Journal of Cellular Biochemistry 99:319–329 (2006)

Repression of Transcription by TSGA/Jmjd1a, a Novel Interaction Partner of the ETS Protein ER71

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Abstract Testis-specific gene A (TSGA) was originally identified in rat and shown to be expressed within the testes. Here, we have cloned the murine homolog [also known as jumonji domain-containing 1a (Jmjd1a)] and for the first time characterized the TSGA protein and its functions. Although murine TSGA is expressed in testes, its mRNA is also present in many other tissues, including heart, thymus, liver, and skin. Immunostaining revealed that TSGA is a nuclear protein, whose N-terminus contains a putative nuclear localization signal. TSGA displays significant homology to a suspected tumor suppressor and coactivator (5qNCA), to a thyroid hormone receptor interacting protein (TRIP8) and to the corepressor Hairless, pointing at a role of TSGA in transcription regulation. Indeed, TSGA contains several functional transcription repression domains. In addition, TSGA interacts both in vitro and in vivo with ER71 (ETS related 71), a transcription factor that is expressed in the testes of adult mice and during embryogenesis. Specifically, the N-terminus of TSGA and the C-terminus of ER71 are primarily engaged in their complex formation. Furthermore, TSGA impairs the ability of ER71 to activate transcription from the matrix metalloproteinase-1 promoter. Thus, TSGA may modulate the function of ER71 and thereby affect spermatogenesis as well as embryonic development. J. Cell. Biochem. 99: 319–329, 2006.

Key words: ER71; ETS protein; jmjC domain; Jmjd1a; jumonji; transcription; TSGA

E26 transformation specific (ETS) proteins are characterized by a ~85 amino acids-long, winged helix-turn-helix domain that mediates binding to DNA. The ETS proteins are versatile transcription factors that perform many functions during development, cell proliferation, cell differentiation, or in the response to mitogenic and stress signals [Janknecht and Nordheim, 1993; Janknecht et al., 1995; Sharrocks, 2001; Oikawa and Yamada, 2003; Hsu et al., 2004]. Presently, 27 different ETS genes have been identified within the human genome and their gene products often share extensive homology beyond the ETS domain. However, a few ETS proteins appear to be unique, sharing no homology outside their DNA binding domain with other ETS proteins. One such protein is ER71 (ETS related 71), which in mice is encoded by the Etsrp71 gene on chromosome 7 and which appears to be expressed in adults selectively in the testes [Brown and McKnight, 1992; De Haro and Janknecht, 2005].

The ER71 protein specifically interacts with DNA sites that conform to the 5′-(G/C)(G/C)(C/A)GGA(A/T)(G/A)(T/C)C-3′ consensus sequence [Brown and McKnight, 1992]. The promoter of the matrix metalloproteinase-1 (MMP-1) gene contains such a site and binding of ER71 to this site and corresponding upregulation of the MMP-1 promoter by ER71 has been demonstrated. This upregulation of gene transcription by ER71 is dependent on its potent N-terminal transactivation domain, but also supported by its C-terminal 21 amino acids that stimulate the DNA binding activity of the ER71 ETS domain [De Haro and Janknecht, 2002]. In order to gain more insight into ER71's modes of action, we attempted to identify its interaction partners utilizing a yeast two-hybrid screen. One potential binding partner obtained in this screen has been testis-specific gene A (TSGA), a protein

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Grant sponsor: Deutscher Akademischer Austauschdienst.

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Received 16 January 2006; Accepted 8 March 2006

DOI 10.1002/jcb.20945

which was previously identified in rat and shown to be expressed in rat testes [Höög et al., 1991].

Testis-specific gene A contains a jumonji C (jmjC) domain, a cupin metalloenzyme-like domain that is present in many DNA or chromatin binding proteins, suggesting that the jmjC domain is involved in the regulation of chromatin structure [Clissold and Ponting, 2001]. Originally, the jmjC domain was identified in the Jumonji family of transcription factors [Balciunas and Ronne, 2000]. The protagonist of this family, the Jumonji protein, is an AT-rich interaction domain transcription factor which can bind directly to DNA and contains a transcriptional repression domain [Kim et al., 2003]. Consistent with its important function during cardiac development [Jung et al., 2005b], Jumonji represses cardiac transcription factors such as Nkx2.5, GATA4, and MEF2A. Interestingly, this repression appears not to require DNA binding of Jumonji, but rather its ability to physically interact with the aforementioned transcription factors [Kim et al., 2004; Kim et al., 2005]. Furthermore, ectopic expression of Jumonji suppresses cell proliferation, most likely entailing Jumonji's interaction with and augmentation of the ability of the retinoblastoma protein to downregulate cyclin D1 and Cdc2 expression [Toyoda et al., 2000; Toyoda et al., 2003; Jung et al., 2005a].

In contrast to Jumonji and other jmjC domain-containing proteins, the function of TSGA has remained unresolved. Thus, we have characterized the TSGA protein in this report with a special emphasis on its ability to modulate the ER71 transcription factor.

MATERIALS AND METHODS

Northern Blotting

A Northern blot containing 2 μg mouse polyA$^+$ mRNA (Origene) was utilized. The respective mRNA was derived from mixed male and female Swiss Webster mice, whose average age was 9–10 months (for thymus, 8–12 weeks). The blot was hybridized with a ^{32}P-labeled murine TSGA cDNA fragment that encodes for amino acids 907–1209. Hybridization of the Northern blot and washing procedures were according to the manufacturer's recommendations. As a control for equal loading of mRNA, the Northern blot was stripped and rehybridized with a human beta-actin cDNA probe.

Cell Transfection

Cells were transiently transfected by the calcium phosphate coprecipitation method essentially as described [Janknecht and Hunter, 1997; Bosc and Janknecht, 2002]. Briefly, cells were seeded in 6-cm dishes at 25% confluency and then transfected with a total of 9 μg of DNA. Eight to ten hours later, cells were washed twice with phosphate-buffered saline and then incubated for another 36 h at 37°C in a humidified atmosphere containing 10% CO_2.

Immunofluorescence

Cells were grown on coverslips and transfected with 1.5 μg of expression vector(s). Two days after transfection, cells were fixed with formaldehyde and stained as described before [Papoutsopoulou and Janknecht, 2000]. Thereafter, cells were visualized in a confocal laser microscope [Janknecht et al., 1998].

Luciferase Assays

Cell were transfected with 1 μg of luciferase reporter gene plasmid and indicated amounts of expression vectors. Thirty-six hours after transfection, cells were lysed in 25 mM Tris (pH 7.8), 2 mM EDTA, 10% glycerol, 2 mM DTT, 1% Triton X-100. After removal of debris by centrifugation, the supernatant was utilized for measurement of luciferase activity in a Berthold Lumat LB 9507 [Janknecht, 2001; Wu and Janknecht, 2002]. Shown are averages from three experiments.

GST Pull-Down Assay

Glutathione S-transferase (GST) and the GST-ER71$_{23-358}$ fusion protein were produced in *E. coli* and affinity-purified according to standard procedures. Purified proteins were then bound to glutathione-agarose beads and incubated with whole cell extracts of 293 T cells transfected with Myc-tagged TSGA expression vectors essentially as described [Janknecht, 2003]. Bound TSGA proteins were then revealed by Western blotting using anti-Myc mouse monoclonal antibodies (9E10).

Coimmunoprecipitation

Two hundred and ninety three T cells were cotransfected with indicated amounts of expression vectors. Thirty-six hours after transfection, cells were lysed at 4°C in 650 μl of 50 mM Tris (pH 8), 150 mM NaCl, 50 mM NaF, 0.5% Igepal

CA-630, 0.2 mM DTT, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM Na_3VO_4. The cell lysate was briefly vortexed, tumbled for 45 min and debris removed by centrifugation. Then, the supernatant was precleared with 15 μl of protein A-agarose beads for 1 h. After removal of the protein A-agarose beads, 0.5 μl of monoclonal antibodies (anti-Myc 9E10 or anti-Flag M2) were added and 2 h later, 15 μl of protein A-agarose beads. After another 1.5 h of tumbling, beads were washed four-times with lysis buffer, boiled in Laemmli sample buffer and subjected to SDS polyacrylamide gel electrophoresis. Proteins were detected on Western blots with 1:5,000 dilutions of monoclonal antibodies employing chemiluminescence.

RESULTS

Cloning and Expression of Murine TSGA

We were interested in identifying interaction partners of ER71. Thus, we performed a yeast two-hybrid screen with ER71 amino acids 23–358 as a bait. From a mouse testis library, we thereby isolated a fragment of TSGA (amino acids 1131–1209) in a prey clone. In order to obtain a complete cDNA for murine TSGA, we performed RT-PCR on murine testis mRNA. Translation of the cloned cDNA revealed a 1,209 amino acids-long protein (Fig. 1A) which is 95% identical to the described 1,214 amino acids-long rat TSGA protein [Höög et al., 1991].

When searching for homologous proteins of TSGA with the BLAST program, we found two close relatives, 5q nuclear coactivator (5qNCA) and thyroid hormone receptor interacting protein 8 (TRIP8), as well as a more distant relative, the Hairless protein. Specifically, TSGA is 47% or 39% identical to 5qNCA and TRIP8, respectively. Recently, this homology has also been observed upon assembling the full-length reading frame of human TRIP8 in silico [Katoh and Katoh, 2003]. Due to the presence of a jmjC domain, TSGA (Jmjd1a), 5qNCA (Jmjd1b), and TRIP8 (Jmjd1c) are now encompassed within the jumonji domain-containing 1 (Jmjd1) protein family and accordingly given the systematic names indicated in brackets.

Testis-specific gene A shares significant homology (30% identity) only in its ~700 C-terminal amino acids with Hairless, indicating that Hairless does not belong to the Jmjd1 protein family. Consistently, compared to 5qNCA and TRIP8, Hairless is less identical to TSGA within a putative Zn-finger domain (Fig. 1B) as well as within the jmjC domain (Fig. 1C). Whereas the molecular function of 5qNCA has not been resolved [Hu et al., 2001], both TRIP8 as well as Hairless have been shown to interact with the thyroid hormone receptor, indicating that they are transcriptional cofactors [Lee et al., 1995; Thompson and Bottcher, 1997] and thus implicating TSGA to be a transcriptional cofactor for ER71.

Previously, rat TSGA has been reported to be expressed in testes, but its expression in other tissues has remained untested [Höög et al., 1991]. To address the question whether murine TSGA is also expressed in testes and possibly other tissues, we performed Northern blotting analyses of various murine tissues. Indeed, we observed expression of TSGA in testes (Fig. 2), but much higher expression was present in the thymus and heart. In addition, considerable expression of TSGA was observed in brain, kidney, liver, skin, small intestine and stomach, whereas negligible to no TSGA mRNA was present in the spleen, lung, and muscle tissue. Interestingly, in addition to the major ~4.8 kb TSGA transcript, a longer one of ~8 kb size was observable in thymus and heart (Fig. 2), which might represent alternatively or non-completely spliced TSGA mRNA. In conclusion, TSGA expression is not limited to the testes, but rather TSGA is expressed in several different tissues.

Intracellular Localization of TSGA

One requirement for a transcriptional cofactor is its presence within the cell nucleus. As such, we determined whether TSGA is a nuclear protein. First, we elected to study the intracellular localization of TSGA in a murine testis cell line, MLTC-1. To this end, we transiently transfected MLTC-1 cells with HA-, Flag-, or Myc-tagged TSGA. All three tagged versions of TSGA localized to the cell nucleus (Fig. 3A). No staining of cells was observed when they were mock-transfected or when only secondary antibody was utilized (data not shown). We also studied the intracellular localization of TSGA in two other cell lines, mink lung Mv1Lu (not shown) and human embryonal kidney 293 T cells (see Fig. 5C), and also observed TSGA to reside in the cell nuclei.

A

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MYKSLLDKAGLGAITSVRFLGDQQSVFVSQDLLKPIQDVNSLRLSLTDNQTVSKEFQALIVKHLDESHLLQGDKNLVGSE    80
VKIYSLDPSTQWFSATVVHGNPSSKTLQVNCEEILALKIVDPALIHVEVVHDNFVTCGNSTRTGAVKRKSSENNGSSVSK   160
QAKSCSEASPSMCPVQSVPTTVFKEILLGCTAATPSSKDPRQQNTPQAANSPPNIGAKLPQGCHKQNLPEELSSCLNTKP   240
EVPRTKPDVCKEGLLSSKSSQVGAGDLKILSEPKGSCIQPKTNTDQESRLESAPQPVTGLPKECLPAKTSSKAELDIATT   320
PELQKHLEHAASTSDDLSDKPEVKAGVTSLNSCAEKKVEPSHLGSQSQNLKETSVKVDNESCCTRSSNKTQTPPARKSVL   400
TDPDKVRKLQQSGEAFVQDDSCVNIVAQLPKCRECRLDSLRKDKDQQKDSPVFCRFFHFRRLQFNKHGVLRVEGFLTPNK   480
YDSEAIGLWLPLTKNVVGTDLDTTKYILANIGDHFCQMVISEKEAMSTIEPHRQVAWKRAVKGVREMCDVCDTTIFNLHW   560
VCPRCGFGVCVDCYRMKRKNCQQGAAYKTFSWIRCVKSQIHEPENLMPTQIIPGKALYDVGDIVHSVRAKWGIKANCPCS   640
NRQFKLFSKPALKEDLKQTSLSGEKPTLGTMVQQSSPVLEPVAVCGEAASKPASSVKPTCPTSTSPLNWLADLTSGNVNK   720
ENKEKQLTMPILKNEIKCLPPLPPLNKPSTVLHTFNSTILTPVSNNNSGFLRNLLNSSTAKTENGLKNTPKILDDIFASL   800
VQNKTSSDSSKRPQGLTIKPSILGFDTPHYWLCDNRLLCLQDPNNKSNWNVFRECWKQGQPVMVSGVHHKLNTELWKPES   880
FRKEFGEQEVDLVNCRTNEIITGATVGDFWDGFEDVPNRLKNDKEKEPMVLKLKDWPPGEDFRDMMPSRFDDLMANIPLP   960
EYTRRDGKLNLASRLPNYFVRPDLGPKMYNAYGLITPEDRKYGTTNLHLDVSDAANVMVYVGIPKGQCEQEEEVLRTIQD  1040
GDSDELTIKRFIEGKEKPGALWHIYAAKDTEKIREFLKKVSEEQGQDNPADHDPIHDQSWYLDRSLRKRLYQEYGVQGWA  1120
IVQFLGDVVFIPAGAPHQVHNLYSCIKVAEDFVSPEHVKHCFWLTQEFRYLSQTHTNHEDKLQVKNVIYHAVKDAVAMLK  1200
ASESSLGKP                                                                           1209
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B

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TSGA/Jmjd1a:    548 CDVCDTTIFNLHWVCPRCGFGVCVDC 573
5qNCA/Jmjd1b:  1031 CDVCETTLFNIHWVCRKCGFGVCLDC 1056
TRIP8/Jmjd1c:  1838 CDACEATLFNVHWVCRKCGFVACLDC 1863
Hairless:       595 CSRCHHGLFNTHWRCSHCSHRLCVAC 620
Zn-finger:          C  C      H  C  C    C  C
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C

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TSGA/Jmjd1a:    946 MPSRFDDLMANIPLPEYTRRDGKLNLASRLPNYFVRPDLGPKMYNAYGLITPEDRKYGTTNLHLDV
5qNCA/Jmjd1b:  1498 MPTRFEDLMENLPLPEYTKRDGRLNLASRLPSYFVRPDLGPKMYNAYGLITAEDRRVGTTNLHLDV
TRIP8/Jmjd1c:  2265 MPTRYEDFLRCLPLPEYCNPEGKFNLASHLPGFFVRPDLGPRLCSAYGVAAAKDHDIGTTNLHIEA
Hairless:       939 DASRVQNLASSLPLPEYCAHQGKLNLASYLPLGLTLHPLEPQLWAAYG-VNSHRGHLGTKNLCVEV

TSGA/Jmjd1a:        SDAANVMVYVGIPKGQC-EQEEEVLRTIQDGDSDELTIKRFIEGKEKPGA----LWHIYAAKDTEK
5qNCA/Jmjd1b:       SDAVNVMVYVGIPIGEG-AHDEEVLKTIDEGDADEVTKQRIHDGKEKPGA----LWHIYAAKDAEK
TRIP8/Jmjd1c:       SDVVNVLVYVGIAKGNGVLSKAGILKKFEEEELDDVLRKRLKDSSEIPGA----LWHIYAGKDVDK
Hairless:           SDLISILVHAEAQLPPW-YRAQKDFLSGLDG----------EGLWSPGSQTSTVWHVFRAQDAQR

TSGA/Jmjd1a:        IREFLKKVSEEQGQDNPADHDPIHDQSWYLDRSLRKRLYQEYGVQGWAIVQFLGDVVFIPAGAPHQ
5qNCA/Jmjd1b:       IRELLRKVGEEQGQENPPDHDPIHDQSWYLDQTLRKRLYEEYGVQGWAIVQFLGDAVFIPAGAPHQ
TRIP8/Jmjd1c:       IREFLQKISKEQGLEVLPEHDPIRDQSWYVNRKLRQRLLEEYGVRACTLIQFLGDAIVLPAGTLHQ
Hairless:           IRRFLQMVCPA----GAGTLEPGAPGSCYLDAGLRRRLREEWGVSCWTLLQAPGEAVLVPAGAPHQ

TSGA/Jmjd1a:        VHNLYSCIKVAEDFVSPEHVKHCFWLTQEFR 1169
5qNCA/Jmjd1b:       VHNLYSCIKVAEDFVSPEHVKHCFRLTQEFR 1721
TRIP8/Jmjd1c:       VQNFHSCVQVTEDFVSPEHLVQSFHLTQELR 2489
Hairless:           VQGLVSTISVTQHFLSPETSALSAQLYHQGA 1150
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Fig. 1. **A**: Amino acid sequence of murine TSGA/Jmjd1a. The corresponding cDNA sequence has been deposited with the EMBL/GenBank data libraries under accession no. DQ323991. **B**: Comparison of the putative Zn-fingers of murine TSGA/Jmjd1a, human 5qNCA/Jmjd1b (Accession No. NP_057688), murine TRIP8/Jmjd1c (Accession No. XP_912801) and murine Hairless (Accession No. NP_068677). The conserved cysteine and histidine residues that may bind to Zn^{2+} are indicated. Amino acids identical to those of TSGA are highlighted by shading. **C**: Similar, alignment of jmjC domains.

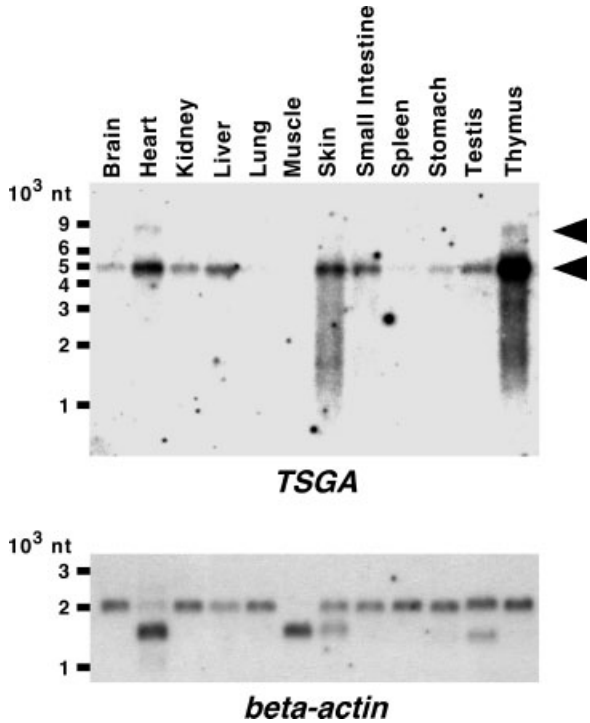
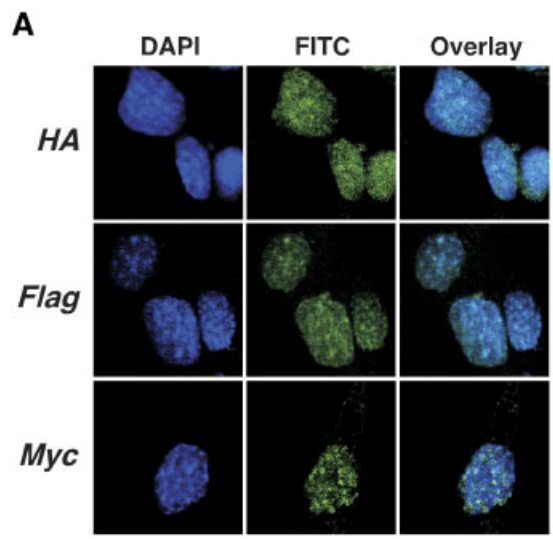


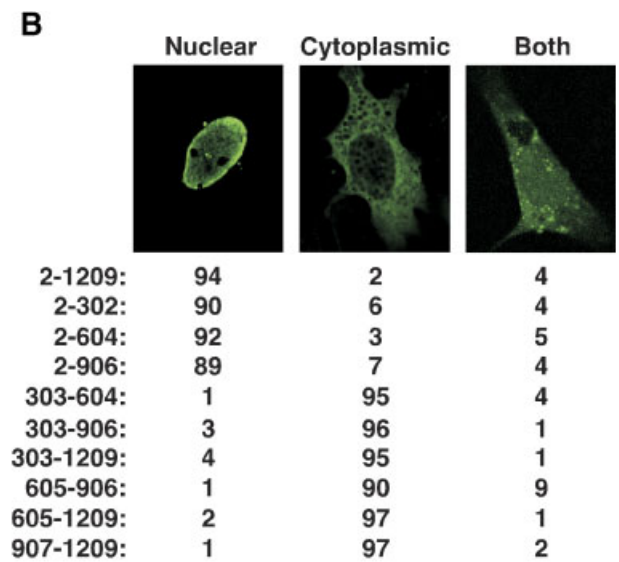
Fig. 2. Tissue-specific expression of TSGA. A mouse multiple-tissue Northern blot was hybridized with a TSGA cDNA probe (**upper panel**), or with a beta-actin probe (**lower panel**) as a control for equal loading of mRNA.

Next, we assessed which portion of TSGA is responsible for its nuclear translocation. To this end, we generated various truncations of TSGA and studied their intracellular localization in MLTC-1 cells (Fig. 3B). Predominantly nuclear localization was retained in all truncations that contained amino acids 2–302, whereas loss of these amino acids resulted in predominantly cytoplasmic staining. Thus, nuclear localization is mediated by the first 302 amino acids of TSGA.

Transcriptional Repression Domains Within TSGA

Testis-specific gene A does not contain any obvious DNA binding domain. Thus, to study its role in transcription regulation, we fused TSGA to the DNA binding domain of the yeast protein GAL4 and assessed its ability to regulate transcription with a GAL4 binding site-driven luciferase reporter construct, $GAL4_2$-tk80-luc [Janknecht et al., 1993]. When expressed in Mv1Lu cells, the GAL4-TSGA fusion protein significantly repressed gene transcription compared to the GAL4 moiety alone (Fig. 4A). However, this appears to be cell type-specific, since no repression was observed in 293 T or rabbit kidney RK13 cells.





2-1209:	94	2	4
2-302:	90	6	4
2-604:	92	3	5
2-906:	89	7	4
303-604:	1	95	4
303-906:	3	96	1
303-1209:	4	95	1
605-906:	1	90	9
605-1209:	2	97	1
907-1209:	1	97	2

Fig. 3. Intracellular localization of TSGA. **A**: MLTC-1 cells were transfected with either HA-, $Flag_3$-, or Myc_6-$TSGA_{2-1209}$. Cells were then stained with respective anti-HA, anti-Flag, or anti-Myc monoclonal antibodies followed by FITC-coupled secondary anti-mouse antibodies. DNA was simultaneously stained with DAPI. **B**: MLTC-1 cells were transfected with the indicated Myc_6-tagged TSGA constructs and their intracellular localization determined by immunostaining. One hundred stained cells were counted and classified into predominantly nuclear, predominantly cytoplasmic, or localized in both the nucleus and cytoplasm, as exemplified by the three immunofluorescent pictures. Underneath, the number of cells in each of these three categories is given. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Next, we dissected the TSGA protein in order to map potential repression domains. Whereas amino acids 2–302 did not mediate transcriptional repression, amino acids 303–604, 605-906, and 907–1209 were capable of repressing transcription when fused to the GAL4 DNA binding domain (Fig. 4B). The strongest repression (more than ninefold) was observed with amino acids 605–906. Thus, multiple repression domains exist within TSGA.

Interaction of TSGA With ER71

Our interest in TSGA was originally raised by its ability to interact with ER71 in a yeast two-hybrid screen. To validate that TSGA indeed binds to ER71, we first performed GST pull-down experiments. To this end, GST or a GST-ER71 fusion protein were bound to glutathione-agarose beads and challenged with Myc_6-$TSGA_{2-1209}$. As shown in Figure 5A, TSGA specifically interacted with GST-ER71.

To corroborate this in vitro result, we also assessed in vivo complex formation between TSGA and ER71. To this end, Flag-tagged TSGA was coexpressed with Myc-tagged ER71, anti-Myc immunoprecipitations performed and any coprecipitated TSGA revealed by anti-Flag Western blotting. Our data demonstrate that TSGA coimmunoprecipitated solely in the presence of ER71 (Fig. 5B, left panels). We additionally performed a reciprocal experiment and observed that ER71 also coimmunoprecipitated with TSGA (Fig. 5B, right panels). Thus, TSGA and ER71 are capable of forming complexes in vivo.

We also assessed in how far TSGA and ER71 colocalize within 293 T cells. To this end, TSGA and ER71 proteins were coexpressed and each immunostained with a different color (Fig. 5C). Both ER71 and TSGA localized to the cell nucleus, and the noticeable extent of yellow color in an electronic overlay demonstrates that a significant proportion of ER71 and TSGA colocalize. This supports the notion that a substantial degree of complex formation between ER71 and TSGA occurs in vivo.

Mapping of Interaction Domains in TSGA and ER71

To determine the region via which TSGA interacts with ER71, we utilized various TSGA deletion mutants and coexpressed them with ER71. Whereas the N-terminal half of TSGA coimmunoprecipitated with ER71 (Fig. 6B, left panels), the C-terminal amino acids 605–1,209 that contain the jmjC domain (see Fig. 6A for a sketch of TSGA) did not. This is consistent with our in vitro pull-down assays showing that $TSGA_{2-604}$ but not $TSGA_{605-1209}$ interacted with GST-ER71 (see Fig. 5A). Upon subdivision

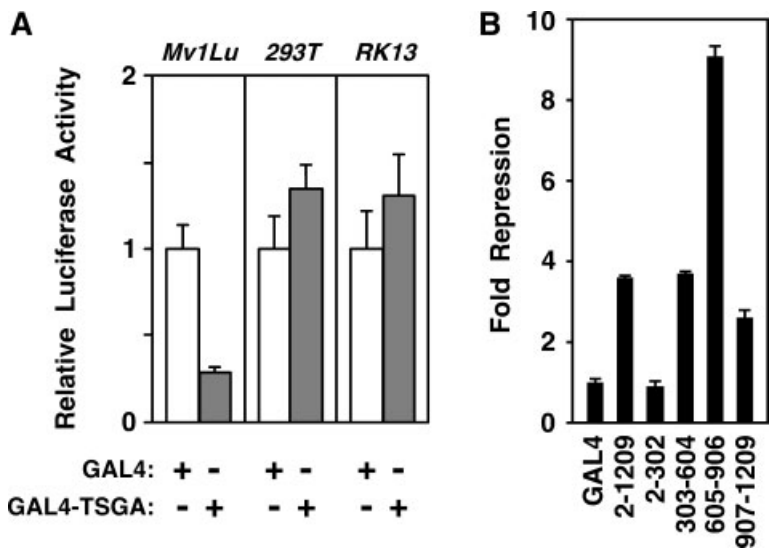


Fig. 4. Transcriptional repression mediated by TSGA. **A**: Mv1Lu, 293 T, or RK13 cells were transfected with the GAL4 DNA binding domain or GAL4-$TSGA_{2-1209}$. Resultant activity of a cotransfected GAL4 binding site-driven luciferase reporter construct ($GAL4_2$-tk80-luc) was measured. **B**: Repression mediated by various GAL4-TSGA fusion proteins in Mv1Lu cells. Protein levels of GAL4-TSGA fusions were made comparable by adjusting the amount of expression plasmids transfected (data not shown). Please note that in contrast to A, we chose here to display the fold repression of the GAL4 binding site-driven reporter to clearly accentuate differences in repression potential.

of the N-terminal half of TSGA into amino acids 2–302 and 303–604, we observed that both fragments interacted with ER71 (Fig. 6B, right panels); however, amino acids 2–302 appear to bind more avidly to ER71 than amino acids 303–604. Thus, TSGA amino acids 2–302 are primarily responsible for the interaction with ER71. Please note that our yeast two-hybrid screen showed that TSGA amino acids 1131–1209 may also interact with ER71, whereas our GST pull-down and coimmunoprecipitation experiments did not reveal an interaction between the C-terminus of TSGA and ER71. One explanation for this discrepancy is the fact that even transient, very weak protein-protein interactions can be detected with the yeast two-hybrid system, but not with GST pull-down or coimmunoprecipitation assays.

Conversely, we assessed which region in ER71 is responsible for its interaction with TSGA. The N-terminal transactivation domain spanning amino acids 23–179 (see Fig. 6C for a sketch of ER71) did not interact with TSGA (Fig. 6D, left panels). Amino acids 23–228 only weakly interacted with TSGA, and also $ER71_{23-337}$ interacted less strongly than $ER71_{23-358}$, indicating that the C-terminal 21 amino acids of ER71 modulate binding to TSGA. On the other hand, deleting amino acids from the N-terminus revealed that the first 229 amino acids are dispensable for the interaction with TSGA (Fig. 6D, right panels). These results suggest that amino acids 230–358, which encompass the ETS domain and the C-terminal modulatory domain of ER71, are primarily mediating the interaction of ER71 with TSGA.

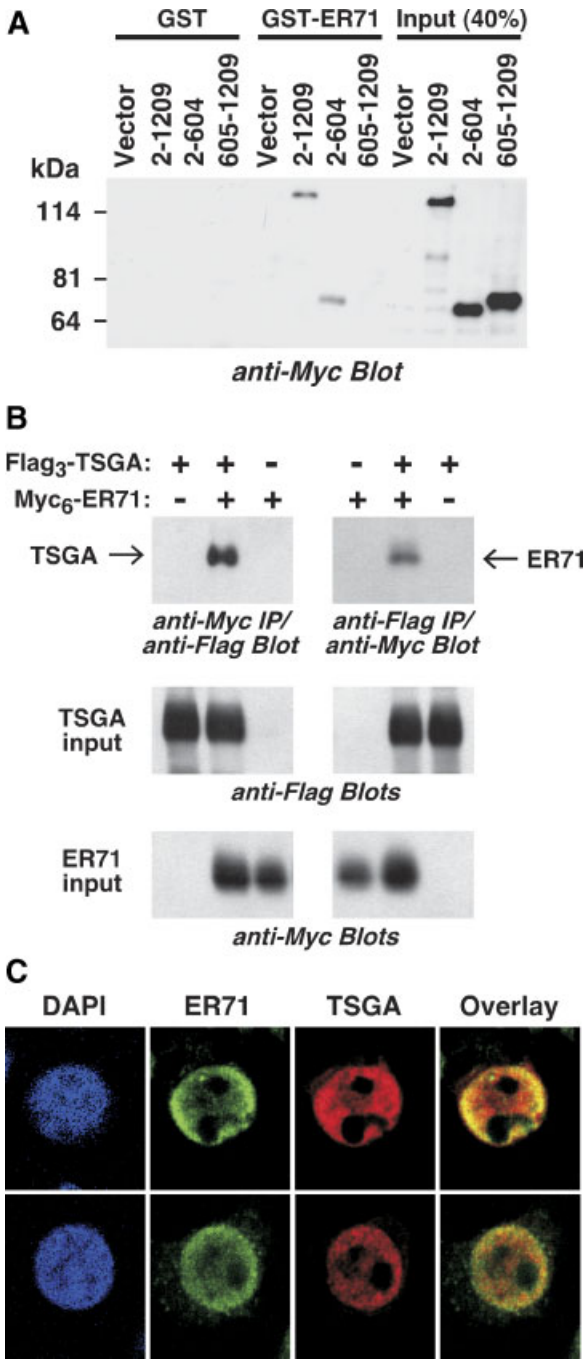


Fig. 5. Interaction of TSGA with ER71. **A**: GST pull-down assay. Myc_6-tagged $TSGA_{2-1209}$, $TSGA_{2-604}$, or $TSGA_{605-1209}$ was incubated with GST or GST-$ER71_{23-358}$ and bound TSGA proteins were detected by anti-Myc Western blotting. **B**: Coimmunoprecipitation of TSGA and ER71. 293 T cells were transfected with 0.5 μg of $Flag_3$-$TSGA_{2-1209}$ and/or 0.5 μg of Myc_6-$ER71_{2-358}$. The top panels show coimmunoprecipitated proteins, whereas the bottom panels show input levels of $Flag_3$-$TSGA_{2-1209}$, and Myc_6-$ER71_{2-358}$. **C**: Colocalization of TSGA and ER71. 293 T cells were transfected with HA-$TSGA_{2-1209}$ and Myc_6-$ER71_{23-358}$. Proteins were immunostained with rabbit anti-Myc and mouse anti-HA antibodies, followed by FITC-coupled donkey anti-rabbit and Texas Red-coupled donkey anti-mouse secondary antibodies. DNA was additionally visualized by DAPI staining. The right panels show the merger of FITC and Texas Red staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Repression of ER71 by TSGA

ER71 has been shown to bind to and activate the MMP-1 promoter [De Haro and Janknecht, 2002]. Thus, we assessed how TSGA affects the transcriptional activity of ER71 utilizing an MMP-1 luciferase reporter gene, pGL2-MMP-1(−525/+15) [Bosc et al., 2001]. On its own, TSGA had little effect on the MMP-1 promoter, whereas $ER71_{23-358}$ strongly activated the MMP-1 promoter in Mv1Lu cells (Fig. 7A).

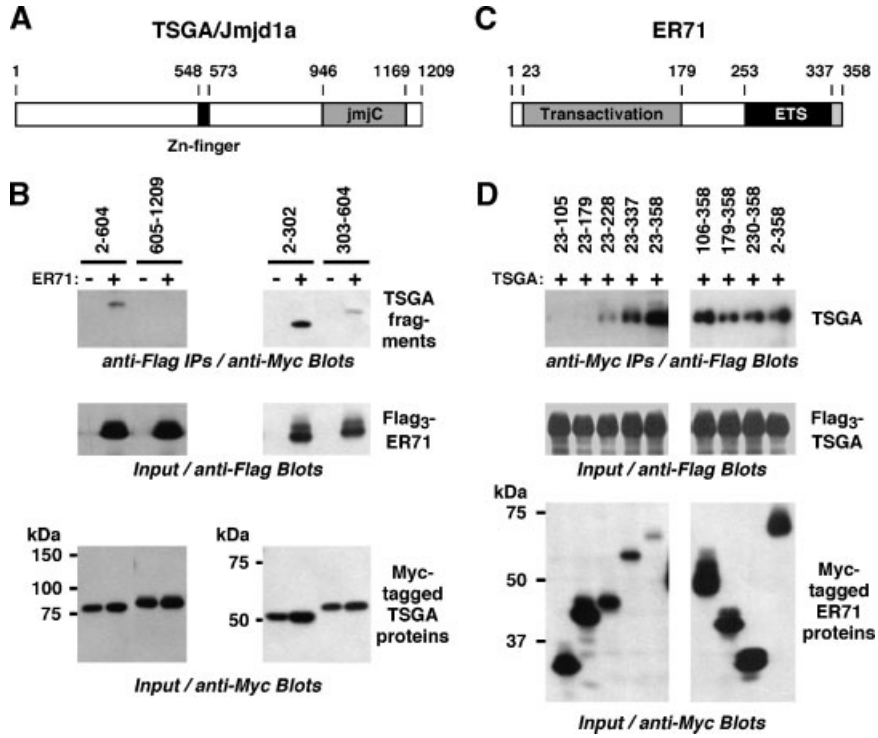


Fig. 6. Mapping of interaction domains in TSGA and ER71. **A**: Scheme of TSGA with its putative Zn-finger and jmjC domain. **B**: $Flag_3$-$ER71_{23-358}$ expression vector (1.5 μg) were cotransfected with 3 μg of indicated Myc_6-tagged TSGA deletion plasmid into 293 T cells. After anti-Flag immunoprecipitation, coprecipitated TSGA proteins were revealed by anti-Myc Western blotting. The bottom panels show input levels. **C**: Scheme of ER71. Due to two start methionines, two ER71 isoforms exist encompassing either amino acids 1–358 or 23–358. **D**: $Flag_3$-$TSGA_{2-1209}$ (0.5 μg) expression vector was cotransfected with 0.5 μg of indicated Myc_6-tagged ER71 truncation plasmid into 293 T cells. After anti-Myc immunoprecipitation, coprecipitated TSGA was revealed by anti-Flag Western blotting. The bottom panels show input levels.

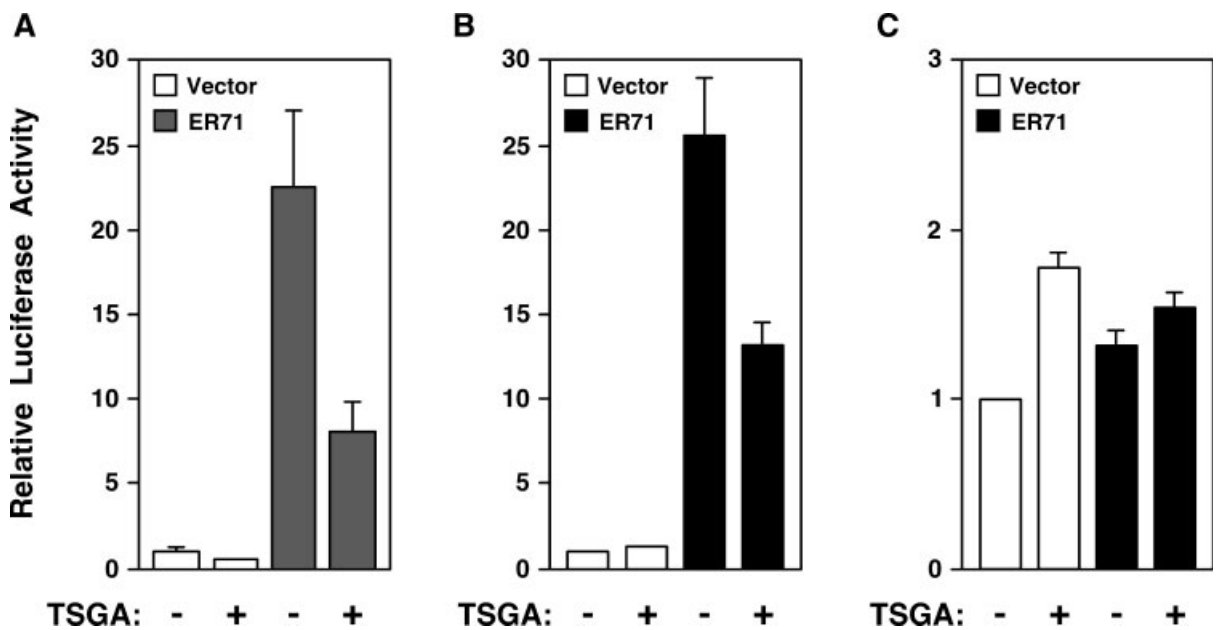


Fig. 7. TSGA suppresses ER71-mediated MMP-1 activation. **A**: 250 ng of $ER71_{23-358}$ expression plasmid or empty vector pEV3S were transfected with 2 μg of HA-$TSGA_{2-1209}$ expression vector or 2 μg of pEV3S into Mv1Lu cells. An MMP-1 luciferase reporter plasmid was cotransfected and resultant luciferase activities are depicted. **B**: Analogous with 50 ng of $ER71_{2-358}$ expression plasmid or empty vector pEV3S. **C**: The CMV-luciferase reporter gene is not repressed by TSGA (2 μg of HA-$TSGA_{2-1209}$ expression vector) in the presence or absence of $ER71_{2-358}$ (50 ng expression vector) in Mv1Lu cells.

Moreover, when TSGA was coexpressed with $ER71_{23-358}$, activation of the MMP-1 promoter was severely reduced from ~23 to ~8 relative luciferase units; this appears not to be due to a reduction of ER71 protein levels upon TSGA coexpression (see the comparable levels of ER71 in the presence and absence of TSGA in Figure 5B). We also confirmed that the longer isoform of ER71, $ER71_{2-358}$, is repressed by TSGA (Fig. 7B). Finally, we assessed whether TSGA unspecifically represses transcription. However, when utilizing a CMV (cytomegalovirus) enhancer/promoter reporter gene construct, CMV-luciferase [Rossow and Janknecht, 2001], which is not regulated by ER71, TSGA expression did not lead to repression of transcription (Fig. 7C). These results strongly indicate that recruitment of TSGA by ER71 leads to the inhibition of ER71's ability to activate gene transcription.

DISCUSSION

In this report, we have characterized the hitherto unexplored TSGA protein. Our data indicate that TSGA is a nuclear protein and that amino acids 2–302 are responsible for its nuclear translocation. Analysis of TSGA amino acids 2–302 revealed a short sequence, 144-GAVKRKSS-151, which resembles the nuclear localization sequence of ER71, GERKRKPG [De Haro and Janknecht, 2002], and contains a string of basic amino acids as does the prototypical nuclear localization signal of the SV40 large T antigen, PKKKRKV [Kalderon et al., 1984]. However, it remains to be determined if this sequence is a bona fide nuclear localization signal or if it is only a part of that since nuclear localization signals are often of a more complex nature than a string of basic amino acids [Christophe et al., 2000; Jans et al., 2000].

Sequence comparison revealed that TSGA shares significant homology with the Hairless protein that is not only critical for hair growth, but also for the maintenance of the cerebellar cortex [Cachon-Gonzalez et al., 1994; Ahmad et al., 1998; Cichon et al., 1998; Garcia-Atares et al., 1998; Kruse et al., 1999; Sprecher et al., 1999]. Hairless has been shown to act as a transcriptional corepressor for several nuclear receptors and contains, like TSGA, multiple repression domains [Potter et al., 2001; Hsieh et al., 2003]. In particular, Hairless interacts with histone deacetylases and the corepressors Groucho and CtBP [Potter et al., 2001; Barolo et al., 2002; Potter et al., 2002], and it is tempting to speculate that TSGA may be capable of doing the same in order to repress transcription. However, the ability of TSGA to repress transcription may be cell type-specific, since GAL4-TSGA repressed transcription in Mv1Lu but not in 293 T and RK13 cells. Possibly, 293 T and RK13 cells lack a necessary corepressor of TSGA, whereas Mv1Lu cells have a sufficiently high intracellular concentration of this putative corepressor; or 293 T and RK13, but not Mv1Lu cells express a protein(s) that interacts with TSGA and thereby precludes the recruitment of corepressors to TSGA.

Testis-specific gene A shares a greater degree of homology with TRIP8 and 5qNCA than with Hairless. Since a fragment of TRIP8 interacts with ligand-bound thyroid hormone receptor [Lee et al., 1995], TSGA may also interact with this nuclear receptor. However, it is unresolved if TRIP8, and possibly TSGA, activates or represses the thyroid hormone receptor. The homology of TSGA with 5qNCA also suggests that TSGA possesses growth suppressive characteristics as 5qNCA does. Of note, 5qNCA is a potential tumor suppressor encoded within a small region of the q-arm of human chromosome 5 whose deletion is frequently associated with myelodysplasia and acute myeloid leukemia [Lai et al., 2000; Hu et al., 2001].

Here, we have identified TSGA to be an interaction partner of the ER71 transcription factor. Like ER71, TSGA is expressed in testes, but its presence in multiple other tissues that do not express ER71 implies that TSGA binds to different proteins in other tissues; since the ETS domain of ER71 is involved in its interaction with TSGA, such other interaction partners of TSGA may be different ETS proteins. Furthermore, we have shown that TSGA suppresses the activation of the MMP-1 promoter by ER71, demonstrating that TSGA is a functional corepressor of ER71. Due to its ability to attenuate ER71-mediated activation of MMP-1 transcription, TSGA may be a modulator of spermatogenesis that is dependent on the tight regulation of MMPs [Monsees et al., 1997]. However, the expression of ER71 during embryonic development [Brown and McKnight, 1992] points at functions of ER71 other than in spermatogenesis, and by extension of its interaction partner TSGA that may therefore also perform important functions during embryogenesis.

Testis-specific gene A belongs to the family of jmjC domain-containing proteins, many of which have been shown to affect chromatin structure [Clissold and Ponting, 2001]. Of note, it has recently been shown that the jmjC domain of the fission yeast protein Epe1 is critical for its ability to modulate heterochromatin stability [Ayoub et al., 2003]. More recently, it has even been suggested that the jmjC domain may be involved in histone demethylation [Trewick et al., 2005], and indeed demethylation of histone 3 on lysine residue 36 by the jmjC domain of JHDM1/FBXL11 has been reported [Tsukada et al., 2006]. Altogether, these data suggest that TSGA might be involved in the regulation of chromatin structure, possibly by influencing histone modification, which may be the mechanism through which TSGA down-regulates ER71 target genes in adult testes and during embryogenesis.

ACKNOWLEDGMENTS

Julia Knebel was supported by a scholarship from the Deutscher Akademischer Austauschdienst.

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