Targeted Disruption of the GAS41 Gene Encoding a Putative Transcription Factor Indicates That GAS41 Is Essential for Cell Viability*

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The glioma-amplified sequence (GAS) 41 protein has been proposed to be a transcription factor. To investigate its functional role in vivo, we attempted to knock out the GAS41 gene by targeted disruption in the chicken pre-lymphoid cell line DT40. Heterozygous GAS41+/− cell lines generated by the first round of homologous recombination express approximately half the normal level of GAS41 mRNA. However, a homozygous GAS41−/− cell line with both GAS41 alleles disrupted was not obtained following the second round of transfection, indicating that the GAS41 gene is essential for cell viability. Indeed, homozygous GAS41−/− cell lines with two disrupted GAS41 alleles can be generated following substitution of the endogenous gene by stable integration of GAS41 cDNA controlled by a tetracycline-regulated CMV promoter. Inactivation of this promoter by tetracycline withdrawal results in rapid depletion of GAS41, causing a significant decrease in RNA synthesis and subsequently cell death. Thus, our results indicate that GAS41 is required for RNA transcription.

The glioma-amplified sequence (GAS) 41 gene was identified for the first time as an amplified sequence in the chromosome region 12q13–15, a region known to be involved in gene amplification in human gliomas. The gene was found to be amplified in 23% of glioblastomas and in 80% of grade I astrocytomas, suggesting that gene amplification occurs also in amplified in human gliomas. The gene was found to be present for the first time as an amplified sequence in the chromosome region 12q13–15, a region known to be involved in gene amplification in human gliomas. The gene was found to be amplified in 23% of glioblastomas and in 80% of grade I astrocytomas, suggesting that gene amplification occurs also in human gliomas.

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¶ The abbreviations used are: GAS, glioma-amplified sequence; CMV, cytomegalovirus; IMDM, Iseove’s modified Dulbecco’s medium; NuMA, nuclear mitotic apparatus protein; tet, tetracycline; tetO, tetracycline operator; RT, reverse transcription; wt, wild-type; PBS, phosphate-buffered saline; TF, transcription factor.

Specifically to matrix attachment regions in vitro (5). In mitotic cells, NuMA is associated with the spindle poles (4). Immunofluorescence microscopic studies revealed a punctate distribution of GAS41 restricted to the nucleus in interphase cells, suggesting that the protein is associated with the nuclear matrix. In mitotic cells, however, GAS41 is found throughout the cell, in apparent contrast to NuMA located at the spindle poles (3).

Sequence comparison revealed high homology of GAS41 to AF-9 and ENL in the proline-rich N-terminal region (2, 3). These nuclear proteins containing a transcriptional activation domain at the C-terminal 90 amino acids are believed to represent a new class of transcription factors (6, 7). GAS41 has been predicted to exhibit α-helical structures containing a significantly above average percentage (27%) of acidic amino acids in the 60-amino acid C-terminal region (2). Negatively charged α-helical structures are present in transcriptional activation domain of several eukaryotic transcription factors (8). In contrast to AF-9 and ENL, GAS41 lacks a typical DNA-binding domain (2, 8). It may be that the protein activates transcription by interacting with components of transcription complexes.

Although GAS41 has been well characterized by in vitro studies, less is known about the role of this protein in vivo. In this study, we therefore tried to understand its role in the chicken B-cell line DT40. In this cell line, targeted integration by homologous recombination has been shown to occur at high frequency (9). Using this gene targeting technique, we attempted to disrupt both GAS41 alleles in DT40 cells. We show that the gene encoding GAS41 is essential for cell viability. Targeted disruption of both alleles of GAS41 could be only achieved following substitution by GAS41 cDNA under control of a tetracycline (tet)-regulated CMV promoter. Inactivation of this promoter by tet withdrawal caused depletion of GAS41, which was accompanied by a decrease in cellular RNA synthesis and subsequent cell death. Thus, the data indicate that GAS41 is required for RNA transcription.

MATERIALS AND METHODS

Cell Culture—The chicken pre-B-cell line DT40 (10) and its derivatives were maintained in Iseove’s modified Dulbecco’s medium (IMDM), supplemented with 10% fetal calf serum (Roche, Mannheim, Germany), 5% chicken serum (Sigma, Taufkirchen, Germany), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM 2-mercaptoethanol, at 41 °C and 6% CO2. The chicken myelomastocytic cell line HD11 (11) and the hepatic cell line DU249 (12) were grown in IMDM, supplemented with 8% fetal calf serum and 2% chicken serum, but without 2-mercaptoethanol, at 37 °C and 6% CO2.

Plasmid Constructs—All plasmids used in this study were constructed by standard procedures (13). Neomycin phosphotransferase and puromycin-N-acetyltransferase genes, both under the control of the chicken β-actin promoter (9, 14), were inserted into the BamHI site of pBluescript IISK+ (Stratagene, Amsterdam, Netherlands). To construct...
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RNA Preparation and Northern Blot Analysis
RNA was isolated using the RNeasy midi kit from Qiagen (Hilden, Germany). To eliminate traces of genomic DNA, samples (50 μg each) were digested by RNase-free DNase, phenol-extracted, and ethanol-purified as described previously (17). Poly(A)⁺ RNA was purified using oligo(dT)-

Fig. 1. Expression of the GAS41 gene located downstream of the lysozyme gene. A, schematic map of the lysozyme-gas41 locus with the relative position of the probes used. Filled boxes denote the four exons of the lysozyme gene and seven exons of the GAS41 gene, whereas open boxes indicate introns. The CpG island is indicated by the black bar. The boxes in the lower part of the figure mark the relative position of the probes used for hybridization. Probe a (a) is lysozyme cDNA. B, poly(A)⁺ RNA purified from HD11 cells (lanes 1) and DT40 cells (lanes 2) was electrophoretically fractionated on 1.4% agarose gels and blotted onto nylon membranes. Blots were hybridized to the indicated 32P-labeled probes (panels a–e), and autoradiographed. No GAS41 mRNA was detected by hybridization to lysozyme cDNA (probe a) and to probe e.

construct targeting plasmids. SacI and EcoRI fragments containing up-stream and downstream flanking sequences of the GAS41 gene were sequentially cloned into the SacI and EcoRI sites upstream and downstream, respectively, of the selection marker genes. The resulting targeting plasmids were designated neo-gas and puro-gas. To construct the gas41 expression vector CMV-gas to substitute for the genomic homologue, a BamHI-XhoI fragment containing the full-length gas41 cDNA was inserted into pcDNA/TO (Invitrogen, Karlsruhe, Germany) digested with BamHI and XhoI. The expression of gas41 cDNA was controlled by the human cytomegalovirus immediate-early (CMV) promoter containing two tetracycline operator sequences tetO2. pcDNA6/TR from Invitrogen contains the tet repressor gene under the control of the human CMV promoter.

DNA Transfections—Transfections of DT40 cells were performed by electroporation using a protocol described by Buerstedde and Takeda (9). Briefly, for each transfection, cells (10⁶) were washed once in ice-cold phosphate-buffered saline (PBS) and resuspended in 0.8 ml of PBS containing 25 μg of linearized plasmid DNA. The suspension was transferred into a 0.4-cm cuvette (Bio-Rad, Munich, Germany) and kept on ice for 10 min. Electroporation was carried out at room temperature at 550 V and 25 microfarads using the Gene Pulser apparatus from Bio-Rad. Following another 10-min incubation on ice, cells were suspended in 40 ml of IMDM and plated in four 24-well plates (0.4 ml/well). After 24 h of plating, cells were selected by adding 0.4 ml/well IMDM containing 2% agarose gel. For plasmid cloning, the PCR product was cloned into plasmid pCR2.1 by the TA cloning kit from Invitrogen according to the instructions of the manufacturer. GAS41 cDNA was sequenced using the T7 Sequenase kit (Amersham Biosciences, Braunschweig, Germany).

Expression and Purification of Chicken GAS41—GAS41 cDNA generated by RT-PCR was amplified using primers 5'-cgggtgccatatgttcaag-3' (sense) and 5'-cgggtgccatatgttcaag-3' (antisense) containing Ndel and BamHI restriction sites (underlined). After digestion with Ndel and BamHI, the full-length cDNA was cloned in the correct orientation and reading frame, into vector pET-16b (Calbiochem-Novabiochem, Schwalbach, Germany). The resulting plasmid (pET16b-GAS41) which expresses GAS41 with an N-terminal His tag, was transformed into the Escherichia coli BL21(DE3)/pLysS. A positive colony was used to inoculate 100 ml of LB medium containing 34 μg/ml chloramphenicol and 50 μg/ml ampicillin. Bacteria were grown at 37 °C to an optical density at 600 nm of 0.6, and then expression was induced by adding 0.2 ml of 0.5 μM isopropyl-1-thio-β-D-galactopyranoside, followed by further shaking for 3 h at 35 °C. His-tagged GAS41 was purified from the lysate under denaturing conditions (6 M guanidine hydrochloride) using nickel-nitritotriacetic acid matrices from Qiagen following the protocol supplied by the manufacturer.

Anti-GAS41 Antiserum and Western Blot Analysis—For immunization, 12-week-old New Zealand White rabbits were initially injected with 0.2 mg of bacterially expressed chicken GAS41 emulsified with an equal volume of Freund's complete adjuvant (Sigma). Four weeks later, the animals were boosted at 2-week intervals with 0.1 mg of the antigen. The specificity of the antisera was tested by Western blotting of chicken GAS41 with the use of Western blot analysis of bacterially expressed GAS41. For Western blot analysis, cells (10⁶) washed twice with PBS were lysed in 50 μl of 2× Laemmli loading buffer. Following sonication and boiling for 5 min, 4 μl of each sample were subjected to a 15% SDS-polyacrylamide gel. After electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and GAS41 was detected by anti-GAS41 antiserum as described previously (17) or using the enhanced chemiluminescence detection system (Amersham Biosciences).

Preparation of [³H]Labeled RNA—For [³H]uridine labeling, 1 ml of each cell suspension (5 × 10⁶ cells) was incubated with 50 μCi of [³H]uridine for 30 min at 41 °C. Cells were then washed twice with PBS, and total RNA was isolated using the high pure RNA isolation kit (Roche). Following precipitation by ethanol, radioactivity of the isolated RNA was quantified by scintillation counting using Rotiszint 22 (Roth, Karlsruhe, Germany).

RESULTS
The Chicken GAS41 Gene Is Located Downstream of the Lysozyme Gene—We have previously mapped a CpG island at the 3' end of the chicken lysozyme gene (20, 21), whereas most CpG islands are found at the 5' end of genes (22). Therefore, we wished to determine whether an as yet unidentified gene lies immediately downstream of the lysozyme CpG island. Indeed, Northern blot analysis of poly(A)⁺ RNA using DNA probes b–d
located progressively downstream of the lysozyme gene revealed mRNA expressed from this region in HD11 and DT40 cell lines (Fig. 1). In contrast, this mRNA was not detected, when the blots were hybridized to lysozyme cDNA (probe a) or to the further distantly located probe e, indicating that the coding sequence of a novel gene lies within a region bounded by the 3’ end of the lysozyme gene and probe e (Fig. 1).

We next cloned the mRNA of this gene by RT-PCR using primers that were chosen from known sequences containing the putative start and stop codons. Sequencing of the cloned cDNA identified the gene as the chicken homologue of a previously described gene, GAS41 (1–3). The chicken GAS41 gene contains 7 exons embedded into a 2.7-kb region. Sequence comparison shows that human and chicken GAS41 are 97% identical at the amino acid level and 80% identical at the nucleotide level (2, 3).

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**TABLE I**

| Transfections     | Plasmid | Neo-gas | Puro-gas |
|-------------------|---------|---------|----------|
| First round       |         | 2/24    | 2/52     |
| Second round      |         | 0/68*   | 0/81*    |

* Cells are #27 cells containing a targeted puro-gas locus.

* Cells are #12 cells containing a targeted neo-gas locus.

To investigate the role of GAS41 in vivo, we started gene targeting experiments to disrupt the GAS41 gene. Wild-type (wt) DT40 cells were transfected by electroporation with the targeting plasmids neo-gas and puro-gas linearized by KpnI. Following selection with G418 and puromycin, drug-resistant clones were

FIG. 2. **Targeted disruption of one GAS41 allele.** A, schematic map of the chicken lysozyme-gas41 locus showing restriction sites EcoRI (E), SacI (S), and XbaI (X), and targeting plasmids neo-gas and puro-gas used for homologous recombination. wt DT40 cells were transfected by electroporation with the targeting plasmids, followed by selection with 2 mg/ml G418 or 0.5 µg/ml puromycin for 10 days. Genomic DNA isolated from wt DT40 cells, G418-resistant clones (B), and puromycin-resistant clones (C) were digested with XbaI. The digested DNA was fractionated on 0.7% agarose gels, blotted onto nylon membranes and hybridized to probe 1. Clones containing targeted integration of neo-gas and puro-gas are indicated by asterisks. Marker fragments were HindIII-digested λ-DNA.

FIG. 3. **GAS41 expression in wt DT40, #12, #27, and DU249 cells.** Poly(A)+ RNA from DT40, #12, #27, and DU249 cells was electrophoretically fractionated on an 1.4% agarose gel and blotted onto a nylon membrane. The blot was sequentially hybridized to GAS41 cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (38).
Southern blot hybridization was performed using an EcoRI-cutter in Z3#3.1 and Z3#4.1 cells. 42-kDa carbonic anhydrase. Control (His-GAS41) antiserum. Eighty mg of isolated His-tagged GAS41 were served as control (His-GAS41). Protein markers used are 30-kDa myoglobin and 42-kDa carbonic anhydrase.

To test whether the endogenous expression of GAS41 is reduced in cells containing only one GAS41 allele, the steady-state level of GAS41 mRNA in heterozygous #12 and #27 cells was studied by Northern blot analysis using the 285-bp DNA probe d (see Fig. 1). As shown in Fig. 3, GAS41 mRNA levels in #12 and #27 cells were decreased to approximately half of that in wild-type DT40 cells, indicating that targeted disruption of one GAS41 allele resulted in reduction of endogenous GAS41 expression.

The GAS41 Gene Is Essential for DT40 Cell Viability—We next performed the second round of gene targeting to disrupt the other GAS41 allele in the #12 and #27 cell lines. These cells were transfected by electroporation with puro-gas and neo-gas, respectively, followed by selection in medium containing both G418 and puromycin. DNA isolated from resistant clones was digested with XbaI and analyzed by Southern blotting. A summary of the results is shown in Table I. A total of 122 clones were selected for dual drug resistance; 81 of these were derived from #12, and 41 were from #27. None of them was determined to have both alleles disrupted by homologous recombination, although the targeting plasmids used have been shown to be efficient in disrupting one GAS41 allele. These results strongly suggest that disruption of both GAS41 alleles is lethal, and thus the GAS41 gene is an essential gene in DT40 cells.

Targeted Disruption of Two GAS41 Alleles Can Be Made by Substitution for GAS41 following Stable Transfection of Exogenous GAS41 cDNA—If GAS41 is essential for DT40 cell viability, one would expect a successful double targeting to inactivate GAS41 gene following prior substitution for the endogenous GAS41. To supply exogenous GAS41, the expression vector pcDNA6/TR were split into two suspensions with 1.25 × 10^6/ml, and these were grown in the absence and presence of 1 μg/ml tet, respectively. A, after 24 h of growth, cell extracts were prepared from 20 ml of each cell suspension and analyzed for GAS41 using an anti-GAS41 antiserum. His-tagged GAS41 was served as control (His-GAS41). B, after 24 and 48 h, the cell density was determined by counting with a hemacytometer using trypan blue. Data shown are means from three experiments. Standard deviations are less than 20%.

Depletion of GAS41 causes cell death. #31 cells (10^5) transfected with 25 μg of the tet repressor expression vector pcDNA6/TR were split into two suspensions with 1.25 × 10^6/ml, and these were grown in the absence and presence of 1 μg/ml tet, respectively. A, after 24 h of growth, cell extracts were prepared from 20 ml of each cell suspension and analyzed for GAS41 using an anti-GAS41 antiserum. His-tagged GAS41 was served as control (His-GAS41). B, after 24 and 48 h, the cell density was determined by counting with a hemacytometer using trypan blue. Data shown are means from three experiments. Standard deviations are less than 20%.

Isolated and screened for homologous recombination. Genomic DNA isolated from these clones was digested with XbaI, and Southern blot hybridization was performed using an EcoRI-XbaI fragment (probe 1) that is contained in neither targeting construct used (Fig. 2A). Southern blot analysis of 12 each of the drug-resistant clones are shown in Fig. 2 (B and C). Hybridization of XbaI-digested genomic DNA from wild-type DT40 cells or from clones with nonhomologous integration to the probe shows a single 6.9-kb DNA band. Targeted integration is revealed by the appearance of an additional diagnostic band: a 6.45-kb band from the targeted neo-gas locus and a 4.15-kb band from the targeted puro-gas locus. In total, 24 G418-resistant and 52 puromycin-resistant clones were analyzed, and 2 clones from each group were identified to have targeted integration via homologous recombination. Thus, the approximate frequencies of homologous recombination obtained in DT40 cells with the targeting plasmids neo-gas and puro-gas were 1/12 and 1/26, respectively.
Next, cells from Z3#3.1 were transfected with the targeting plasmid puro-gas, and selected in medium containing G418 and puromycin, and, to prevent a loss of the integrated GAS41 cDNA, in the presence of zeocin. DNAs isolated from triple drug-resistant clones and digested with XbaI were analyzed by Southern blotting using probe 1 as described above. Clones with double targeting are identifiable by the complete disappearance of the parental band at 6.9 kb and the appearance of the diagnostic band at 4.15 kb for the targeted puro-gas locus in addition to the diagnostic band at 6.45 kb for the targeted neo-gas locus (see clone #31 in Fig. 5). From 224 clones analyzed, 4 clones were identified to contain double targeted integration at the GAS41 locus. We obtained a lower frequency of 1/56 for the second round of homologous recombination with puro-gas, compared with 1/26 for the first round, probably because cells in which the puro-gas plasmid recombined with the already targeted neo-gas locus did not survive during selection with G418. Taken together, our data show that disruption of both alleles of the GAS41 gene did not result in cell death following substitution by exogenous GAS41.

Depletion of GAS41 Results in Decrease in RNA Synthesis and Subsequent Cell Death—The CMV promoter of the GAS41 expression vector CMV-gas contains two tetO2 downstream of the TATA element; thus, its transcriptional activity is repressible by the tet repressor (23). De-repression occurs following inactivation of the tet repressor by tet. Therefore, #31 cells, in which two GAS41 alleles were disrupted in the presence of the GAS41 transgene, were transfected with the tet repressor expression vector pDNA6/TRA. To test for repressible synthesis of GAS41 by tet repressor, the cells were analyzed for GAS41 following tet withdrawal by Western blotting. As shown in Fig. 6A, GAS41 was not detected in cells grown for 24 h in the absence of tet.

We next examined cell growth and viability following depletion of GAS41 by tet withdrawal. Cells transfected with pDNA6/TRA were split into two suspensions with 1.25 x 10⁵/ml, and these were grown in the presence and absence of tet, respectively. By counting with trypan blue, in the absence of tet cells were grown to a density of 4.5 x 10⁶/ml by 24 h, but by 48 h nearly all cells were dead. In contrast, in the presence of tet, cells were grown reaching a density of 1.4 x 10⁶/ml by 48 h (Fig. 6B). These data demonstrated that the absence of tet results in depletion of GAS41 and subsequent cell death.

GAS41 has been proposed to represent a new class of transcription factors (3, 25). We therefore examined RNA synthesis following depletion of GAS41 by tet withdrawal. Cells (#31) transfected with the tet repressor expression vector pDNA6/TRA were grown in the presence or absence of tet for increasing lengths of time and then labeled with [3H]uridine for 30 min. Fig. 7A demonstrates that cells grown in the presence of tet show an increase in RNA synthesis proportional to cell growth (see Fig. 6B). In contrast, RNA synthesis gradually decreased in cells grown in the absence of tet for longer than 18 h; by 36–48 h, cells ceased to synthesize RNA. Next, RNA rescue experiments were performed by adding tet to cells grown in the absence of tet for different time periods. After another 48 h, RNA synthesis was examined by incorporation of [3H]uridine. As shown in Fig. 7B, RNA synthesis was fully rescued within 18 h of the absence of tet. Taken together, these data show that depletion of GAS41 results in cell death, preceded by a decrease in cellular RNA synthesis, and indicate that GAS41 plays an essential role in RNA transcription.

DISCUSSION

In this study, we used a gene targeting technique by homologous recombination to investigate the role of the GAS41 gene
in the chicken pre-B-cell line DT40. This cell line has been shown to exhibit homologous recombination at very high frequency. Depending on the vector used, targeted integration at frequencies as high as 80–100% have been reported (9, 24). In contrast to these data, using the targeting vectors neo-gas and puro-gas, we obtained homologous recombination at the GAS41 gene locus at lower frequencies of 1/12 and 1/26, respectively. A possible explanation is that our vectors were constructed for targeted deletion of a great part of the GAS41 gene and are thus less efficient in achieving homologous recombination. We provide several lines of evidence indicating that the gene encoding GAS41 is essential for cell viability. Targeted disruption of both alleles of GAS41 was made possible only by prior stable integration of a GAS41-encoding CDNA under control of a CMV promoter to substitute for the endogenous gene. Furthermore, the CMV promoter containing two tetO2 is trans-repressible by tet repressor, whereas de-repression occurs in the presence of tet. Indeed, transfection with a tet repressor expression vector leads to a total depletion of GAS41 after 24 h of tet withdrawal. This agrees with the short half-life of ~30 min of GAS41 as determined by Western blotting following treatment with cycloheximide (data not shown). Successively, depletion of GAS41 causes cell death after 48 h of tet withdrawal. We note, however, that heterozygous cells containing only one GAS41 allele (#12 and #27), which express GAS mRNA at ~50% reduced level, did not show any decrease in growth rate compared with wild-type DT40 cells. This suggests that the amount of GAS41 in DT40 cells is not limited, and that the cells can tolerate a moderate reduction of GAS41 level.

In addition to establishing the essential role of GAS41 in cell viability, our results demonstrated that GAS41 is involved in RNA transcription. The suggestion that GAS41 is a transcription factor is based on the facts (i) that the protein displays high sequence homology to two putative transcription factors, AF-9 and ENL (26–28); (ii) that it contains a negatively charged α-helical structure, which is found in the transcriptional activation domain of several eukaryotic transcription factors (2, 3, 8); and (iii) that GAS41 is located in the nucleus of interphase cells (3, 25). In this study, we provide evidence for the first time that GAS41 is required for RNA transcription that in turn is essential for cell viability. Our data demonstrate that depletion of GAS41 causes a general decrease in RNA synthesis in vivo and thus seem to agree with the suggestion that GAS41 is involved in basal cellular transcription. Interestingly, GAS41 contains a tf2f domain originally described in a yeast transcription factor, TFG3/ANC1, which, like GAS41, shows significant similarities to AF-9 and ENL, two proteins involved in human acute leukemia (25–28). TFG3/ANC1 has been shown to be identical to the transcription factor TFIIF small subunit and to be an integral component of TFIIID and TFIIIF, two transcription factor complexes required for basal transcription by RNA polymerase II (26, 29, 30). Furthermore, GAS41 has been demonstrated to interact specifically with the nuclear NuMA protein, a component of the nuclear matrix of interphase cells (3, 5). The interaction of RNA polymerase II, transcription factors, and nascent RNA with the nuclear matrix has been already reported (31–37). It is therefore tempting to speculate that GAS41 is an essential component of transcription factor complexes required for basal transcription, and that it bridges the assembly of these complexes on the nuclear matrix to facilitate efficient RNA transcription.

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