

Complexity of Translationally Controlled Transcription Factor Sp3 Isoform Expression*

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Sp3 is a ubiquitous transcription factor closely related to Sp1. Both proteins contain a highly conserved DNA-binding domain close to the C terminus and two glutamine-rich domains in the N-terminal moiety. Immunoblot analyses of Sp3 reveal a striking complex protein pattern of up to eight distinct species. This pattern is not observed in Sp3-deficient cell lines showing that all signals reflect Sp3 antigen. In this study, we have unraveled the complexity of Sp3 expression. We show that four isoforms of Sp3 that retain different parts of the N terminus are expressed *in vivo*. The four isoforms derive from alternative translational start sites at positions 1, 37, 856, and 907. An upstream open reading frame located at position –47 to –18 regulates expression of the two long isoforms. Unlike Sp1, none of the Sp3 isoforms is glycosylated. However, all four isoforms become SUMO-modified *in vivo* and *in vitro* specifically and exclusively at lysine residue 551. The transcriptional activity of the two long isoforms strongly depends on the promoter settings, whereas the small isoforms appear to be inactive. The transcriptional activity of all the Sp3 isoforms is regulated by SUMO modification. Our results demonstrate that Sp3 has many unique features and is not simply a functional equivalent of Sp1.

The transcription factor Sp3 is a ubiquitously expressed member of the Sp family of transcription factor that is involved in the expression and regulation of many genes, including housekeeping genes, tissue-specifically expressed genes, viral genes, and cell cycle-regulated genes (1, 2). Sp3 contains a highly conserved DNA-binding domain close to the C terminus and two glutamine-rich activation domains in the N-terminal moiety. The expression pattern, the structure, and the DNA-binding properties of Sp3 are very similar to Sp1, which suggested originally that these two proteins exert similar functions. The physiological roles of Sp1 and Sp3, however, appear to be significantly different. Sp1 knock-out mouse embryos are severely retarded in growth, and die after day 10 of embryonic development (3). Sp3-deficient embryos develop until birth, but die invariably of respiratory failure immediately after birth (4). In addition, late tooth and bone developmental processes are impaired in Sp3^{–/–} mice (4, 5).

Functional analyses of the transcriptional properties of Sp1

and Sp3 also revealed significant differences between these two transcription factors (6). On many reporter constructs containing multiple Sp-binding sites Sp3 is, unlike Sp1, inactive or acts only as a weak activator (7). The molecular basis for the inactivity of Sp3 under these conditions has been mapped to an inhibitory domain located between the second glutamine-rich activation domain and the zinc finger region (8). More recently, it was shown that Sp3 is post-translationally modified by the small ubiquitin-like modifier (SUMO)¹ within its inhibitory domain and that SUMO modification leads to inactivation (9, 10).

All previously published studies with Sp3 (more than 500 citations in Medline) were performed with N-terminally truncated versions of Sp3. In addition, unraveling the transcriptional role of Sp3 was complicated by the fact that several isoforms exist (2, 11).

For long it was thought that full-length Sp3 protein is derived from translational initiation at a non-AUG codon, whereas two small Sp3 isoforms derive from internally initiated translation at AUG start sites (11). However, recently, human genomic DNA sequences were identified that encompass three exons coding for additional 85 N-terminal amino acids of Sp3 (12).

Here, we report the cloning of the full-length Sp3 open reading frame that starts with a classic AUG initiation codon. The full-length Sp3 cDNA enabled us to unravel the complexity of the Sp3 antigen pattern observed in immunoblots. We show that *in vivo* four isoforms of Sp3 are expressed that differ in the extent of the N-terminal part. Detailed mutational analyses suggest that all four isoforms derive from alternative translational start sites. Moreover, an upstream open reading frame regulates expression of the two long isoforms. None of the Sp3 isoforms are glycosylated. However, all four isoforms become SUMO-modified *in vivo* as well as *in vitro* specifically and exclusively at lysine residue 551. The two long isoforms of Sp3 can act as transcriptional activators on certain promoter settings, whereas the two small isoforms appear to be always inactive. Mutational analyses of the SUMO acceptor site in the context of different isoforms show that their transcriptional inactivity is regulated by SUMO modification. Our results demonstrate that Sp3 has many unique molecular features and is not simply a functional equivalent of Sp1.

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¹ The abbreviations used are: SUMO, small ubiquitin-like modifier; CMV, cytomegalovirus; HA, hemagglutinin; GFP, green fluorescent protein; EGFP, enhanced GFP; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; MEF, mouse embryonic fibroblast; WGA, wheat germ agglutinin; uORF, upstream open reading frame; WT, wild-type; eIF, eukaryotic translation initiation factor; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.

EXPERIMENTAL PROCEDURES

Plasmids—The missing 5' part of the Sp3 cDNA was cloned by reverse transcription-PCR of HeLa RNA using the primers 5'-actcggaattcCTTTTGTGTTTCCCGACAGTCA-3' and 5'-CTGTGCAGAAGC-CAAATCACCTGT-5'. The resulting 470-bp product was cut with EcoRI (artificial site at the 5'-end of the forward primer) and NotI (site within the Sp3 cDNA sequence) and cloned into EcoRI/NotI-restricted pSPT18-Sp3 plasmid that contained the original published Sp3 sequences (13, 14). For *in vitro* transcription/translation assays of wild-type Sp3 and mutants, the T7/Sp6 promoter containing pSPT18 plasmid was used. Transient expression of Sp3 variants in *Drosophila* Schneider SL2 cells (15) was driven by the *Drosophila* actin 5C 5'-flanking region. For transient expression of Sp3 variants in mammalian cells the CMV promoter containing pN3, pMCS-HA, and pHA-MCS plasmids was used. pN3 was constructed by removing the GFP moiety from pEGFP-N3 (Clontech) with BamHI and NotI. The plasmids pMCS-HA and pHA-MCS (16) are derivatives of pEGFP-N1 and pEGFP-C1 (Clontech), respectively, in which the GFP part of pEGFP is replaced by the HA epitope. Point mutations were introduced into pSPT18-Sp3 using the QuikChange XL site-directed mutagenesis kit (Stratagene). Mutagenized Sp3 cDNAs were subsequently recloned into pPac, pN3, pMCS-HA, or pHA-MCS vectors. Detailed information on the construction of individual plasmids will be provided upon request.

Cell Culture and Transient Transfection Assays—For activation studies SL2 cells were transfected by the calcium phosphate method as described previously (15). Every plate received 4 μ g of reporter plasmid (BCAT-2 or pGL3) and 2 μ g of the β -galactosidase expression plasmid p97b as internal reference. Variable amounts of pPacSp3 expression plasmids were compensated with the plasmid pPac. 24 h after addition of DNA, the medium was changed and 24 h later the cells were washed twice with PBS prior to harvest. Chloramphenicol acetyltransferase (CAT) enzyme expression was assayed by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Roche Applied Science). Luciferase and β -galactosidase assays were carried out as described previously (17, 18). Expression of CAT or luciferase enzymatic activity was normalized to β -galactosidase activity. Each transfection was repeated at least three times. For analyses of Sp3 isoforms SL2 and mammalian cells were transfected with the FuGENE6 reagent according to the manufacturer's instructions (Roche Applied Science). Lysate preparation was as described before (10).

Immunofluorescence and Microscopy—Stable transfected Sp3^{-/-} KO MEFs or SL2 cells were plated on 13-mm \varnothing round coverslips in 24-well tissue culture plates and 24-h post-transfection (SL2 cells) fixed for 25 min at room temperature with 4% paraformaldehyde in PBS. Cells were washed twice for 5 min with PBS and subsequently permeabilized with 0.2% Triton X-100 in PBS for 15 min. After blocking with 3% bovine serum albumin in PBS for 1 h, cells were incubated with rabbit anti-Sp3 (Santa Cruz Biotechnology) for 1 h at room temperature. Coverslips were washed three times for 5 min with PBS and incubated with a fluorescein isothiocyanate (Jackson ImmunoResearch)- or Alexa Fluor[®] 594 (Molecular Probes)-conjugated secondary goat anti-rabbit IgG antibodies for 1 h at room temperature in a dark cabinet. After three washings with PBS for 5 min, coverslips were mounted onto glass slides using Vectashield mounting media with 4,6-diamino-2-phenylindole and sealed with nail polish. Fluorescence images were obtained on Leica DMLB microscope with a Leica N Plan 100 \times /1.25 oil objective.

Immunoblotting—Proteins resolved by SDS-PAGE were electrotransferred to phenylmethylsulfonyl fluoride membranes and probed with the appropriate antibodies. The following antibodies with the indicated dilutions in 20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, 1% skim milk were used: rabbit anti-Sp3 polyclonal IgG (Santa Cruz Biotechnology), 1:2,000; rabbit anti-HA (Santa Cruz Biotechnology), 1:2,000; rabbit anti-Sp1 polyclonal IgG (7), 1:5,000; rat anti-HA monoclonal IgG (Roche Applied Science), 1:2,000; and horseradish peroxidase-conjugated donkey anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-rat IgG (Amersham Biosciences), 1:10,000. An enhanced chemiluminescence detection system (Amersham Biosciences) was used to visualize the peroxidase reaction.

In Vitro Transcription/Translation and Autoradiography—For *in vitro* transcription/translation of Sp3 proteins the TnT T7-coupled reticulocyte lysate system (Promega) was used according to the manufacturer's instructions. Radioactive labeled reaction products were separated through 5% SDS-polyacrylamide gels. Gels were subsequently treated with fixing solution (10% acetic acid, 25% 2-propanol) and Amplify reagent (Amersham Biosciences) prior to drying and exposure to x-ray films (Kodak).

In Vitro SUMOylation Assay—SUMO modification reactions of *in vitro* transcribed/translated Sp3 proteins were carried out at 30 °C for 90 min in a total volume of 20 μ l of reaction buffer (20 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 0.05% Tween 20, 0.4 mg/ml ovalbumin, 1 mM ATP, 5 mM dithiothreitol) containing 5 μ l of ³⁵S-labeled substrate protein, 25 ng of E1 enzyme (Aos1/Uba2), 75 ng of E2 enzyme (Ubc9), 50 ng of E3 ligase (GST-PIAS1), and 200 ng of SUMO-1 (10). Reactions were stopped by adding 2 \times SDS Laemmli buffer (Sigma).

Wheat Germ Agglutinin Affinity Chromatography—293 cells expressing endogenous Sp proteins or transfected SL2 cells expressing Sp1, Sp3WT, or the Sp3K551R mutant were harvested in lysis buffer (40 mM Tris/HCl, pH 6.8, 1% SDS, 2.5% β -mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture). 100 μ l of whole cell extracts (~1 mg of protein) was diluted 1:10 in binding buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture) and incubated with 50 μ l of equilibrated agarose wheat germ lectin (Amersham Biosciences) for 1 h at 4 °C. Matrix-bound glycosylated proteins were washed extensively in binding buffer before adding 2 \times SDS Laemmli buffer (Sigma). For competition experiments, 100 mM *N*-acetyl D-glucosamine (Roth) was added to the binding buffer. Binding of Sp proteins to agarose wheat germ lectin was analyzed by Western blotting.

RESULTS

Complexity of Sp3 Isoforms—High resolution immunoblot analyses with antibodies directed to the C terminus of Sp3 resulted in a striking complex protein pattern. Four distinct proteins, two slow migrating species of more than 100 kDa and two fast migrating species of ~72 kDa (Fig. 1A, lane 5) were observed in nuclear extracts or whole cell extracts prepared with radioimmune precipitation assay buffer. Additional bands appear, when cells were lysed directly in SDS containing buffer. Under these conditions seven to eight Sp3 species are visualized in immunoblots (Fig. 1A, lanes 3, 4, and 6). All signals are specific for Sp3, because extracts prepared from Sp3-deficient embryonic stem cells did not cause any background (Fig. 1A, lane 2).

In the past, analyses that could clarify the nature of the various Sp3 species were hampered due to the lack of a full-length Sp3 cDNA clone. The original Sp3 cDNA sequences (13, 14) did not contain an AUG translational start codon, and it was postulated that translation of the Sp3 mRNA starts at a non-AUG codon (14). In the course of a comparative sequence analysis among the evolutionary related Sp transcription factors Sp1, Sp2, Sp3, and Sp4 (19), it was suggested that the 5'-end of Sp3 is absent in the published cDNA clones. Moreover, human genomic DNA sequences were identified that encompass three exons coding for additional 85 N-terminal amino acids of Sp3 (12). Based on these data, we cloned the missing 5' part of Sp3 by reverse transcription-PCR with primer pairs specific for sequences in exons 1 and 3 (GenBankTM accession number AF494280) (12) and fused it to the Sp3 coding region. The resulting Sp3 cDNA codes for 781 amino acids when translated from the first AUG codon.

Expression of the four Sp3 isoforms observed in immunoblots can be reconstituted in a coupled *in vitro* transcription/translation assay using the full-length Sp3 cDNA clone (Fig. 1B, lane 2). Moreover, the additional Sp3 species observed exclusively in SDS-lysed cell extracts can also be reconstituted when *in vitro* translated Sp3 proteins were subjected to an *in vitro* SUMO modification reaction (10) (Fig. 1B, lane 1). This result strongly suggests that the additional signals observed in SDS-lysed cell extracts represent SUMO-modified versions of the four Sp3 isoforms observed in non-denatured nuclear extracts. Very likely, highly active isopeptidases present in nuclear extracts readily cleave the isopeptide bond between SUMO and Sp3 unless special precautions are taken to inactivate these enzymes.

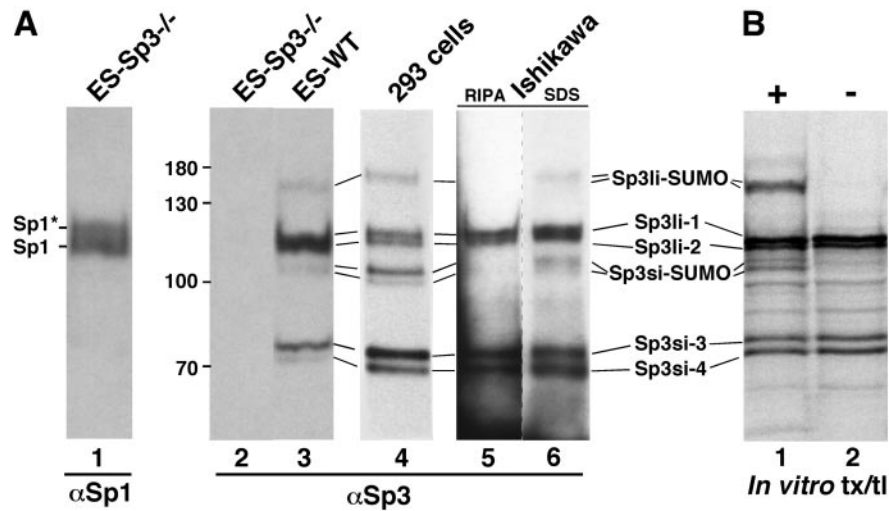


FIG. 1. Complexity of endogenous Sp3 protein expression. *A*, Western blot analyses of Sp1 and Sp3 in wild-type ES (ES-WT, lane 3), Sp3-deficient ES (ES-Sp3^{-/-}, lanes 1 and 2), 293 (lane 4), and Ishikawa cells (lanes 5 and 6). Cells were lysed with SDS-containing buffer (lanes 1–4 and 6) or in radioimmune precipitation assay buffer (lane 5), and proteins were separated through 6% SDS-polyacrylamide gels. After blotting, phenylmethylsulfonyl fluoride membranes were incubated with Sp1- or Sp3-specific antibodies as indicated. *B*, *in vitro* transcribed/translated Sp3 proteins radiolabeled with ³⁵S were subjected to *in vitro* SUMOylation reactions using purified recombinant E1 enzyme (Aos1/Uba2), Ubc9, and PIAS1 (10) in the presence (lane 1, +) or absence (lane 2, -) of recombinant SUMO-1. Reaction products were separated through 5% SDS-PAGE and visualized by autoradiography. Abbreviations are: Sp3li-1 and -2, long isoforms of Sp3; Sp3si-3 and -4, small isoforms of Sp3; Sp3li-SUMO and Sp3si-SUMO, SUMO-modified long and small isoforms of Sp3 (see Fig. 2). For Sp1, the asterisk indicates the phosphorylated form.

All Four Sp3 Isoforms Are Derived from Alternative Translational Start Sites—The entire Sp3 coding region contains four AUG in frame codons at position 1, 37, 856, and 907 relative to the first AUG. To analyze whether the four isoforms (designated Sp3li-1, Sp3li-2, Sp3si-3, and Sp3si-4) observed *in vitro* derive from these potential translational initiation sites, we mutated each of the four AUGs in the context of the full-length Sp3 cDNA (Fig. 2A). Each AUG mutation resulted in the lack of the appropriate isoform in an *in vitro* transcription/translation system (Fig. 2B, lanes 3–6). Thus the four different isoforms derive from translational initiation at the four AUG sites leading to translation products of 781, 769, 496, and 479 amino acids that differ in their N-terminal end (Fig. 2C). We also analyzed an Sp3 cDNA mutant in which the first two AUGs were mutated (Fig. 2B, 1.+2.AUGm in lane 8). In addition to the two small isoforms Sp3si-3 and Sp3si-4, this mutant produced several new long protein products not synthesized from the wild-type cDNA. We speculate that these proteins arise from non-AUG initiation codons. Inspection of the cDNA sequence revealed indeed the presence of three potential non-AUG initiation codons in a strong context (gacGUGga at position 52, gcgGUGg at position 133, and gauUUGg at position 289). We also asked whether expression of any of the smaller isoforms is dependent on the context of the first AUG codon. The wild-type sequence acuAUGa was replaced by an optimal Kozak sequence (gccAUGg). *In vitro*, this mutation did not impair expression of any of the four isoforms (Fig. 2B, lane 7), which is in contrast to the *in vivo* situation presented below.

Next we asked whether expression of the four Sp3 isoforms could be reconstituted *in vivo* after transfection of appropriate expression constructs. We cloned the full-length Sp3 cDNA and mutants into CMV promoter-driven expression vectors (pN3 and pMCS-HA vectors, see “Experimental Procedures” for details) useful for mammalian cells as well as in actin promoter-driven expression vectors (pPac) useful for insect Schneider cells (SL2 cells). After transfection of the wild-type Sp3 cDNA both long isoforms of Sp3 were expressed in 293 cells and SL2 cells similar to the situation *in vitro*. The two small isoforms of Sp3 were produced in 293 cells at low level but were not detectable in extracts from transfected SL2 cells (Fig. 2, D and E). It should be noted that SUMO-modified isoforms were ob-

served in all cases where SDS extracts were prepared for Sp3 expression analyses (Fig. 2D). In those cases where we generated nuclear extracts, these post-translational modified proteins were not observed (Fig. 2E), because hydrolases presumably cleaved the Sp3-SUMO isopeptide bond.

Mutation of any of the four AUG codons in the context of the full-length cDNA resulted in the absence of the appropriate isoform in 293 cells (Fig. 2D, lanes 2, 4, 7, and 8). In SL2 cells mutation of the first and second AUG codon also resulted in the lack of the corresponding isoform (Fig. 2E, left, lanes 3 and 5). These results strongly suggest that *in vivo* the four isoforms of Sp3 derive also from alternative usage of the four in-frame AUG start codons present in the Sp3 mRNA.

Additional point and 5'-deletion mutants support this conclusion. Deletion of the 5'-non-coding sequences and the first AUG codon (Δ 1.AUG) resulted in the absence of the Sp3li-1 isoform, whereas expression of the second Sp3 isoform was unchanged (Fig. 2E, lane 7). Deletion of the first 85 codons (Δ 1.+2.AUG in Fig. 2E, lane 6) resulted in exclusive expression of the two small isoform (predominantly of Sp3si-4).

Upon mutation of the first two AUG codons in the context of full-length Sp3 (1.+2.AUGm in Fig. 2D, lane 5) as well as upon deletion of 5'-non-coding sequences and the first AUG and simultaneous replacement of the second AUG by AGC (Δ 1.AUG/2.AUGm, in Fig. 2E, lane 8), several new long protein products were observed. Similar to the *in vitro* situation, these new proteins likely arise from non-AUG start codons.

The constructs used in the above analyses did not contain the full 3'-non-coding sequences. To test the influence of the 3'-non-coding sequences on the expression of different isoforms, we generated constructs with the complete 3'-untranslated region. The ratio of the four isoforms was not altered by the presence of the 3'-non-coding sequences neither *in vitro* nor *in vivo* (data not shown).

Expression of the Long Isoforms Is Regulated by an Upstream Open Reading Frame—During the course of our studies, we realized that the 5'-non-coding region of the Sp3 mRNA contains a short upstream open reading frame (uORF) at position -47 to -18 (Fig. 3A). Because uORFs can participate in translational control (20), we asked whether the Sp3mRNA uORF is involved in the regulation of Sp3 isoform expression. At first,

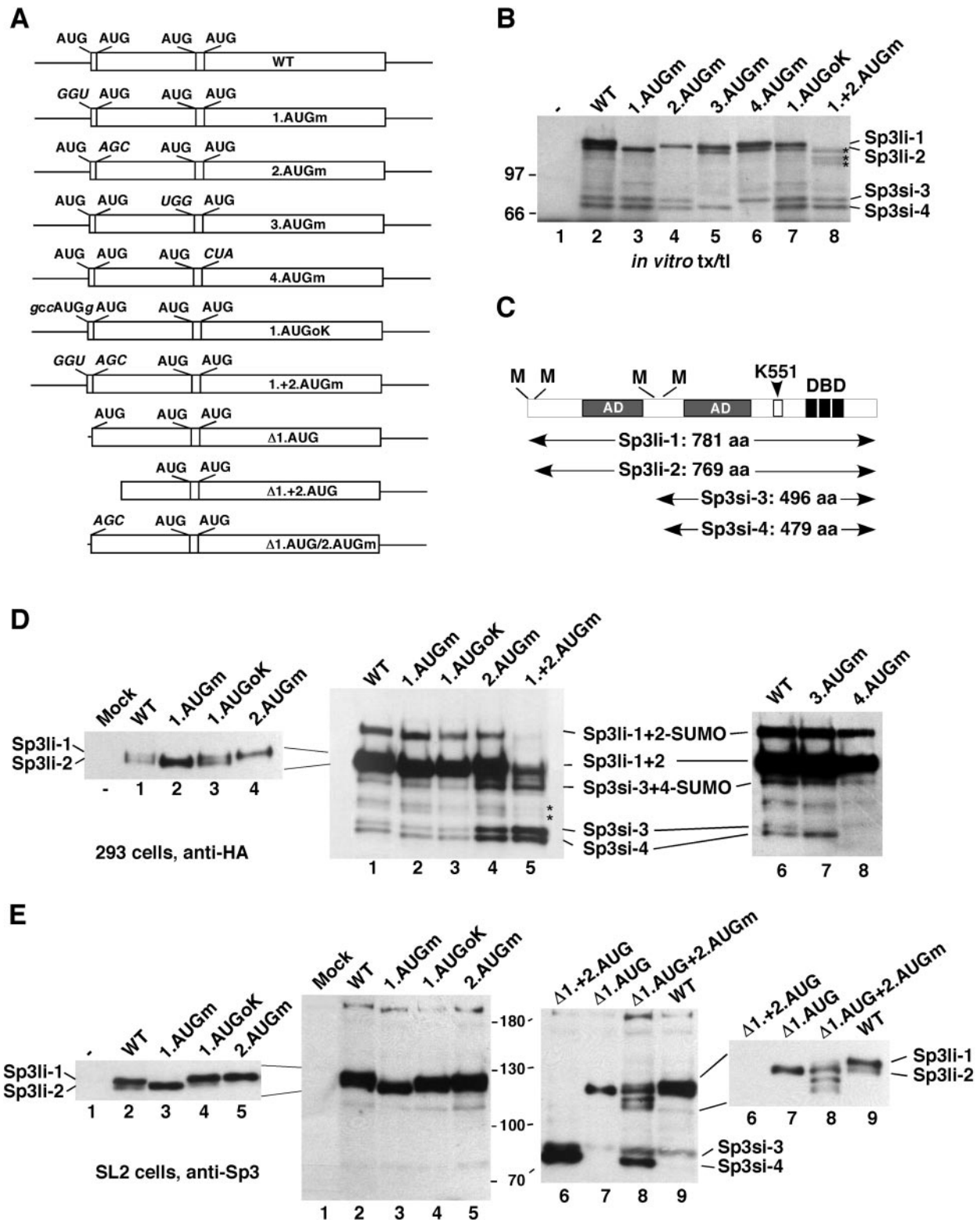


FIG. 2. The four isoforms of Sp3 derive from four in-frame AUG codons. *A*, schematic drawing of Sp3 cDNA; *e.g.* RNA mutants cloned in a vector appropriate for *in vitro* transcription/translation (T7- and Sp6-promoter-containing pSPT18 vector), transient expression in SL2 cells (*D. melanogaster* actin promoter-driven pPac vectors), and transient expression in mammalian cells (CMV-driven C-terminal HA-epitope vectors). Mutant codons are depicted in *italic letters*. *B*, coupled *in vitro* transcription/translation reactions. Sp3 wild-type cDNA and mutants were *in vitro* transcribed/translated. Subsequently, ^{35}S -radiolabeled proteins were separated through 5% SDS-PAGE and visualized by autoradiography. *C*, schematic drawing of the Sp3 domain structure and the four isoforms. The two long isoforms of Sp3 (*Sp3li-1* and *Sp3li-2*) contain both two glutamine-rich activation domains (*AD*, indicated in *gray*); the two small isoforms contain only one activation domain. *M* depicts the four methionine residues. The DNA-binding domain (*DBD*) consists of three zinc fingers indicated as *black boxes*. Lys-551 indicates the lysine residue that is targeted by SUMO. *D*, Western blot analyses of transiently transfected 293 cells. Cells were transfected with 3 μg of expression plasmids for C-terminally HA-tagged Sp3 mutants as indicated. Cells were lysed in SDS-containing buffer, separated through 6% SDS-PAGE, and Sp3 isoform expression was analyzed by Western blot using anti-HA antibodies. To separate the two long isoforms, an independent long gel run was performed (*left*). *E*, Western blot analyses of transiently transfected SL2 cells. Cells were transfected with 1 μg of expression plasmids for Sp3 mutants as indicated. Nuclear extracts were prepared, and 10 μg of protein extract was analyzed by Western blotting using anti-Sp3 antibodies.

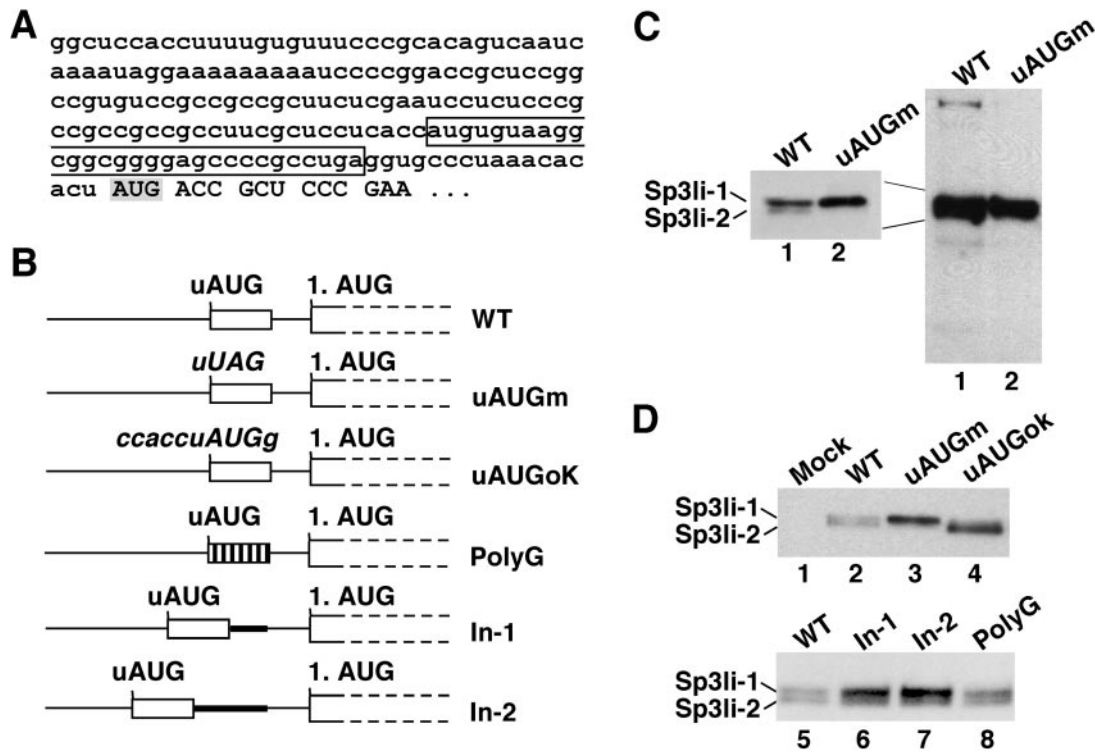


FIG. 3. An upstream ORF regulates expression of the Sp3 long isoforms. *A*, sequence of the 5'-non-translated region of the Sp3 mRNA. The uORF is boxed, and the first start codon is highlighted in gray. *B*, schematic drawing of uORF mutants. *C* and *D*, Western blot analyses of transfected SL2 (*C*) and 293 (*D*) cells. Cells were transfected with 1 μ g of expression plasmids for Sp3WT, Sp3uAUGm, Sp3uAUGoK, Sp3In-1, Sp3In-2, or Sp3PolyG mutants as indicated. 10 μ g of nuclear extract (SL2 cells) or SDS extracts (293 cells) or SDS extracts (293 cells), respectively, was separated through 6% SDS-polyacrylamide gels and analyzed by Western blotting using anti-Sp3 (*C*) or anti-HA (*D* and *E*) antibodies.

we generated two Sp3 cDNA mutants. In one of them the uORF initiation site was replaced by UAG (uAUGm), whereas in the other the uAUG, normally in a suboptimal context, is placed in an optimal Kozak sequence (uAUGoK) (Fig. 3*B*). These mutations did not alter Sp3 isoform expression in an *in vitro* transcription/translation assays (data not shown). In contrast, upon transfection in SL2 or 293 cells, the uAUGm mutation almost entirely abolished translation from the second AUG and enhanced initiation from the first AUG (Fig. 3*C*, lane 2 and Fig. 3*D*, lane 3). *Vice versa*, upon increasing the uAUG strength, the Sp3 isoform 1 and 2 ratio shifted toward the second isoform (Fig. 3*D*, lane 4, uAUGoK). Expression of the small isoforms of Sp3 was not significantly altered by these mutations.

We also asked whether initiation site selection is dependent on the sequence of the uORF. The wild-type codons of the uORF were replaced by codons for glycine residues without changing the length of the uORF (*PolyG* mutant in Fig. 3*B*). Compared with wild-type, this mutation did not alter the ratio of the two long isoforms (Fig. 3*D*, lane 8). In conclusion, the peptide that might be translated from the uORF is not involved in initiation site selection. We also generated mutants in which the distance of the uORF stop codon to the first AUG start codon was increased by 17 or 35 nucleotides (insertion mutants *In-1* and *In-2* in Fig. 3*B*). These mutations shifted expression of the long Sp3 isoforms toward the first isoform (Fig. 3*D*, lanes 6 and 7). Hence, the distance of the uORF to the first AUG start codon appears to be important for initiation site selection.

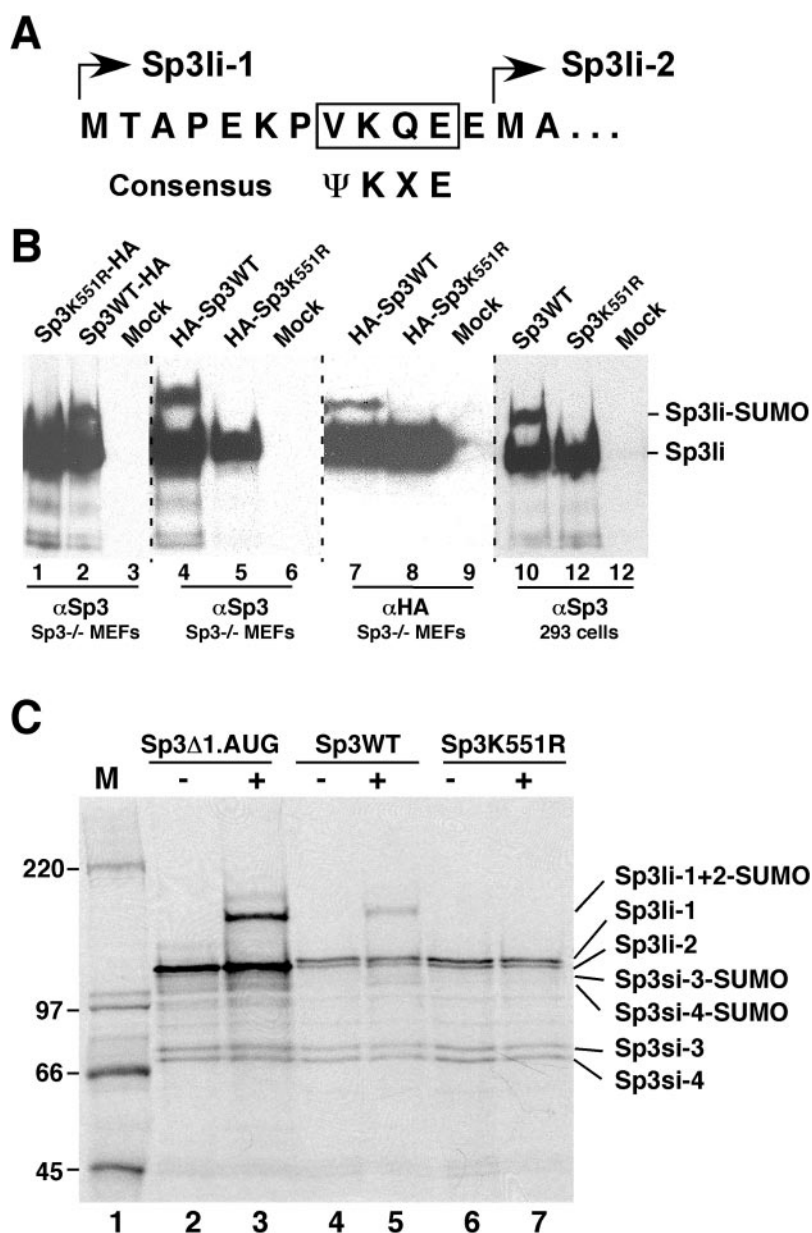
The SUMOylation Motif Present Exclusively in the Long Isoform of Sp3 Is Not a Target for SUMO Modification—Sp3 is post-translationally modified by SUMO at lysine 551 within the inhibitory domain of Sp3 (10). Close inspection of the N-terminal amino acid sequence revealed another potential site for SUMOylation present exclusively in the first Sp3 isoform (Fig. 4*A*). Lysine residue Lys-9 lies within the sequence VKQE

(amino acids 8–11) that fits well with the general SUMO conjugation motif Ψ KXE (10, 21). Obviously, an intriguing idea would be that the first and the second long Sp3 isoforms differ in their capacity to become post-translationally modified by a SUMO moiety at the N terminus. We asked whether lysine 9 is a target for SUMO modification *in vivo* or *in vitro*. Full-length versions of wild-type Sp3 or the K551R mutant HA-epitope-tagged at either the N or C terminus were transfected into Sp3^{-/-} MEFs and 293 cells and subjected to immunoblot analyses. SUMO-modified Sp3, visualized as a slower migrating species, was detected with the wild-type Sp3 construct but not with the Sp3K551R construct (Fig. 4*B*). This result strongly suggests that exclusively Lys-551 becomes SUMO-modified but not Lys-9 or any other lysine residue of the Sp3 sequence. This conclusion is supported by results obtained *in vitro*. *In vitro* translation products of wild-type Sp3 as well as of the Sp3 Δ 1.AUG mutant lacking the first isoform of Sp3 but not of the Sp3K551R mutant are targets for SUMOylation *in vitro* (Fig. 4*C*). All together, these results show that lysine 9 within the VKQE motif, which is only present in the longest Sp3 isoform, is not a target for SUMO modification, neither *in vivo* nor *in vitro*.

Unlike Sp1, Sp3 Is Not Glycosylated—The transcription factor Sp1 is post-translationally modified by glycosylation (22). Because Sp3 is evolutionarily and structurally very closely related to Sp1, we asked whether any of the isoforms of Sp3 are also modified by glycosylation. Glycosylated proteins bind strongly to wheat germ agglutinin (WGA), and WGA affinity chromatography has been used in the past as a major step to purify Sp1 from cell extracts (23).

Whole cell extracts of untransfected 293 cells and of SL2 cells transfected with Sp1, wild-type Sp3, or the SUMOylation-deficient Sp3K551R mutant were incubated with the wheat germ agglutinin matrix. Subsequently, bound proteins were sub-

FIG. 4. Post-translational modification of Sp3 by SUMO. *A*, N-terminal amino acid sequence of the Sp3 protein. The potential SUMO target sequence VKQE present in the longest Sp3 isoform (Sp3li-1) is boxed. The SUMO target consensus sequence reads Ψ KXE. *B*, *in vivo* Sp3 becomes modified by SUMO exclusively at lysine 551. CMV-driven expression plasmids for N-terminally HA-tagged (*HA-Sp3WT* and *HA-Sp3K551R*), C-terminal HA-tagged (*Sp3WT-HA* and *Sp3K551R-HA*) or untagged Sp3 (*Sp3WT* and *Sp3K551R*) were transfected into Sp3^{-/-} MEFs (lanes 1–9) or 293 cells (lanes 10–12). Cells were lysed with SDS-containing buffer, and Sp3 expression was analyzed by Western blotting using anti-Sp3 or anti-HA antibodies as indicated. *C*, *in vitro* SUMOylation of Sp3WT, Sp3K551R, and Sp3 Δ 1.AUG (see Fig. 2) proteins. *In vitro* transcribed/translated ³⁵S-labeled Sp3 proteins were subjected to *in vitro* SUMOylation reactions (10) using recombinant E1 enzyme, Ubc9, and PIAS1 in the presence (+) and absence (–) of SUMO-1. Reaction products were separated by SDS-PAGE and visualized by autoradiography. Molecular weight markers (*M*) are also shown.



jected to immunoblot analyses using antibodies to Sp1 and Sp3. As expected, endogenous Sp1 present in 293 extracts (Fig. 5, lane 2) as well as transiently (Fig. 5, lane 16) or stably (not shown) expressed Sp1 in SL2 cells bound strongly to wheat germ agglutinin (Fig. 5). Binding was specific, because it was competed by an excess of *N*-acetyl-D-glucosamine (Fig. 5, lane 4). Surprisingly, the small, the long, and the SUMO-modified isoforms of Sp3 did not bind to wheat germ agglutinin (Fig. 5, lanes 6 and 14). The same holds true for the SUMO modification-deficient Sp3K551R mutant (Fig. 5, lane 12). These results clearly show that the transcription factor Sp3, unlike Sp1, is not a target for post-translational modification by glycosylation.

Functional Differences between the Various Isoforms—All published data analyses on Sp3 activation capacity were performed with N-terminally truncated versions of Sp3. Thus it was obvious to ask whether full-length Sp3 or the various isoforms of Sp3, respectively, differ in their capacity to activate transcription. The transcriptional activity of various Sp3 isoforms and mutants was analyzed by transiently transfecting Sp3 expression constructs along with reporter constructs in

Schneider insect cells that lack endogenous Sp transcription factors. Gel shift experiments showed that all Sp3 isoforms bind GC-boxes specifically (Fig. 6A and data not shown). The BCAT-2 reporter contains two GC-boxes fused to the E1b TATA box. Upon transfection of the wild-type Sp3 construct or cDNA mutants from which predominantly the first isoform (2.AUGm), the second isoform (1.AUGm), or exclusively the third and fourth isoforms (Δ 1.+2.AUG) were expressed, the BCAT-2 reporter became non-activated (Fig. 6B). This result shows that all four isoforms of Sp3 are unable to activate the BCAT-2 reporter.

The SV40 promoter bearing five GC-boxes becomes activated upon co-transfection of the wild-type Sp3 construct (WT) as well as by mutants from which predominantly the first isoform (2.AUGm) or the second isoform (1.AUGm) are expressed. The small isoforms of Sp3 expressed upon transfection of the Δ 1.+2.AUG construct did not activate this reporter construct (Fig. 6B).

The promoter-specific activation capacity of the long isoforms of Sp3 (781 and 769 amino acids) basically reiterate results obtained previously with an N-terminally tagged trun-

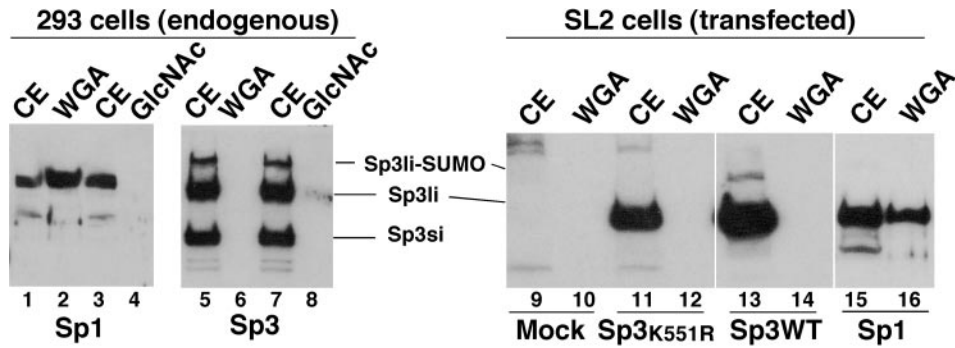


FIG. 5. Unlike Sp1, Sp3 is not post-translationally modified by glycosylation. Whole SDS cell extracts (CE) prepared from 293 cells or transfected SL2 cells were subjected to wheat germ agglutinin (WGA) affinity chromatography and subsequently analyzed by Western blotting using anti-Sp1 or anti-Sp3 antibodies as indicated. In lanes 1, 3, 5, 6, 9, 11, 13, and 15, 100 μ g of whole cell extract protein was separated. In lanes 2, 4, 6, 8, 10, 12, 14, and 16 wheat germ agglutinin-bound material was analyzed. In lanes 4 and 8 100 mM *N*-acetyl D-glucosamine (GlcNAc) was included in the WGA binding reaction.

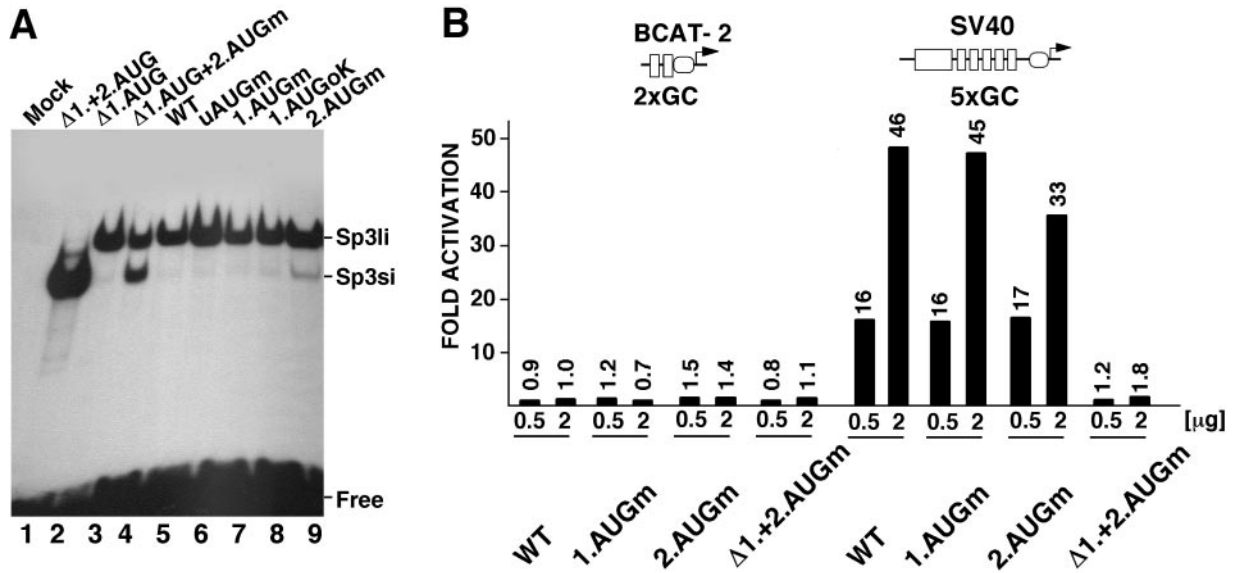


FIG. 6. Functional analyses of Sp3 isoforms. A, DNA binding capacity of Sp3 mutant proteins in SL2 cells. Gel retardation assays were performed with crude nuclear extracts from SL2 cells transfected with 1 μ g of expression plasmids for Sp3 isoform mutants as indicated. All reactions contained 0.2 ng of 32 P-labeled GC-box oligonucleotide and 5 μ g of protein extract. B, SL2 cells were transfected with 4 μ g of the BCAT-2 reporter plasmid or the SV40 promoter-driven luciferase reporter plasmid pGL3 along with 0.5 μ g or 2 μ g of expression plasmids for wild-type Sp3 or Sp3 mutants as indicated. The -fold activation values represent mean values of at least two independent transfections.

cated version (697 amino acids) (8). Thus the two long isoforms of Sp3 do have the capacity to activate transcription from certain promoters, for instance the SV40 promoter, but are inactive on other promoter settings exemplified here by BCAT-2. In contrast, both promoters were not activated by the small Sp3 isoforms.

The capacity of Sp3 to activate transcription is silenced by SUMO modification at lysine Lys-551 (9, 10). These studies also have been performed with N-terminally truncated, epitope-tagged versions of Sp3 that, according to the results described above, mimicked the activity of the long isoforms. Because all four isoforms of Sp3 are SUMO-modified, we asked whether the inactivity of the full-length Sp3 construct on the BCAT-2 reporter as well as the inactivity of the small isoforms on both the BCAT-2 and the SV40 promoter is dependent on the presence of the SUMO target lysine Lys-551.

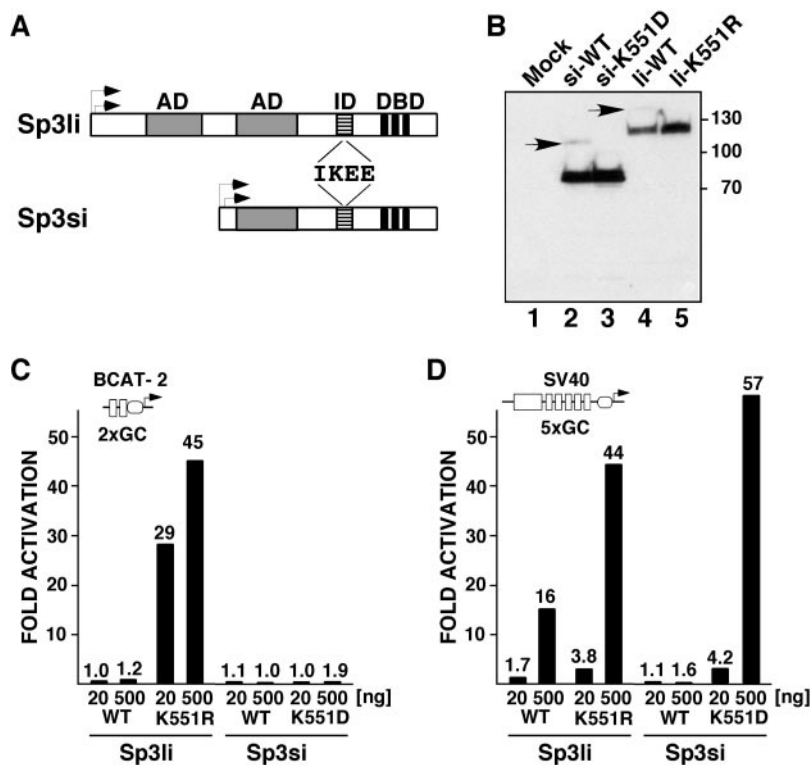
To this end, we introduced mutations that prevent SUMOylation (Lys-551 mutations) into the full-length Sp3 construct from which only the two long isoforms are expressed and into the Sp3 Δ 1.+2.AUG construct from which only the two small isoforms are expressed after transfection (Fig. 7A). Western blot analyses demonstrate that the small isoforms are expressed at higher level as compared with the long isoforms (Fig.

7B). Different to the wild-type long isoforms, the Sp3li-K551R mutant strongly activated the BCAT-2 reporter (Fig. 7C). Similarly, on the SV40 promoter, the Sp3li-K551R mutant is a 2- to 3-fold stronger activator compared with the Sp3liWT (Fig. 7D). Like the wild-type, the SUMOylation-deficient small isoforms of Sp3 (si-Sp3K551D) were unable to activate BCAT-2 (Fig. 7C). However, the SV40 promoter that is not activated by the wild-type small isoforms becomes strongly activated by the Sp3si-K551D mutant (Fig. 7D). This result shows also that the inactivity of the small Sp3 isoforms that contain a single glutamine-rich activation domain is to some extent due to post-translational modification by SUMO.

Subcellular Localization of Sp3 Isoforms—We wanted to know whether the apparent differences in regulatory properties of the small and long isoforms of Sp3 may reflect differences in their subcellular localization. Sp3-deficient KO MEFs and SL2 cells were transfected with appropriate expression constructs for the long and the small wild-type Sp3 isoforms, as well as with the corresponding SUMOylation-deficient mutants. Immunostaining with anti-Sp3 antibodies revealed that in both cell types the wild-type isoforms and the SUMOylation-deficient mutants were located in the nucleus exhibiting a sponge-like, diffuse appearance (Fig. 8). These results show

FIG. 7. Point mutations that prevent SUMO modification strongly enhance Sp3 activation capacity. **A**,

schematic drawing of Sp3 long (*Sp3li*) and small (*Sp3si*) isoforms. The glutamine-rich activation domains (AD), the SUMOylation site (IKEE), and the DNA-binding domain (DBD) are indicated. Mutations that replace lysine 551 were introduced in expression constructs for wild-type Sp3 and the $\Delta 1.+2$ AUGm mutant. **B**, transient expression of Sp3 long and short isoforms and lysine 551 mutants in SL2 cells. Western blot analyses was performed with whole cell extracts (SDS lysis) from SL2 cells transfected with 1 μ g of expression plasmid for the wild-type Sp3 short isoforms (si-WT, lane 2), the Sp3K551D short isoform mutant (si-K551D, lane 3), wild-type long isoforms (li-WT, lane 4), and Sp3K551R long isoform mutant (li-K551R, lane 5). The arrows point to the SUMO-modified short and long WT Sp3 isoforms (lanes 2 and 4). **C** and **D**, SL2 cells were transfected with 20 and 500 ng of expression plasmids for the long (*Sp3li*) and short (*Sp3si*) Sp3 isoforms and corresponding lysine 551 mutants along with 4 μ g of the BCAT-2 plasmid (**C**) or 4 μ g of the SV40 promoter driven luciferase reporter plasmid pGL3 (**D**).



that the differences in the activation capacity of the various isoforms and mutants are not due to differences in their subcellular or subnuclear localization.

DISCUSSION

Four Different Isoforms of the Sp3 Exist That Derive from Different Translational Initiation Sites—Twelve years after the original cloning of the transcription factor Sp3 (13, 14), we unraveled the puzzling complex Sp3 antigen pattern observed in immunoblots. Sp3 is expressed in four different isoforms that differ in their N-terminal extension. In addition, all four isoforms are targets of post-translational modification by SUMO, which accounts for the eight Western blot signals.

The four isoforms of Sp3 derive from four AUG translational start sites. At this stage, we can only speculate on the mechanisms governing different translational initiation within the Sp3 mRNA. The second AUG (GaaAUGG) and the fourth AUG (GcuAUGG) but not the neighboring first AUG (AcaAUGa) and the third AUG (AcaAUGa), respectively, are embedded in an optimal context. Thus, leaky scanning at the first AUG leading to initiation at the second AUG, and leaky scanning at the third AUG leading to initiation at the fourth AUG are likely to occur. How then can initiation at the internal third AUG be explained? An internal ribosomal entry site might be present in the Sp3 coding region that recruits ribosomes internally, although there is a strong debate whether cellular internal ribosome entry sites exist at all (24, 33). Secondary structure prediction using the Mfold web server (25) revealed striking stable RNA hairpins involving an extreme GC-rich Sp3 mRNA region between nucleotides +58 and +279 relative to the transcriptional start site. Possibly, some ribosomes stall at these hairpins, shunt, and subsequently reinitiate at the downstream third and fourth AUGs. Stable secondary structures might also explain why simultaneous mutation of the first two AUG codons resulted in the appearances of a number of new long isoforms initiating at non-AUG start codons. Scanning ribosomes may stall at the hairpin structures thereby initiating at non-AUGs that are in an optimal context.

An Upstream Open Reading Frame Is Involved in Regulating Sp3 Isoform Expression—Initiation site selection at the first and second AUG is dependent on a short 30-nucleotide uORF. Mutation of the uAUG codon leads to exclusive initiation at the first AUG. Thus, the uORF is essential for the synthesis of the second long Sp3 isoform. Another mutation that brings the uAUG in an optimal context leads to predominant initiation at the second AUG start codon. This result could be explained also by a leaky scanning and reinitiation model. The uAUG is in a suboptimal context. Thus, leaky scanning over the uAUG would lead to initiation at the first AUG. Other ribosomes translate the uORF and reach the terminator codon. According to current models, the 40 S subunit may hold on to the mRNA, resume scanning, and reinitiate at a downstream AUG (26). Reinitiation, however, is most efficient when the uORF terminates some distance before the next AUG to re-acquire Met-tRNAi-eIF-2 for downstream AUG recognition (26). Because the distance between the uORF stop codon and the next AUG is only 17 nucleotides, these ribosomes would preferentially use the second AUG. Such a scenario would explain why predominantly the second AUG was used, when the uAUG codon was placed in an optimal Kozak sequence (more ribosomes initiate at the uAUG). In addition, this model explains also the shift toward initiation at the first AUG when the distance between the uORF stop codon and the first AUG was increased (insertion mutants described in Fig. 3).

Functional Differences between Individual Isoforms—The most obvious difference between the two long isoforms and the two small isoforms of Sp3 is the presence of two and one single glutamine-rich domains, respectively. Both Gln-rich domains can act as strong activation domains on their own when fused to a heterologous DNA-binding domain (8). Accordingly, the long isoforms containing two activation domains do have a stronger activation potential than the short isoforms. The SV40 promoter for instance becomes activated upon expression of the long Sp3 isoforms but not by the small isoforms that contain a single activation domain. However, in other promoter settings

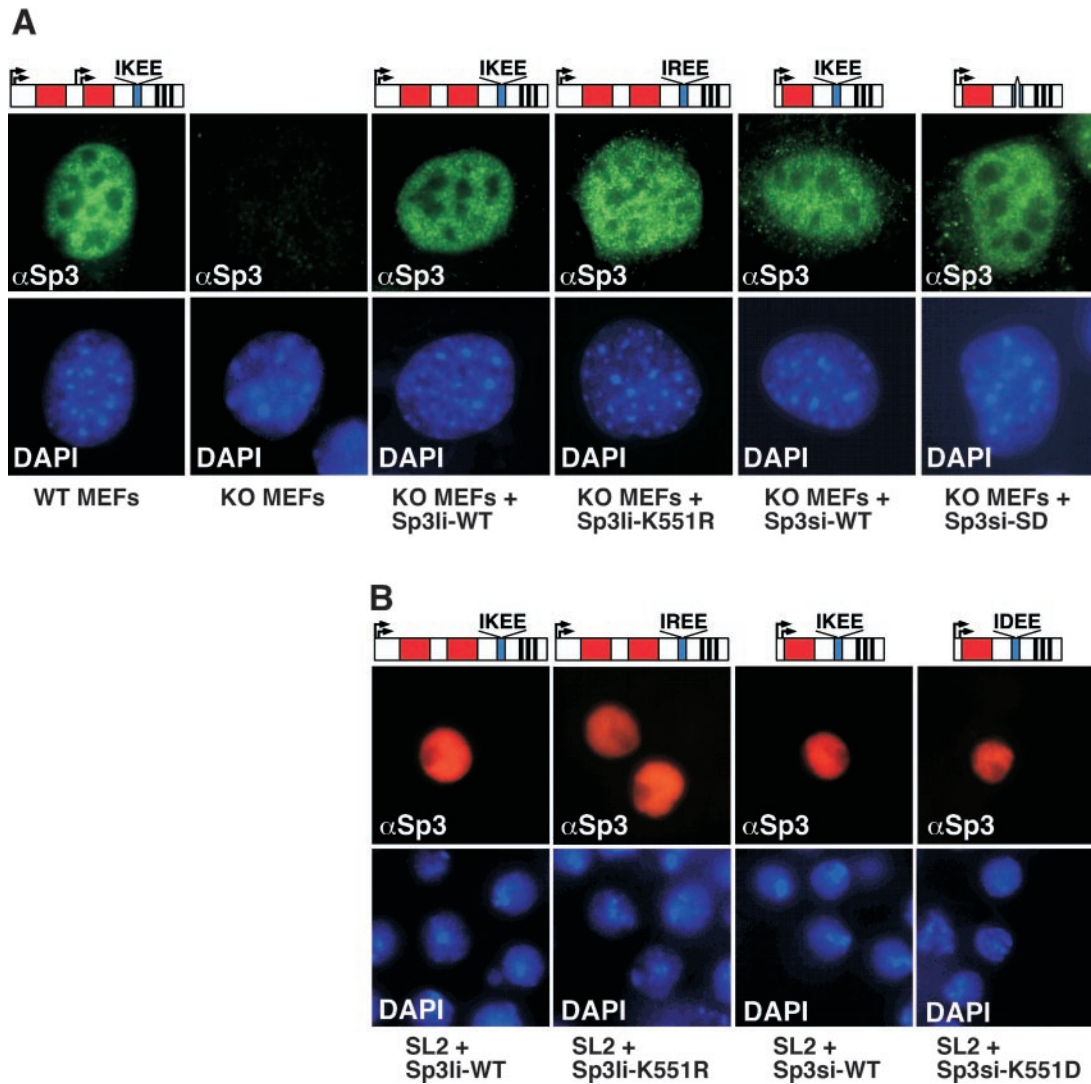


FIG. 8. Subcellular localization of Sp3 isoforms and corresponding SUMOylation-deficient mutants in MEFs and SL2 cells. Sp3 was detected by immunostaining with a rabbit anti-Sp3 antibody and a fluorescein isothiocyanate (in *A*)- or AF594 (in *B*)-conjugated secondary antibody. Control stainings of nuclei were performed with 4,6-diamino-2-phenylindole. *A*, Sp3^{-/-} MEFs stably transfected with expression constructs for the long or the small isoforms (*Sp3li-WT* and *Sp3si-WT*, respectively) and corresponding SUMOylation-deficient mutants (*Sp3li-K551R* and *Sp3si-SD*) are shown. The Sp3siSD mutants contain a 13-amino acid deletion, including the IKEE SUMOylation motif. WT MEFs and mock-transfected Sp3-deficient KO MEFs were included in the analyses. *B*, SL2 cells were transiently transfected with 1 μ g of expression constructs for Sp3 isoforms and mutants as indicated.

exemplified by BCAT-2 the long isoforms of Sp3 are also inactive. Thus far, we do not understand the different activation capacities of Sp3 on different promoter settings. Clearly, it is not simply the number of Sp3 binding sites or the spacing of the Sp3 binding sites that is decisive for the activation by the long isoforms. A promoter that contains only a single GC-box becomes also activated by the long Sp3 isoforms (Ref. 8 and data not shown). BCAT-2 derivatives with variable distances of the two GC-boxes can also not be activated by Sp3 isoforms.² We speculate that different chromatin structures that become established on different promoter settings are responsible for the observed activation differences.

A major determinant of Sp3 activity is the post-translational modification by SUMO. SUMOylation takes place exclusively at lysine Lys-551 present in all four isoforms. Mutation of Lys-551 strongly enhanced transcriptional activity of the long Sp3 isoforms. However, the inactivity of the small isoforms also depends on SUMO modification. The small Sp3 isoforms in

which Lys-551 was mutated strongly activated the SV40 promoter. Two additional SUMO acceptor motifs (Ψ KXE) are present exclusively in the longest isoform (VK9QE) or in both long isoforms (IK120DE), respectively. Both sites, however, do not become SUMOylated either *in vivo* or *in vitro*.

At this stage we do not know the functional relevance of having two long Sp3 isoforms. Based on transfection results, their activation capacities are similar. This does not mean that their function *in vivo* on natural promoters is identical and that both isoforms occupy the same promoter at the same time. For instance, the transcription factor C/EBP β exists in three isoforms. The two long isoforms of C/EBP β , designated LAP* and LAP, differ in only 21 amino acids at the N terminus. Both isoforms are strong activators on plasmid-based gene transcription. Nevertheless, LAP* and LAP are strikingly different in their ability to activate chromatin-embedded chromosomal genes, because the extreme N terminus present exclusively in LAP* recruits the SWI/SNF complex involved in chromatin remodeling (27). Sequence comparison of the N terminus of LAP* with the N terminus of Sp3 revealed no obvious similarities. It remains to be established whether the two long iso-

² A. Sapetschnig, F. Koch, G. Rischitor, T. Mennenga, and G. Suske, unpublished data.

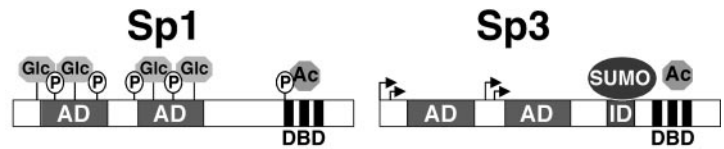


FIG. 9. Comparison of known structural, biological, and biochemical features of the transcription factors Sp1 and Sp3 (see "Discussion" for details).

KO phenotype	Embryonic lethal (E10)	Neonatal lethal
Transcriptional activity	++	+/-
Posttranslational modifications		
Phosphorylation	+	?
Glycosylation	+	-
Acetylation	+	+
SUMOylation	-	+
Isoforms	1	4

forms of Sp3 exhibit distinct activities on chromatin-embedded endogenous genes. Generating knock-in mice that express only single isoforms of Sp3 might also help to clarify the function of individual isoforms.

Is Sp3 Isoform Expression Altered under Certain Conditions?—Sp3 isoform expression is to some extent reminiscent of the isoform expression described for the transcription factors C/EBP α and C/EBP β (28) as well as of the transcription factor SCL (29). In all three cases isoforms arise from unique mRNAs by differential initiation of translation. Like Sp3 isoforms, C/EBP isoforms retain different parts of the N terminus and display different functions in gene regulation. Moreover, upstream open reading frames regulate initiation from different translation initiation sites. In the case of C/EBP α and β , deregulated translational control interfered with terminal differentiation and induced cell transformation in 3T3-L1 adipocytes (28). Thus far, we do not know whether and how isoform expression of Sp3 is regulated *in vivo*. Serum removal or serum addition did not change the relative ratio of the different isoforms² making it unlikely that isoform expression changes during the cell cycle.

Initiation of translation is affected by a number of pathways that control the activity and level of eukaryotic translation initiation factors (eIFs) (30). For instance, at high eIF-2 and eIF-4E activity comparatively more truncated C/EBP isoforms are expressed, whereas at lower activity of these factors expression of long isoforms dominates (28). We have transfected full-length Sp3 along with expression constructs for the eukaryotic initiation factors eIF2 and eIF4E and subsequently analyzed Sp3 isoform expression. Under our experimental conditions, the isoform ratio did not change. Moreover, we grew cells in the presence or absence of rapamycin and 2-aminopurin. Rapamycin diminishes the activity of eIF4E, and 2-aminopurin enhances the activity of eIF2 α . Again, we could not detect a change in Sp3 isoform expression (data not shown). Thus at this stage we do not know under which physiological conditions Sp3 isoform ratio alterations may take place. A significant shift toward the long isoforms of Sp3, however, is observed in Sp1^{-/-} ES cells² demonstrating that Sp3 isoform expression principally can change *in vivo*. In addition, this observation suggests that the long isoforms of Sp3 may take over Sp1 functions under Sp1 knock-out conditions.

Sp1 versus Sp3—The structural similarity between Sp1 and Sp3 as well as their ubiquitous expression suggested originally that they have similar properties and exert similar functions. Detailed biochemical and biological studies, however, highlighted significant differences, including different knock-out phenotypes, different post-translational modifications, and expression of different isoforms (Fig. 9). These studies establish

Sp3 as a transcription factor with properties considerably distinct from Sp1. Most obviously, Sp3 is expressed as four isoforms that can all become post-translationally modified by a SUMO moiety. In addition, unlike Sp1, Sp3 is not glycosylated. Moreover, Sp1 is highly phosphorylated. Whether any of the isoforms of Sp3 can be a substrate for phosphorylation under certain conditions remains to be established. A modification that has been described for both molecules is acetylation. Acetylation of Sp1 likely occurs at lysine residues within the DNA-binding domain (31), whereas the location of acetylation of Sp3 has not yet been precisely determined. Previously, we have shown that mutation of the SUMO lysine 551 impaired acetylation of Sp3 *in vivo* (32). This finding suggested originally that the same lysine residue that is a target for SUMOylation is also a target for acetylation. However, antibodies highly specific for the acetylated IKEE motif of Sp3 did not recognize endogenous Sp3 or Sp3 overexpressed in 293 cells or SL2 cells.² These results suggest that it may not be Lys-551 that becomes acetylated *in vivo*. We speculate, rather, that SUMO-modified Sp3 might recruit acetyltransferases that in turn acetylate other lysine residues within the Sp3 protein.

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