Identification of survival motor neuron as a transcriptional activator-binding protein

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Spinal muscular atrophy (SMA) is an inherited neuro-muscular disease characterized by specific degeneration of spinal cord anterior horn cells and subsequent muscle atrophy. Survival motor neuron (SMN), located on chromosome 5q13, is the SMA-determining gene. In the nucleus, SMN is present in large foci called gems, the function of which is not yet known, while cytoplasmic SMN has been implicated in snRNP biogenesis. In SMA patients, SMN protein levels and the number of gems generally correlate with disease severity, suggesting a critical nuclear function for SMN. In a screen for proteins associated with the nuclear transcription activator ‘E2’ of papillomavirus, two independent SMN cDNAs were isolated. The E2 and SMN proteins were found to associate specifically \textit{in vitro} and \textit{in vivo}. Expression of SMN enhanced E2-dependent transcriptional activation, and patient-derived SMN missense mutations reduced E2 gene expression. Our results demonstrate that SMN interacts with a nuclear transcription factor and imply that SMN may serve a role in regulating gene expression. These observations suggest that SMA may in part result from abnormal gene expression and that E2 may influence viral gene expression through SMN interaction.

\section*{INTRODUCTION}

Spinal muscular atrophy (SMA) is a common autosomal recessive disorder characterized by degeneration of \(\alpha\)-motor neurons of the spinal cord anterior horn with resultant muscle atrophy. Three clinical manifestations of SMA (types I, II and III) are based on age of onset and clinical course (1). The gene responsible for all three types was mapped to chromosome 5q13 (2–4) and termed survival motor neuron (SMN) (5).

The genetics of SMA are complex due to the instability of the SMA locus. The genomic region encompassing SMN is duplicated and inverted and contains two SMN genes: a telomeric (\textit{SMN}tel) and a centromeric (\textit{SMN}cen) copy (4–7). Presence of disease correlates only with loss or mutation of \textit{SMNtel}, and the subsequent decrease in SMN protein levels. Loss of \textit{SMNtel} occurs by deletion or by gene conversion to \textit{SMNcen} (6). The copy number of \textit{SMNcen} genes appears to modulate disease severity (8,9). Complete lack of both \textit{SMNtel} and \textit{SMNcen} has not been identified, presumably because an \textit{SMN} null is lethal \textit{in utero}, as is the case in mouse (10). Five reported nucleotide differences between the two \textit{SMN} genes allow them to be distinguished (5), and both genes encode identical products; however, the majority of \textit{SMNcen} mRNA undergoes alternative splicing, producing an isoform lacking exon 7, \textit{SMNΔ7} (5,11). Therefore, SMA patients with homozygous deletion of \textit{SMNtel} alleles still produce SMN proteins although the level of full-length \textit{SMN} is greatly reduced (5,12). While most patients have \textit{SMNtel} deletions, several have been identified who carry \textit{SMN} missense mutations (reviewed in ref. 6), producing proteins that are presumably functionally defective (13).

The major detectable product of \textit{SMN} genes is a 38 kDa protein expressed in all cell types examined to date (12,14,15). \textit{SMN} is found in distinct compartments including the cytosol (16–18) and the nucleus (19). In the cytosol, \textit{SMN} is complexed with Sm proteins and \textit{SMN}-interacting protein-1 (SIP1) (18). Disruption of SIP1–\textit{SMN} complexes in Xenopus oocyte extracts decreased snRNP formation (17), suggesting that SIP1–\textit{SMN} functions to control snRNP biogenesis and implying an indirect role for \textit{SMN} in RNA processing. \textit{SMN} recently has been shown to function in the distribution and regeneration of the pre-mRNA splicing machinery (20). Exon 6, a 37 amino acid region containing several Y–G elements (21), is absolutely required for self-association (13). A series of point mutations clustered in or immediately adjacent to the oligomerization domain have been isolated from types I, II and III SMA patients, and mutant \textit{SMN} protein self-association activity correlates with disease severity (13). \textit{SMNΔ7} protein also has significantly reduced self-association activity. Recently identified biochemical functions of \textit{SMN} are intriguing beginnings to understanding the molecular nature of SMA; however, it is unclear how \textit{SMN} defects result in the highly specific \(\alpha\)-motor neuron degeneration associated with SMA.
Within the nucleus, SMN is concentrated in discrete subnuclear foci called gems, whose only identified components are SMN and SIP-1 (18,19). A correlation between the presence of SMA and gem number has been observed. Gems are virtually absent from SMA type I patients and gem number is significantly decreased in SMA II and III patients, although cytoplasmic SMN protein is readily detectable in all types (12,15). Identification of SMN with a nuclear protein of known function might identify a novel role for SMN and provide insight into gem function.

A two-hybrid screen to identify cellular factors which functionally interact with bovine papillomavirus (BPV) E2 transactivator resulted in the isolation of SMN. Papillomavirus gene expression is largely under the control of one major viral-encoded protein, the E2 transactivator. BPV E2 is a 410 amino acid nuclear protein with a separable transcriptional activation domain (TAD; residues 1–216) and DNA-binding domain (DBD; amino acids 285–410) (22). It activates transcription through interactions with multiple cellular factors, including TATA-binding protein (TBP), transcription factor IIB (TFIIB), Sp1 and AMF-1 (23–26). The TAD contains two acidic domains and several ‘bulky hydrophobic’ motifs identified by genetic screens and mutational analyses that are critical for E2 transcriptional activity (27). Subtle point mutations have been identified that disrupt binding to E2 by a discrete subset of transcription factors and subsequent inactivation of transcription while maintaining the overall integrity of the TAD (27). This is evidenced by the ability of those E2 mutants to retain transcription factor binding in domains outside the mutated domain. This ability to disrupt E2 TAD function significantly has been exploited recently by allowing E2 TAD point mutants to serve as bait in two-hybrid screens for additional E2-interacting proteins (26). Papillomaviruses also exploit several post-transcriptional levels of regulation (28,29), the mechanisms of which are not thoroughly understood. E2 conceivably could coordinate these post-transcriptional events.

Experiments described here demonstrate a direct and specific interaction in vitro and in vivo between SMN and E2, which is conserved among divergent papillomavirus E2 proteins. Transient expression of SMN protein stimulated E2-dependent gene activation, while SMA patient-derived missense SMN proteins inhibited E2 activity. The association of SMN with a nuclear transactivator and its ability to alter gene expression suggest that SMN plays a role in cellular gene expression.

RESULTS

A yeast two-hybrid screen for interacting partners of BPV-1 E2 transactivator protein identified SMN

A two-hybrid screen was used to identify cellular factors that interact with the BPV-1-encoded proteins. An E6–E2 fusion protein served as bait in the HeLa cell library screen (30). Two independent cDNA isolates were obtained from ~1.0 × 10⁶ colonies and subsequently identified as the C-terminal portion of the SMN gene (largest encoding amino acids 182–293) fused to the VP16 TAD. The VP16–SMN isolate, but not controls, reacted with E2 in the absence of E6 at comparable levels with another previously identified E2-binding protein, AMF-1 (26) (Table 1). To validate the interaction, the full-length SMN cDNA was cloned and additional two-hybrid analyses were performed with full-length, 410 amino acid E2 (Table 2). Wild-type E2 is a potent activator in yeast and is therefore not a suitable bait for two-hybrid analysis; therefore, a full-length, transcription-defective E2 point mutant (E2:Q15H) was used in the two-hybrid analysis. This protein is as stable as wild-type E2 and was chosen for this assay because it retains binding activity for other E2 TAD-binding factors required for E2 transactivation, probably due to the separable nature of the E2 TAD functions. Additionally, the Q15H mutation, which retains AMF-1 and BPV E1 binding, is outside of the E2 peptide used in the original two-hybrid screen and would therefore be predicted to retain SMN binding. An E2 DBD (amino acids 286–410) bait that excluded the total TAD (amino acids 1–216) did not interact with SMN. An E2 construct (E2:115–410) containing a portion of the TAD interacted with VP16–SMN, demonstrating that the E2 TAD mediated the association. Identical results were obtained using VP16 TAD fusions with the full-length SMN and the original partial cDNA (data not shown). To determine whether the TAD was sufficient to support the SMN–E2 association, portions of the E2 TAD were fused to the bacterial LexA DBD and assayed in a complimentary approach with a LexA-dependent reporter. LexA–E2:1–286, encoding the entire E2 TAD, and LexA–E2:115–215 interacted specifically and at comparable levels with VP16–SMN, thereby confirming the

Table 1. Quantitative β-galactosidase assays of the interaction of E2 with the original partial SMN construct (Δ1–125)

<table>
<thead>
<tr>
<th>Bait*</th>
<th>Prey*</th>
<th>+VP16</th>
<th>+VP16–SMN Δ1–125</th>
<th>+SMN Δ1–125</th>
<th>+VP16–AMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 wt (115–215)</td>
<td>1–125</td>
<td>1.2</td>
<td>16</td>
<td>1.2</td>
<td>16</td>
</tr>
<tr>
<td>Vector alone*</td>
<td>1–125</td>
<td>1.2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1</td>
</tr>
</tbody>
</table>

*The reporter was E2 based.

*VP16 alone, VP16 fused to the SMN isolate Δ1–125, Δ1–125 alone or a second VP16-tagged E2-binding protein, VP16–AMF.

*Empty vector control.
SMN-interacting domain with E2 as the 115–215 region of the E2 TAD. The interaction of E2 with VP16–SMN was specific, as the LexA DBD and VP16 TAD failed to interact with VP16–SMN and LexA–E2, respectively.

Table 2. Qualitative two-hybrid analysis of the SMN–E2 interaction

<table>
<thead>
<tr>
<th>Bait</th>
<th>Relative colony color intensities</th>
<th>+VP16 SMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 wt (1–410)</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>E2 (Q15H)</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>E2 (115–410)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E2 (286–410)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LexA–E2 (1–286)</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>LexA–E2 (115–215)</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>LexA</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Reporters were E2 based (1–410, 115–410, 286–410 and 286–410) and LexA based (1–286 and 115–215).

*Results from expression of only the ‘bait’.

*Results of co-expression of ‘bait’ and VP16 activation domain-tagged SMN.

SMN binds directly to E2

To determine whether SMN associates directly with E2, a series of in vitro binding assays were performed. Radiolabeled, in vitro translated (IVT) SMN was reacted with a glutathione-S-transferase (GST)–E2 fusion protein purified from Saccharomyces cerevisiae. GST–E2 captured ~5% of SMN input, while binding to GST alone was not detected (Fig. 1A). To demonstrate conclusively that SMN and E2 directly interact, in vitro binding assays were performed with bacterially produced and purified recombinant SMN and E2 proteins. SMN was expressed as a GST fusion and E2 was N-terminally tagged with six histidines (6×His:E2) and purified through a nickel–bead slurry. SMN specifically bound a high level of E2, ~10% of input, while GST did not bind detectable levels (Fig. 1B), demonstrating that SMN and E2 bind directly and that this interaction occurs independently of additional eukaryotic bridging factors.

In reciprocal in vitro binding experiments, GST–SMN specifically captured ~5% of radiolabeled E2 input protein with no detectable binding of GST alone to E2 (Fig. 1C). Luciferase, a non-specific control protein, failed to associate with GST–SMN. The amino acid sequences of E2 proteins are partially conserved between bovine and human papillomaviruses with ~25% identity (22). We therefore tested whether E2 from high-risk human papillomaviruses (HPV-16 and -18) associated with cervical cancer and a low-risk HPV (HPV-11) would also bind the SMN protein. HPV-11, -16 and -18 E2 were captured specifically by GST–SMN at levels similar to BPV E2 (Fig. 1C, compare lanes 1–4 and 5–8), suggesting that the ability of divergent E2 species to bind SMN is a conserved property.

SMN binds E2 in vivo

To determine whether SMN and E2 associate in vivo, co-immunoprecipitation experiments were performed. Following transient transfection of E2 and SMN expression vectors into mammalian cells, cellular extracts were immunoprecipitated with an anti-E2 monoclonal antibody (mAb B202), and bound
fractions were probed with the C3 anti-SMN antibody (12). A substantial, E2-dependent association with SMN was detectable from the extracts derived from the E2- and SMN-transfected cells (Fig. 2A, lane 3). Significantly, in the absence of transiently expressed SMN, an E2–SMN association was also detectable between E2 and endogenous SMN protein (Fig. 2B, lane 2). Co-expression of SMN and E2 did not result in aberrant cellular distribution for either protein (data not shown). Additionally, E2 present in transfected mammalian cell extracts associated specifically with GST–SMN (Fig. 2C), suggesting that the GST–SMN molecule used in the previous in vitro binding experiments behaved similarly to the endogenous SMN molecule.

**SMN alters gene expression**

E2 is a potent transcriptional activator, and several critical interactions with cellular transcription factors are mediated by the E2 TAD. Since SMN binds E2 within the TAD, the following series of experiments were designed to determine whether transiently expressed SMN affected E2-dependent transcriptional activation. Mammalian cells were co-transfected with an E2-dependent luciferase reporter in the presence or absence of E2 and SMN expression vectors, as indicated in Figure 3A. For these experiments, a relatively low amount of E2 was transfected since high levels result in general transcriptional repression (23). As expected, E2 activity was strong and resulted in ~60-fold stimulation over basal levels (Fig. 3A). The addition of SMN resulted in even higher reporter gene expression, increasing E2 activity by an additional ~30%. Normal cells contain relatively high levels of endogenous SMN protein. Similar experiments were performed in SMA type I and II patient-derived fibroblasts in an effort to examine E2 transcriptional activity in the context of reduced endogenous SMN protein levels. However, these human primary cells transfect poorly. Nonetheless, E2 was still active in both types of SMA-derived fibroblasts, perhaps due to the readily detectable levels of full-length SMN seen in western blot analysis (data not shown).

The effects of various SMN isoforms and mutants were assessed in analogous E2-dependent reporter assays (Fig. 3B). First, the SMN construct (SMNΔ1–125) was transiently expressed and resulted in ~60% decrease in E2 activity. Next, two naturally occurring SMN spliced isoforms, SMNΔ5 and SMNΔ7, which lack exon 5 or 7, respectively, were transiently expressed in the reporter assay, resulting in ~70 and ~15% decreases of E2 activity, respectively. Finally, two SMA patient-derived SMN point mutations, SMN-Y272C and -G279V, were assayed similarly, and shown to result in greater than an ~80% decrease in E2 reporter activity. Taken together, these results demonstrate that SMN associates in vivo and in vitro with a nuclear transcription factor, and suggest that SMN can alter gene expression.

**DISCUSSION**

In this report, we describe the isolation of SMN based on its interaction with a nuclear regulator of viral gene expression, BPV-1 E2. From the original screen, two partial cDNAs coding for SMN were isolated. Yeast two-hybrid analyses demonstrated that the SMN interaction with E2 was mediated by the E2 TAD. This association was confirmed using in vitro binding and in vivo co-immunoprecipitation assays and found to be direct and specific. Interestingly, human papillomavirus E2 proteins that share <30% identity also bind SMN, suggesting that this interaction is a conserved viral function. Furthermore,
SMN stimulated E2 gene expression, while the spliced isoforms including the primary product of SMNcen (SMNΔ7) and patient-derived missense mutations inhibited E2 transactivation. Taken together, these results identify SMN as a viral transcriptional activator-binding factor, and demonstrate a function that may provide insight into the role of SMN within the nucleus.

SMN is a complex molecule, as evidenced by its primary sequence (Fig. 4). Sequence analysis has identified several motifs: a highly basic lysine-rich region in exon 2b, a Tudor homology region in exon 3 (31), a polyproline region in exon 5 and several YG-rich boxes in exon 6 (21). These domains correlate with some SMN properties such as nucleic acid binding (32). Sm and SIP-1 interaction (18), E2 binding and self-association (13). Within the cytosol, SMN interacts with Sm proteins and SIP1 and, in Xenopus oocytes, the SMN–SIP1 interaction was necessary for proper formation of snRNPs (17,18). SMN subcellular biochemistry and function are complicated, as within each compartment SMN is likely to perform different functions. It was of particular interest to obtain a nuclear SMN-interacting factor, since the number of nuclear SMN structures (gems) correlates with SMA disease severity (12,15).

E2 functions in part through interactions with components of the general transcription machinery including TBP, TFII B and Sp1 (23–25,27). SMN is proline rich (Fig. 4), a characteristic of one class of transcription factors including Sp1. It is unlikely, however, that SMN functions as a classic transcription factor since simply tethering SMN upstream of a promoter through a heterologous DBD had no effect on transcription (data not shown). Nonetheless, such a function cannot be completely excluded. SMN, like other RNA-binding proteins, has double-stranded as well as single-stranded DNA-binding activity (32). Interestingly, the missense mutations and similar N-terminal deletion constructs that either decrease DNA- or RNA-binding activity or lack this domain entirely (32) failed to stimulate E2 gene expression. An intact SMN nucleic acid-binding domain seems to be required to actuate its E2 stimulatory activity. SMN may function downstream, perhaps at a transport step, a speculative function based on its RNA-binding activity. SMN is thought to be involved in RNA processing because of its binding to cellular proteins known to participate in RNA metabolism, and gems often co-localize with coiled bodies that may serve to process RNA (19). It has been shown that SMN functions in vitro to enhance mRNA splicing (20). Recent evidence demonstrates that the transcription and mRNA processing machinery function in concert ‘co-transcriptionally.’ Along these lines, Ge et al. (33) recently identified a transcriptional co-activator that also interacts with the essential splicing factor ASF/SF2. We speculate that while E2 acts as a classical transcriptional activator, it may also stimulate viral gene expression through an RNA processing step mediated by SMN.

SMN has been implicated in the biogenesis of snRNPs in the cytoplasm (17,18), and recent evidence suggests that SMN possesses a ‘regenerative’ activity for the pre-mRNA splicing machinery components (20). As snRNPs are required for RNA processing, the effects on E2 gene expression described here might reflect a direct or indirect alteration in post-transcriptional processing. The reporter assays do not distinguish between any of the steps required for proper transcription and translation; however, not all reporter constructs are regulated similarly by SMN (C.L. Lorson and E.J. Androphy, unpublished data). If SMN were to exert its effects on gene expression through altered RNA metabolism or transport, then E2 might utilize this function to alter papillomavirus transcript processing in a similar manner. A connection between SMA and papillomavirus infection has not been observed, nor do papillomaviruses infect spinal motor neurons. However, as SMN has been detected readily in nearly all tissues, a role for SMN–E2 complexes in the papillomavirus life cycle remains a possibility. Current studies are underway to address these possibilities.
Naturally occurring SMN missense mutations Y272C and G279V differ functionally from wild-type as they cannot protect against SMA and are defective for self-association (13). However, binding to wild-type SMN by these mutants is higher than SMN missense self-association (unpublished data). Thus, these mutants may inhibit wild-type SMN function either as hetero-oligomers or as monomers. The dominant-negative transcriptional repression activity described here for Y272C and G29V is in contrast to the recessive nature of SMA. A dominant-negative mutant SMN protein has, however, been identified recently for pre-mRNA splicing. The inhibitory activity described here suggests that the higher levels of mutant SMN protein achieved due to transient expression titrate a limiting factor or at these levels partially overcomes the oligomerization defect, thereby allowing inactive complexes to form. Additionally, since these mutations exhibit reduced SMN self-association, SMN oligomerization is probably a prerequisite for SMN effects on gene expression. In support of this, SMNΔ5 and SMNΔ7 share these oligomerization and gene expression defects. In SMA patients, the SMN-cen copy produces transcripts that consist primarily of the SMNΔ7 isoform. These results provide a potential explanation for why patients with intact SMN-cen show the SMA phenotype.

While several clues to the role of SMN in the pathogenesis of SMA have been suggested recently, it is still not clear how an SMN defect results in an abnormality that is cell-type restricted. Viral model systems have been characterized extensively to provide mechanistic insight into numerous cellular processes, including transcription, DNA replication and protein transport. Viral proteins have evolved molecularly to ‘mimic’ endogenous cellular factors; therefore, it is possible that an endogenous transcription factor is also the target of SMN regulation. The activity of SMN in gene expression allows a model in which specific subsets of genes absolutely required for α-motor neuron maintenance are aberrantly regulated.

**MATERIALS AND METHODS**

**Two-hybrid screen**

A HeLa cDNA library was screened using ‘bait’ consisting of the papillomavirus E6 oncogene fused to the C-terminal two-thirds of BPV E2 (E6–E2) as previously described (30). Yeast plasmids encoding wild-type E2 (YepLac112GE2), E2Δ1–115 (YepLac112GE2 Δ1–115), E2Δ1–286 (YepLac112GE2 Δ1–286), LexA DBD (Yeplac181GLexA), the LexA DBD fused to E2 residues 115–215 (YepLac181GLexA:E2115–215) and E2 missense mutant Q15H (YepLac112GLexA:E2Q15H) and quantitative β-galactosidase assays have been described (26).

To clone the full-length SMN protein, the EcoRI–HindIII fragment of the largest isolate was cloned into pBSSK (Stratagene, La Jolla, CA). The 5′ portion of the cDNA missing from the yeast two-hybrid library isolate was PCR amplified from an anchored human brain cDNA library (Clontech, Palo Alto, CA), and the resultant full-length clone was sequenced. SMN deletion mutant SMNΔ1–125 encodes residues 125–294. Plasmids encoding SMNΔ7 and SMN missense mutants have been described (13). All constructs were subcloned to produce hemagglutinin (HA)-tagged cDNAs in pCDNA3HA. Plasmids pCDNA3-HASMN, -HASMNΔ1–125, -HASMNΔ7, -HASMNΔ5, -HASMNΔ7 and -HASMNΔ272C and -HASMNΔ279V were used for IVT reactions and for mammalian cell expression (13).

Two-hybrid ‘fish’ constructs were made by inserting the SMN cDNA into pSD10a (34) to produce pSDSMN or into pSDVP1610a to produce pSDVP16:SMN. The two-hybrid ‘fish’ construct encoding the original isolate of the E2-binding protein AMF-1 was used as a control (26).

**In vitro binding reactions**

IVT plasmids encoding BPV-1 E2 or HPV-11, -16 and -18 have been described (35). IVT reactions were carried out using the TnT system (Promega, Madison, WI) according to the manufacturer’s instructions. Preparation of GST–SMN fusion protein in Escherichia coli (13) and GST–E2 and GST proteins in S.cerevisiae has been described (26).

In vitro binding reactions contained ~1 μg of purified GST fusion protein (as determined by BCA analysis; Pierce, Rockford, IL) and were incubated with 5 μl of IVT protein in a
total volume of 500 µl of NETN (250 mM NaCl, 0.1% NP-40, 10 mM Tris pH 7.4, 1 mM EDTA) with fresh aliquots of protease inhibitors (phenylmethylsulfonyl fluoride and pepsatin A) for 3 h at 4°C. Samples were washed three times in NETN and twice in NETN with 500 mM NaCl. Bound fractions were analyzed by denaturing 12% polyacrylamide gel electrophoresis. Gels were dried and exposed on a Bio-Rad (Hercules, CA) Molecular Imager. Binding using GST–SMN and 6xHis:E2 was performed with 6 µg of GST–SMN and 2 µg of 6xHis:E2 in 500 µl of NETN [200 mM NaCl + Boehringer (Mannheim, Germany)] complete protease inhibitor cocktail (EDTA)] for 2.5 h at room temperature. Bound fractions were washed five times in 1 ml of NETN (200 mM NaCl) and twice in NETN (300 mM NaCl), and detected by western analysis with anti-E2 mAb B201 using the Pierce chemiluminescence kit. 6xHis:E2 purification was described previously (35).

In vivo association
A total of 1.0 × 106 COS-7 cells were electroporated with 5 µg of pCGE2 (36) in 0.4 ml of Dulbecco’s modified Eagle’s medium (DMEM) + 10% fetal calf serum (FCS) in a 0.4 cm cuvette at 1050µF/220V at room temperature in a GenePulser II apparatus (Bio-Rad). Whole cell extracts (WCE) were prepared by harvesting 24 h post-electroporation in 500 µl of lysis buffer (13) at 4°C for 60 min and centrifuged twice for 30 min at 12 000 g. Supernatant was collected and 150 µl of this was incubated with protein A–Sepharose (Pharmacia, Uppsala, Sweden) and mAb B202, which recognizes BPV-1 E2, for 90 min at 4°C. The beads were collected by centrifugation for 30 s at 500 g and washed five times in lysis buffer. Bound fractions were eluted by boiling in SDS sample buffer and resolved by 12% SDS–PAGE. SMN was detected by western blot as previously described (13).

Approximately 5 µg of GST, GST–SMN or non-specific control fusion protein GST–Fyn were reacted with equivalent amounts of WCE derived from cells transfected with pCGE2 (E2 WCE) or pCGE2 (mock WCE) for 2 h at 4°C. Bound fractions were washed four times in lysis buffer and subsequently probed with E2 mAb B202.

Gene expression assays
The E2-dependent luciferase reporter plasmid, pJS-Luc, was constructed by inserting three high-affinity E2-binding sites into pA3RSV180-Luc (37). The resulting plasmid contains sequentially a transcription termination signal, three E2-binding sites, 180 bp of the Rous sarcoma virus 3’-untranslated region and the luciferase-coding region.

C33a cells were grown in DMEM with 10% FCS and seeded at 1.0 × 106 per 60 mm dish 1 day prior to calcium phosphate-mediated transfection reactions containing 0.5 µg of pJS-Luc, 0.5 µg of E2 and 4.5 µg of SMN expression plasmids in a total volume of 500 µl with a total of 5 µg of DNA. At 20 h post-transfection, cells were harvested in reporter lysis buffer (Promega) and measured in a luminometer (MGM Instruments, Hamden, CT). Cell survival was qualitatively unaffected. Multiple transfections were conducted in triplicate, and representative results are shown.

ABBREVIATIONS
BPV, bovine papillomavirus; DBD, DNA-binding domain; HPV, human papillomavirus; SMA, spinal muscular atrophy; SMN, survival motor neuron; TAD, transactivation domain.

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