter can be stimulated by Myb. In addition, we transfected HD11 cells with these reporter genes and expression vectors encoding the

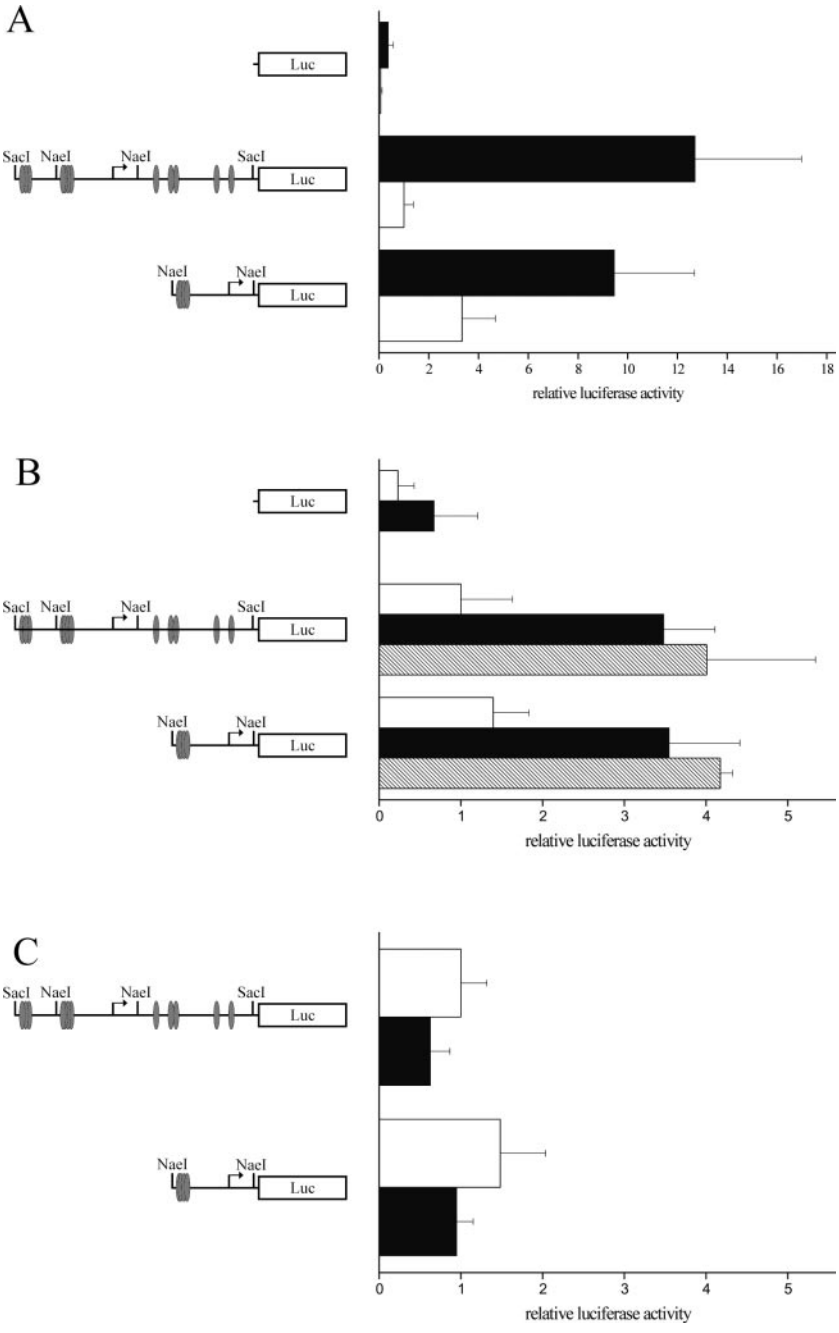

Figure 3. Activation of *GAS41* reporter genes by v-Myb. *GAS41* promoter reporter gene constructs used in transactivation studies are shown schematically at the left of each panel. The SacI and NaeI restriction sites that were used for cloning are marked. Potential *Myb*-binding sites are indicated as grey ovals. (**A**) Transient transfection experiments of the reporter gene constructs in 10.4 cells. Cells were transfected with 5 μg of the reporter gene and 1 μg of the β-galactosidase reference plasmid pCMVβ as described in (24). Cells were cultivated in the presence (black bars) or absence (white bars) of 2 μM β-estradiol. 24 h after transfection the cells were analysed for luciferase and β-galactosidase activity. The columns show the average relative luciferase activity of the reporter gene. Thin lines show the standard deviations. (**B**) HD11 cells were transfected as described in (A). In addition, 1 μg of either AMV Myb or E26 v-Myb expressing vector, pCDAMVv-myb (hatched bars) and pCDE26v-myb (black bars), or of empty expression vector pCDNA3 (white bars) were co-transfected and analysed as described in (A). (**C**) To emphasize that DNA binding of v-Myb is important for transactivation 1 μg of an expression vector for a v-Myb mutant lacking the DNA binding domain (pVM 130, black bars) or 1 μg of the corresponding empty expression vector (pVM 111, white bars) were co-transfected into HD11 cells. Cells were transfected, harvested and analysed as described in (A).

v-Myb proteins of AMV and E26. Since the *lysozyme* gene is activated only by the E26 version of v-Myb but not by that of AMV (19) we wanted to see if AMV and E26 Myb also differ with respect to *GAS41*. As shown in Figure 3B, both v-Myb variants activate the *GAS41* constructs nearly to the same extent, although the induction by v-Myb was somewhat lower in case of the HD11 cells compared to 10.4 cells. To further show that the DNA-binding activity of v-Myb is crucial for activation of the *GAS41* promoter, we used an expression vector for a v-Myb mutant that lacks the DNA binding domain (pVM130) (Figure 3C). In this case transactivation of the reporter gene constructs was not observed indicating that Myb binding is required for the activiation of the *GAS41* promoter. Taken together, the experiments illustrated in Figure 3 support the idea that the *GAS41* promoter is directly activated by Myb.

In vitro binding of v-Myb to the *GAS41* promoter

To show that v-Myb is able to bind to the potential Myb-binding sites located nearest to the *GAS41* transcription start site we performed EMSAs. Figure 4 depicts the pGL3-GAS-0.4NaeI construct that was used for reporter gene assays and the four potential Myb-binding sites therein. Oligonucleotides used in the EMSA experiments are underlined. To show that Myb binds to these sites, *in vitro* recombinant protein was expressed in bacteria and used for binding assays as described in (28). Several major DNA–protein complexes can be distinguished in Figure 4. These complexes are due to partial proteolysis of the v-Myb protein preparation used in this array. The slowest migrating complex is formed by full-length v-Myb. We conclude from this experiment that v-Myb binds *in vitro* to all of the four potential binding sites suggesting that they play a role in Myb induction of the *GAS41* promoter.

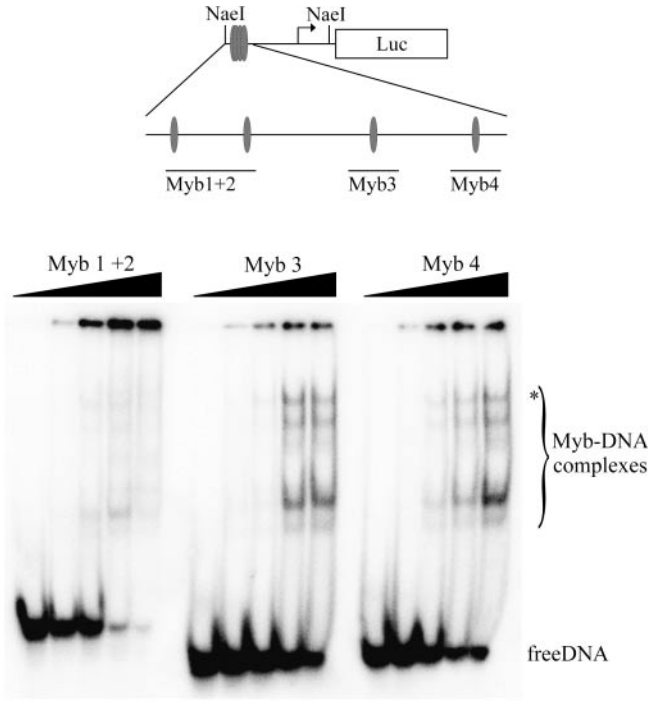

Figure 4. EMSA of the *GAS41* promoter. The pGL3-GAS-0.4NaeI reporter gene construct and positions of Myb binding sites 1 to 4 are depicted (upper panel). The oligonucleotides used for the binding assays are drawn as lines below. Oligonucleotides were radioactively labelled and incubated with increasing amounts (0–11 µl) of bacterially expressed v-Myb as described in (28). Due to partial proteolysis of the bacterial v-Myb protein several Myb–DNA complexes are visible. The complex formed by full-length v-Myb is marked by an asterisk. The intense bands at the bottom are unbound oligonucleotides.

v-Myb is bound to the *GAS41* promoter *in vivo*

To demonstrate that v-Myb is bound to the *GAS41* promoter *in vivo* we performed ChIP experiments using the v-Myb-transformed myeloblast line BM2 and the Myb non-expressing myeloid macrophage line HD11. The cells were formaldehyde-fixed, and the DNA–protein complexes obtained after sonication were immunoprecipitated with two different antibodies against Myb. As a control, parallel incubations with non-immune serum or without any antiserum were performed. After reverse-crosslinking of the immunoprecipitated DNA, the samples were analysed by PCR using primers specific for the *GAS41* promoter region. The result of this experiment is illustrated in Figure 5A–D. The first two lanes of all panels show DNA samples that were precipitated with Myb-specific antibodies. Amplification of the *GAS41* promoter region was only observed in samples derived from BM2 cells that express

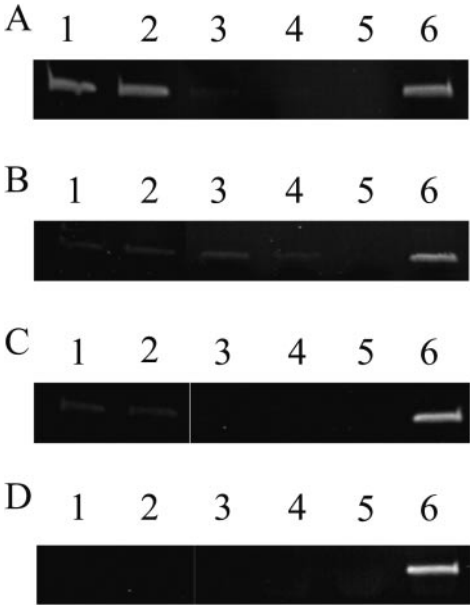

Figure 5. ChIP analysis of the *GAS41* promoter. ChIP analysis was performed as described in Lefevre *et al*. (40). In brief, ~10^8 BM2 (**A** and **C**) or HD11 cells (**B** and **D**) were incubated in 1/10 crosslinking solution, 11% formaldehyde, for 1 h. After washing nuclei were sonicated on ice (4 times for 20 sec in 2 min intervals) in ELB. DNA–protein complexes were incubated with two different Myb-specific antibodies (lanes 1 and 2), normal rabbit serum (lane 3) or water (lane 4). Precipitates were washed in ELB buffer and then eluted. Reverse crosslinking was performed for 6 h at 37 and 65°C, respectively, in a buffer containing proteinase K. The immunoprecipitated DNA was then purified by phenol–chloroform extraction and ethanol precipitation. PCR amplification of immunoprecipitated DNA was performed with the indicated samples (lanes 1–4), with water as no template control (lane 5) and with the sonicated and reverse-crosslinked input DNA (lane 6), using primers specific for the chicken *GAS41* promoter region (A and B) and for the chicken l*ysozyme* intron 2 (C and D). PCR products were resolved on a 10% polyacrylamide gel and stained with ethidium bromide.

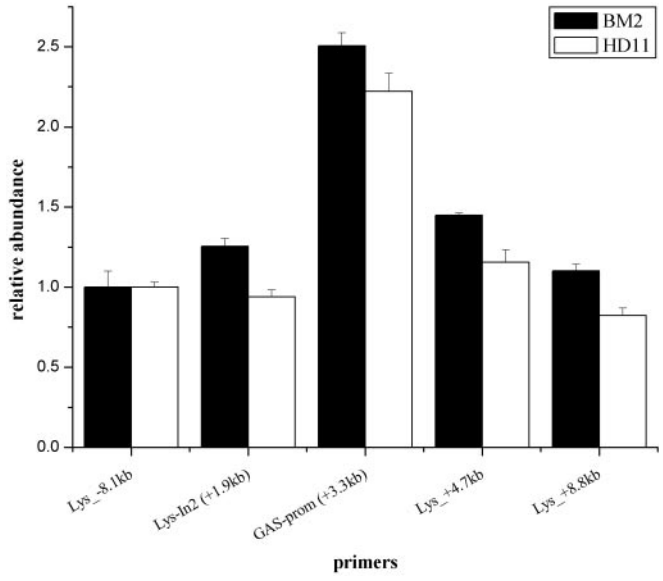

Figure 6. Nascent strand abundance assay of the GAS41 origin. A modified nascent strand abundance assay was used to map GAS41 origin activity within an 18 kb region (accession no. AF410481). For the quantification, real-time PCR was performed. Primer pairs used for PCR are designated corresponding to their position relative to the transcription start of the *lysozyme* gene. Columns show the average abundance of specific sequences in nascent strand preparations of BM2 and HD11 cells relative to the Lys_−8.1 kb primers. Error bars mark standard deviations.

v-Myb (Figure 5A). Samples derived from HD11 cells that lack v-Myb expression did not yield a PCR product (Figure 5B). Analysis of input samples (lanes 6) show that similar amounts of DNA were present before precipitation and negative controls (lanes 3–5) demonstrate the specificity of the assay. As an additional control we performed PCR reactions using primers that are specific for a part of the intron 2 of the chicken *lysozyme* gene. This region is ∼1.4 kb away from the region amplified by the *GAS41* promoter-specific primers and lacks potential *Myb*-binding sites. Figure 5 shows that there was only a very weak signal in samples derived from BM2 cells (C) and none at all in those from HD11 cells (D) when Myb-specific antibodies were used. Therefore, the ChIP experiment clearly shows that v-Myb binds to the *GAS41* promoter *in vivo*.

Effect of v-Myb on *GAS41* origin activity

Since the *GAS41* promoter region contains an origin of DNA replication (33,34) we were interested to know whether the presence of v-Myb effects the activity of the origin. We performed nascent strand abundance assay using BM2 and HD11 cells (Figure 6) to address this issue. These assays confirm the presence of a replication origin in this region; however, there was no significant difference between the two cell types.

DISCUSSION

The results described above provide strong evidence that the *GAS41* gene is a direct Myb target gene in v-*myb* transformed myeloid cells. Amplification of the *GAS41* gene has been implicated in the development of glial tumours (1) and recent analysis of its binding partners in cancer cells directly links GAS41 overexpression to acute leukaemia in human (8). Its interaction with INI1 (Integrase Interactor 1) (8) which is a component of the human SWI/SNF complex suggests that GAS41 might play a role in either chromatin remodelling or in recruiting chromatin remodelling complexes (9). However, its physiological function is not yet fully understood since *GAS41* is expressed in many cell types, including haematopoietic cells (6). Furthermore, knock-out of *GAS41* leads to a global decrease of transcriptional activity and is incompatible with cell viability (11). The role of GAS41 in v-Myb-transformed cells is presently unknown. Further insight into its role will depend on further characterization of the biochemical function.

The observation by Chong *et al*. (6) that the *GAS41* gene is situated immediately downstream of the *lysozyme* gene is quite surprising given that the cell-type-specific expression patterns of both genes are completely different. Despite their close proximity and the absence of any known boundary elements between them, both genes appear to be regulated independently. For example, the upstream region of the *lysozyme* gene contains several enhancers and silencers that direct the cell-type- and stage-specific expression of the *lysozyme* gene in myelomonocytic cells, apparently without affecting the expression of the *GAS41* gene. The effect of Myb on the expression of both genes is also quite different, not only in magnitude but also with respect to the involvement of C/EBP transcription factors. As shown before, the *lysozyme* gene is activated by Myb together with a member of the C/EBP transcription factor family (24,37). In contrast, co-transfection experiments of the *GAS41* promoter with C/EBP expression vectors have not shown any cooperativity between Myb and C/EBP (data not shown). Finally, it is of note that the *GAS41* promoter region also acts as an origin of DNA replication (33,34). The presence of a number of Myb binding sites in this region raises the question of whether Myb, in addition to affecting *GAS41*'s transcription, might also affect the activity of this region as a replication origin. In this respect it is interesting to note that Myb has already been linked to DNA replication in *Drosophila melanogaster* (38). However, our studies showed that there is no significant difference in origin usage between Myb-expressing BM2 and non-expressing HD11 macrophages (Figure 6). This finding is plausible in the context of the observations made by Danis *et al*. (39) since the *GAS41* promoter lies within a CpG island. Therefore, this region is very likely to be easily accessible by DNA polymerase and might provide the origin function in almost all cell types. In summary, our work identifies the *lysozyme–GAS41* gene region as an interesting genomic locus containing two Myb target genes side-by-side. The further analysis of the molecular mechanisms involved in Myb-dependent regulation of both genes will probably provide interesting insight into how Myb activates the expression of cellular genes.

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