Overexpressed human survival motor neurone isoforms, SMNΔexon7 and SMN+exon7, both form intranuclear gems but differ in cytoplasmic distribution

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Received 8 March 2001; accepted 15 March 2001

First published online 3 April 2001

Edited by Jesus Avila

Abstract Homozygous mutations of the telomeric survival motor neurone gene (SMN1) cause spinal muscular atrophy (SMA). The centromeric copy gene (SMN2) generally skips exon 7 during splicing and fails to compensate for SMN1 deficits, so SMA cells have reduced SMN protein and few nuclear gems. To investigate the role of exon 7 in SMN localisation, cDNAs for full-length SMN and SMNΔexon 7 were overexpressed in COS cells, neurones and SMA fibroblasts. Both constructs formed discrete intranuclear bodies colocalising with p80-coilin, but produced more cytoplasmic aggregates in cells overexpressing exon 7. Hence, the exon 7 domain enhances SMN aggregation but is not critical for gem formation.

Key words: Spinal muscular atrophy; Survival motor neuron; cDNA; Gem

1. Introduction

Spinal muscular atrophy (SMA) is caused by degeneration of spinal cord motor neurones leading to muscle denervation and atrophy. SMA is the second most common inherited lethal disorder of childhood and defined as types I–III on the basis of age at onset and disease severity [1–3]. The SMA locus on chromosome 5q13 comprises two survival motor neurone genes (SMN1 and SMN2) differing by just 11 nucleotides [4]. However, the critical nucleotide difference occurs within the exon 7 exonic splicing enhancer causing the copy gene, SMN2, to skip exon 7 in up to 90% of transcripts [5,6]. Over 95% of 5q-linked SMA patients are homozygously deleted for SMN1 or have undergone gene conversion at exons 7 or 8, while the remainder have missense, nonsense or splice site mutations [4,7–9]. SMN2 may be present in multiple copies and increasing copy number correlates with a milder phenotype in SMA. Indeed, one SMN1-deleted individual with eight SMN2 copies remained almost asymptomatic in his eighth decade [10]. Mice have only one SMN gene which, if deleted, is lethal early in embryogenesis [11]. Although this is rescued by expression of either human SMN1 or SMN2 in transgenic embryos, it remains unclear in the case of SMN2 whether amelioration of SMA is due to full-length SMN2 transcripts generating residual intact protein or to a partially deleted SMN protein derived from SMNΔ7 transcripts [12–15].

Although SMN is expressed in many tissues, levels are particularly high in motor neurones, the cells principally affected in SMA. In the nucleus, SMN is associated with spliceosomal proteins in aggregates known as gems, which may represent a site for snRNP processing or transcriptosome assembly [16,17]. These structures are closely associated with coiled bodies (often called Cajal bodies) and it is possible that gems and coiled bodies are identical. SMN is normally diffusely distributed in the cytoplasm and is found in dendritic and axonal processes of motor neurones [18–20].

In order to determine the influence of exon 7 on the subcellular localisation of SMN, cDNAs corresponding to the two principal SMN isoforms (SMN+exon7 and SMNΔexon7) were created and expressed as fusion proteins with green fluorescent protein (GFP) or c-myc tags to distinguish them from endogenous SMN. Expression of high levels of recombinant SMN in COS cells, rat primary cortical neurones and fibroblasts from SMA type II patients demonstrated that gem formation is not dependent on the exon 7 region of SMN and that SMNΔ7 was able to restore gems in fibroblasts from SMN1-deleted patients. However, differences between isoforms were observed in the cytoplasm.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma-Aldrich unless stated otherwise. All restriction enzymes were purchased from New England Biolabs.

2.2. Construction of cDNA vectors

A cDNA for the full coding region of human SMN1 (SMN+7) was generated from human cells by RT-PCR, cloned into the vector pGEM-T (Promega), then sub-cloned into pBluescript SK^+ (Strata-

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Abbreviations: SMA, spinal muscular atrophy; SMN, survival motor neurone; GFP, green fluorescent protein

0014-5793/01/$20.00
PII: S0014-5793(01)02330-4

gene) using *Sac*II and *Hin*dIII restriction sites. Exon 7 was deleted by PCR mutagenesis to create SMNΔ7 and the 3′ stop codon eliminated from both cDNAs to enable expression of 3′ fusion proteins. *Bgl*II/*Hin*dIII fragments of SMN cDNAs lacking a stop codon were cloned into pCMVTag1 (Stratagene) to produce a C-terminal c-myc tag and an N-terminal FLAG epitope. *Bgl*II/*Hin*dIII fragments of SMN cDNAs retaining the stop codon were cloned into the pEGFP-C2 vector (Clontech) to express GFP at the N-terminus. Each construct was sequenced using a di-rhodamine terminator cycle sequencing kit and an ABI 377 sequencer (PE Applied Biosystems). Large quantities of each of the vectors (c-myc-SMN+7, c-myc-SMNΔ7, GFP-SMN+7, GFP-SMNΔ7) were prepared by maxiprep columns (Qiagen) for use in transfections (Fig. 1A).

2.3. Cell culture and transfections

The simian kidney cell line COS-7 was grown in Dulbecco's modified Eagle's medium (DMEM)/10% foetal calf serum (FCS)/2 mM L-glutamine (Life Technologies) with antibiotic/antimycotic. Cells were plated on glass coverslips coated with 2% gelatin at 1×10^5 cells/well and transfected 24 h later using the cationic lipid reagent DOSPER (1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamide; Roche Diagnostics). Lipoplexes were formed by diluting 0.25 μg plasmid DNA in HEPES-buffered saline and mixing with DOSPER in a ratio (w:w) of 5:1 to a final volume of 5 μl then added to wells containing 0.5 ml serum-free DMEM. After 4 h, this medium was replaced by growth medium and cells incubated over a 6–40 h time course before analysis.

Primary cortical neurone cultures were established from E16 rat embryos. The cerebral cortex was dissected free from the meninges, mechanically and enzymatically dissociated before being plated out onto glass coverslips coated with poly-D-lysine and laminin. They were grown in Neurobasal medium with B27 supplement containing 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all Life Technologies). After 7 days the cells were almost exclusively neurones, staining positively for antibodies to neurofilaments and negative for glial fibrillary acidic protein (Dako). Cultures were transfected by calcium phosphate precipitation (Promega Profection kit) using 10 μg of plasmid DNA per well [21].

Human fibroblasts were obtained from two independent patients with SMA type II at the time of spinal surgery and cultured in DMEM/20% FCS. These cells were transfected with GFP-SMN+7 and GFP-SMNΔ7 in DOSPER lipoplex complexes as above.

2.4. SDS–PAGE and Western blotting

Twenty-four hours post-transfection, COS cells were rinsed twice in PBS and centrifuged at 13 000 rpm. Cell pellets were resuspended in 50 μl SDS sample buffer (60 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min. Lysates were run on a 10% polyacrylamide gel for 1.5 h at 120 V alongside a full-range Rainbow™ marker (Amersham Pharmacia) using the Bio-Rad Protean II system. Bands were then transferred to a nitrocellulose filter at 100 V for 1 h. Non-specific antibody binding was blocked by a 1 h incubation with 5% non-fat dried milk in Tris-buffered saline containing 1% Tween 20 (TBS-T) then incubated overnight at 4°C with a mouse monoclonal anti-SMN antibody (Transduction Laboratories), diluted 1:5000 in TBS-T/2% bovine serum albumin/2% horse serum. After further washes in TBS-T and a 5 min block in TBS-T with 0.15% non-fat dried milk, the blot was incubated for 1 h with a horseradish peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia; diluted 1:100) followed by three 10 min washes in TBS-T. Bound antibody was detected using a chemiluminescence system (Renaissance™ Western blot chemiluminescence reagent, NEN Life Science Products).

2.5. Immunocytochemistry

Cells were fixed for 5 min in 100% methanol at −20°C, permeabilised in PBS containing 1% NP40 and incubated for 1 h at room temperature with primary antibodies to: (a) SMN exon 2 (mouse monoclonal MANSMA1, a kind gift from Prof. Glenn Morris, North East Wales Institute; 1:25); (b) c-myc (rabbit polyclonal primary antibody A14 sc-789; Santa Cruz Biotechnology; 1:200); (c) p80-coilin (rabbit polyclonal anti-p80 coilin, a kind gift from Prof. Angus Lamond, University of Dundee; 1:350). Appropriate secondary antibodies used were: biotinylated anti-mouse or anti-rabbit IgG (Amersham-Pharmacia, 1:400), FITC-conjugated anti-rabbit IgG (Dako; 1:75), and streptavidin–Texas red for tertiary detection (Amersham-Pharmacia, 1:400). In all cases, specificity of staining was confirmed by omission of the primary antibody in controls. Stained cells were mounted in fluorescence mounting medium (Dako) with 100 ng/ml DAPI, visualised using a Zeiss Axioplan fluorescence microscope, and photographed using a CCD digital camera with Smartcapture™ software.

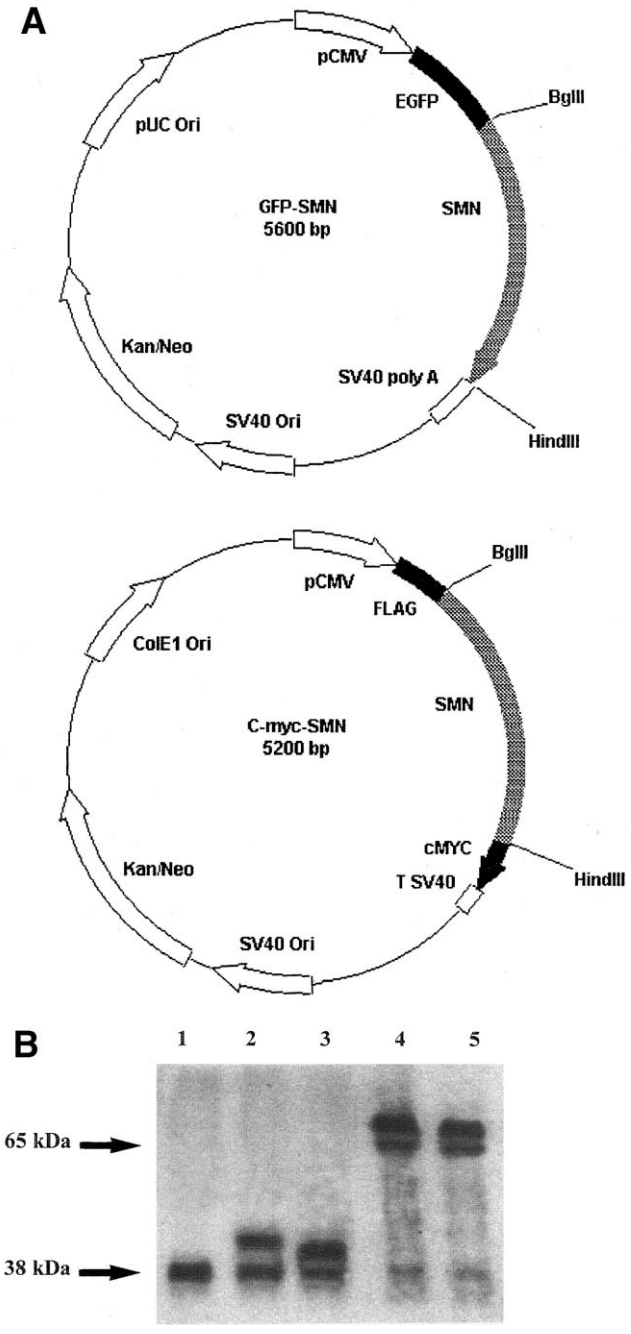

Fig. 1. A: Design of expression vectors for expression of SMN+7 or SMNΔ7 cDNAs fused to either GFP or c-myc tags. B: Detection of SMN in transfected COS cells by Western blot. Untransfected cells express endogenous SMN (lane 1); cells transfected with c-myc-SMN+7 (lane 2) and c-myc-SMNΔ7 (lane 3) showed a shift in molecular weight in the upper band corresponding to loss of the exon 7 domain; cells transfected with GFP-SMN+7 (lane 4) or GFP-SMNΔ7 (lane 5) generated doublets showing a reduction in size in the absence of the exon 7 domain.

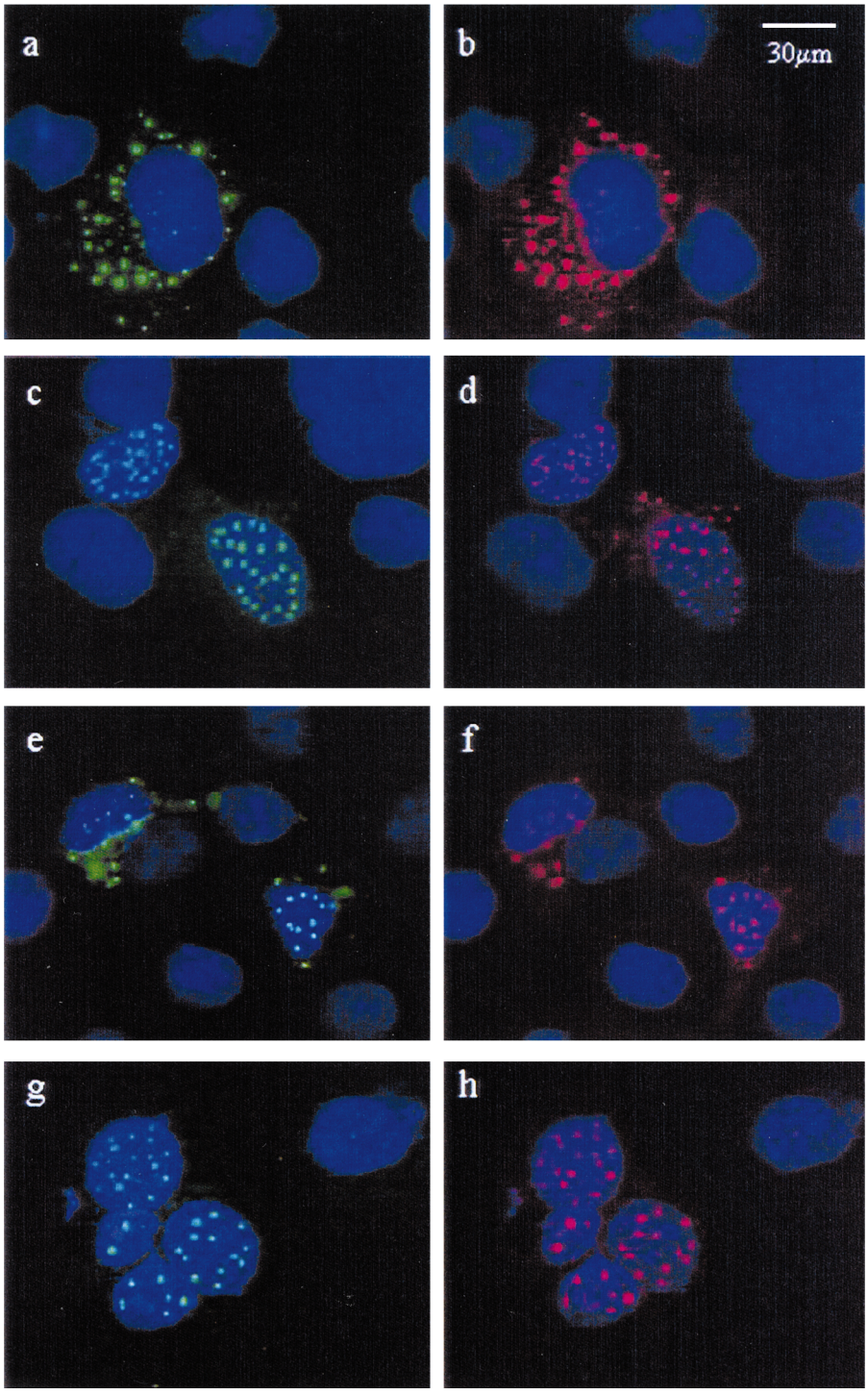

Fig. 2. Subcellular localisation of recombinant SMN in COS cells. High levels of recombinant SMN were detected by anti-SMN antibody MANSMA1 relative to adjacent untransfected cells (b, d, f, h) and colocalised with their GFP (a, c) or c-myc (e, g) tags. The GFP tag appeared to inhibit intranuclear localisation of SMN+7 (a, b). Greater numbers of intranuclear aggregates were observed in cells expressing SMNΔ7 (c, d, g, h) relative to cells expressing full-length SMN (a, b, e, f), which expressed more cytoplasmic SMN. Magnification ×300.

2.6. Quantification of SMN expression over time

Aggregates of recombinant SMN were counted according to GFP or c-myc staining in the nucleus and cytoplasm, after set time intervals ranging from 6 to 40 h. Twenty transfected cells were counted on each coverslip and three to six coverslips were analysed per vector at each time point. The mean numbers of aggregates in transfected cells were calculated and analysed by Student's *t*-test (±S.E.M., Fig. 4).

3. Results

3.1. Expression of SMN fusion proteins in COS-7 cells

All SMN constructs were transcribed from a CMV promoter and contained an SV40 origin of replication, giving relatively high expression levels in transfected COS cells

(Fig. 1A). Western blots probed with anti-SMN antibody detected a prominent band at 44 kDa in cells transfected with pCMVTag/SMN+7 and a slightly smaller band in cells transfected with pCMVTag/SMNΔ7 (Fig. 1B). Samples transfected with pGFP-SMN+7 and pGFP-SMNΔ7 showed doublets at approximately 70 and 65 kDa respectively, probably reflecting conformational isoforms of the SMN-GFP fusion proteins. Untransfected COS cell lysates revealed a band at approximately 38 kDa, representing endogenous SMN. This band was also present in CMVTag-SMN and GFP-SMN lanes, although the latter is unseen in Fig. 1B, as a greater dilution of GFP-SMN lysates was employed to match exposures across samples.

In COS cells transfected with GFP constructs, colocalisation of SMN and GFP expression (Fig. 2a–d) showed that both full-length and Δ7 versions of SMN fused to GFP were able to form intranuclear aggregates. Interestingly, a greater number of nuclear aggregates were observed in cells transfected with GFP-SMNΔ7 than in cells expressing GFP-SMN+7 (26.7 ± 1.2/cell for GFP-SMNΔ7; 4.7 ± 1.2/cell for GFP-SMN+7; t-test, $P < 0.01$), although overall expression levels were similar. In order to test if this was an effect of the 27 kDa GFP moiety, cells were also transfected with SMNΔ7 and SMN+7 linked to a much smaller (10 amino acid) c-myc tag (Fig. 2e–h). Expression of c-myc-SMN+7 restored large numbers of SMN aggregates to the nucleus, suggesting that the GFP moiety may inhibit intranuclear transport. Recombinant SMN proteins were also observed in the cytoplasm both diffusely and in large cytoplasmic aggregates. At 24 h post-transfection, the number of cytoplasmic aggregates was significantly greater in cells transfected with GFP-SMN+7 and c-myc-SMN+7 (Fig. 2a,e; 46.9 ± 3.2 and 29.5 ± 0.5 respectively) relative to those expressing GFP-SMNΔ7 and c-myc-SMNΔ7 (Fig. 2c,g; 3.9 ± 0.6 and 3 ± 0.7 respectively). Cells transfected with GFP constructs lacking SMN showed only diffuse cytoplasmic and nuclear fluorescence (data not shown). Only faint cytoplasmic staining for SMN and few gems were observed in untransfected COS cells.

3.2. *Colocalisation of recombinant SMN with p80-coilin: evidence of gem formation*

COS cells transfected with either GFP-SMN+7 or GFP-SMNΔ7 and immunolabelled with p80-coilin antibody demonstrated a striking colocalisation typical of gems (Fig. 3). Interestingly, the number of p80-positive aggregates was higher in cells transfected with SMN (Fig. 3C) compared to un-

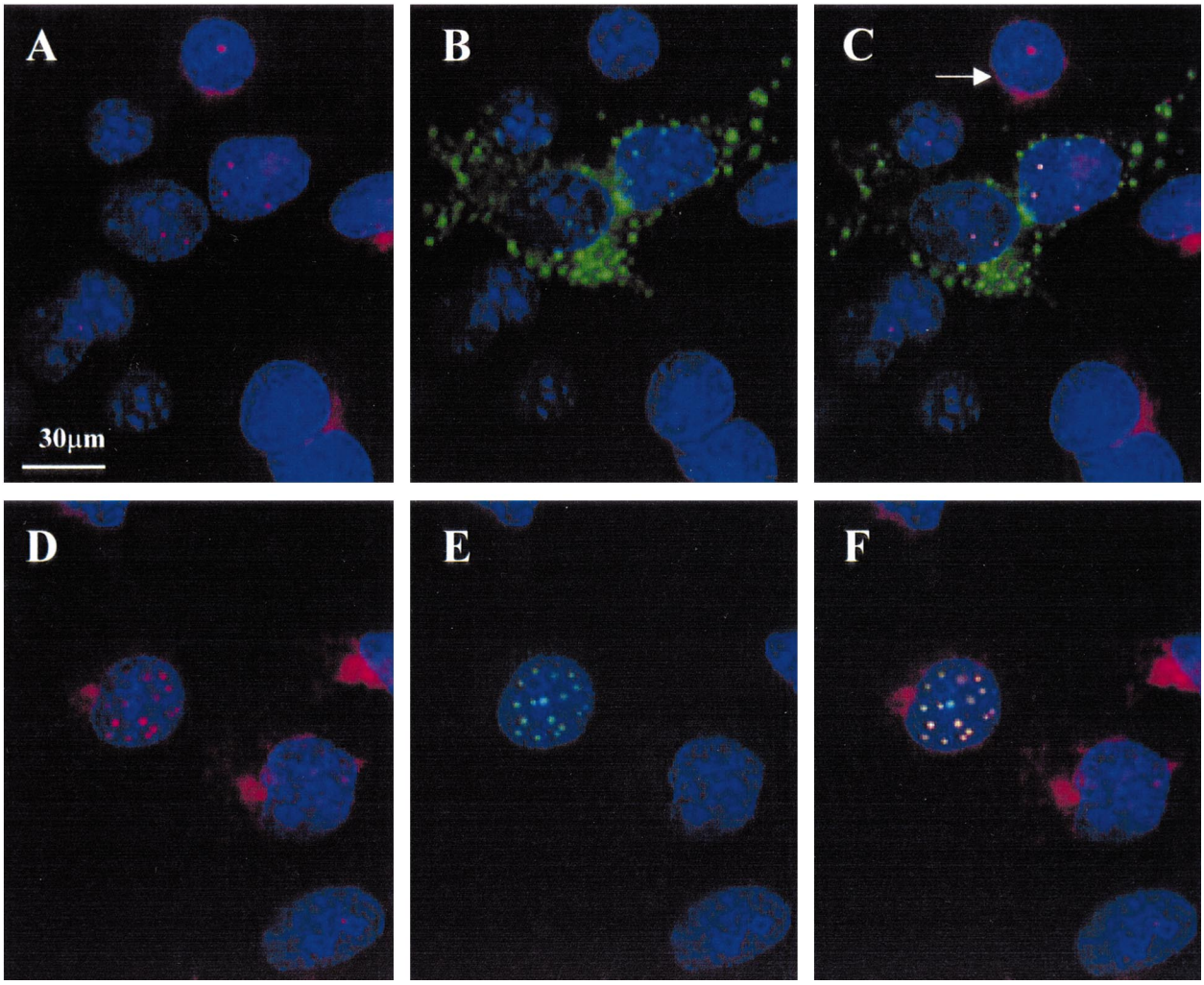

Fig. 3. Colocalisation of GFP-SMN and p80-coilin in transfected nuclei. Cells transfected with either GFP-SMN+7 (A–C) or GFP-SMNΔ7 (D–F) were immunostained for p80-coilin (A, D). Rhodamine and green fluorescence images were merged (C, F) to show intranuclear colocalisation (yellow). Coilin staining in an untransfected cell is arrowed. Magnification ×270.

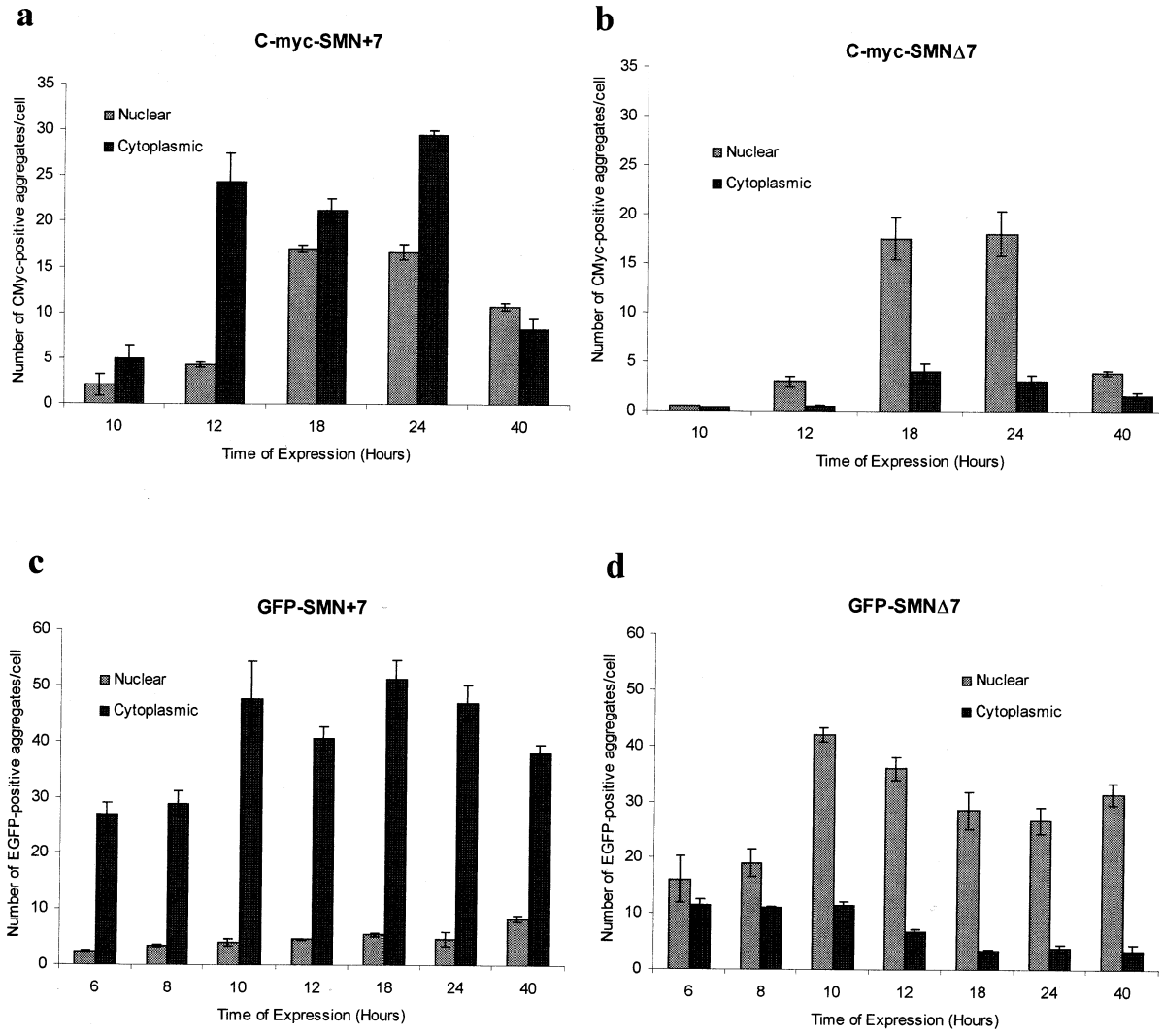

Fig. 4. Time course of recombinant SMN expression in COS cells. Cells transfected with each construct were fixed at time points from 6 to 40 h and examined for green fluorescence or c-myc immunofluorescence. Expression is represented as number of GFP- or c-myc-positive aggregates per cell (nuclear or cytoplasmic). Error bars indicate S.E.M.

transfected cells (Fig. 3C arrowed). This supports the idea that gems and coiled bodies are associated structures and increased expression of one component (SMN) leads to a corresponding increase in p80-coilin aggregation [17]. Consistent with some other studies, occasional aggregates that were SMN-positive but lacked coilin were observed, suggesting that coiled bodies and gems are not identical [17].

3.3. Expression of recombinant SMN in transfected COS-7 cells over time

To further investigate observed differences in the expression of GFP-SMN and c-myc-SMN, a time course study was performed for each of the four vectors (Fig. 4). GFP-SMN was evident 6 h after transfection while c-myc-SMN was not observed before 10 h. No differences were seen between the onset of expression of SMN+7 and SMNΔ7 for either vector. Peak expression of both vectors occurred between 12 and 24 h post-transfection.

Nuclear and cytoplasmic aggregates appeared concurrently in all transfected cells. In cells expressing c-myc-SMN the numbers of intranuclear aggregates were equivalent for SMN+7 and SMNΔ7 proteins at each time point (17.1 ± 1.3 and 17.6 ± 2.1 respectively; t-test, $P > 0.8$; Fig. 4a,b: 18 h time point) indicating that nuclear targeting is not dependent on the exon-7-derived region. However, c-myc-SMN+7-transfected cells had significantly larger numbers of cytoplasmic aggregates than c-myc-SMNΔ7-transfected cells (21.3 ± 1.3 compared to 4 ± 0.9; t-test, $P < 0.05$; Fig. 4a,b: 18 h time point) providing evidence that the exon-7-derived region promotes self-association. Consistent with this, cells expressing GFP-SMN+7 demonstrated high levels of cytoplasmic expression relative to GFP-SMNΔ7 (Fig. 4c,d). A slight decrease in the number of cytoplasmic aggregates over time can be seen with both GFP-linked proteins, probably reflecting loss of episomal plasmid DNA.

3.4. SMN+7 and SMNΔ7 proteins form gems in cultured primary neurones

To examine the subcellular expression of SMN in a cell type relevant to SMA, GFP-SMN constructs were transfected into

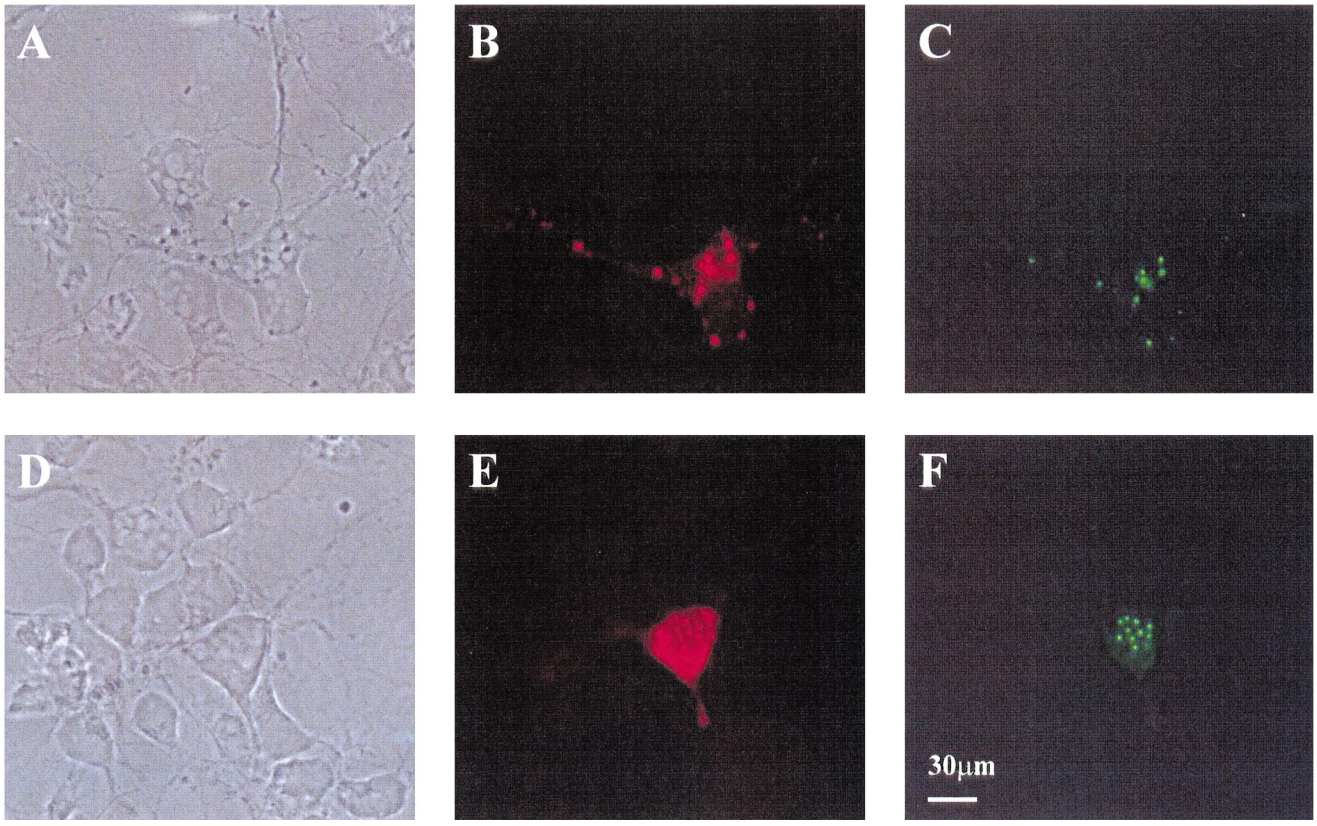

Fig. 5. Expression of GFP-SMN in rat cortical neurone cultures. Phase contrast (A, D), SMN immunostaining (B, E) and green fluorescence images (C, F) are shown. GFP-SMN+7 was mainly localised in the cytoplasm and dendrites (B, C) whereas SMNΔ7 was present in the nucleus and cytoplasm (E, F). Magnification ×228.

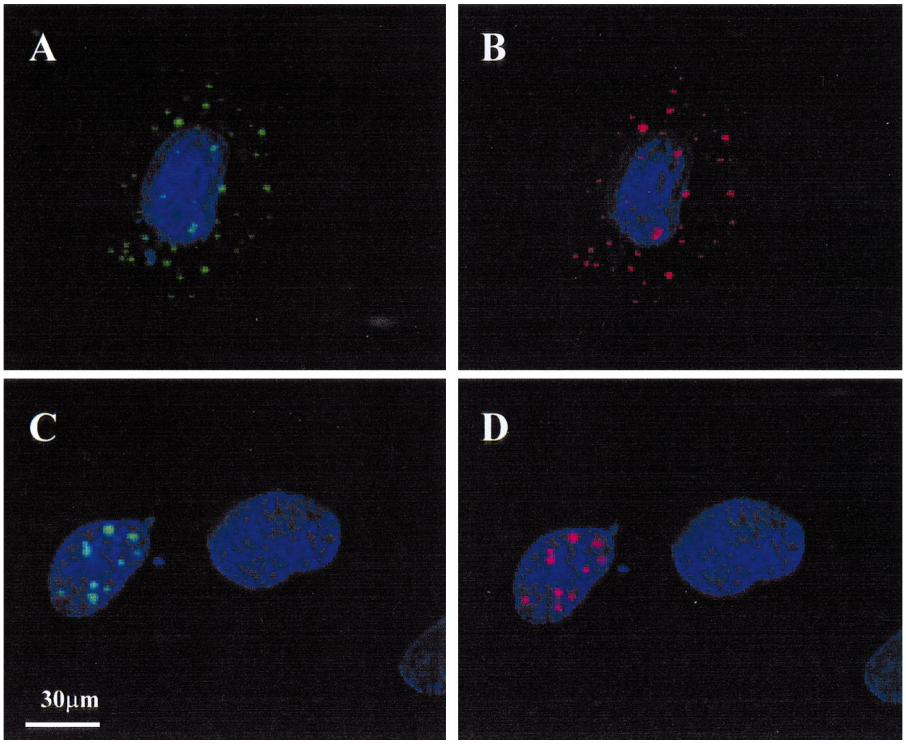

Fig. 6. Expression of GFP-SMN in fibroblasts from SMA type II patients. Green fluorescence (A, C) or immunofluorescence (B, D) for recombinant SMN showed cytoplasmic and intranuclear aggregates in cells transfected with GFP-SMN+7 (A, B) and mainly intranuclear aggregates in cells transfected with GFP-SMNΔ7 (C, D). Magnification ×228.

primary rat cortical neurones (Fig. 5). In confirmation of our findings in COS cells the relative differences in nuclear aggregate formation between SMN+7 and SMNΔ7 were also observed in these cells (Fig. 5C,F). Normally, rodents express very little SMN in the cytoplasm, but cytoplasmic aggregates of GFP-SMN+7 were found throughout the cell, including along the dendrites and axon (Fig. 5B) as previously described in vivo [18,20]. Indeed, while GFP-SMN+7 formed aggregates preferentially in the cytoplasm, with a few visible nuclear gems (Fig. 5B,C), GFP-SMNΔ7 formed numerous gems and a more diffuse cytoplasmic expression pattern (Fig. 5E,F).

3.5. SMN+7 and SMNΔ7 restore gems in the nuclei of cells from SMA patients

Fibroblast cultures were obtained from skin biopsies of two patients with SMA type II and genetically confirmed absence of SMN1. These cells were transfected with GFP-SMN+7 and GFP-SMNΔ7 and immunolabelled for SMN 24 h later, when they showed a substantial increase in nuclear gems relative to untransfected cells (Fig. 6C,D). Consistent with earlier observations in COS cells and cortical neurones, both GFP-SMN+7 (Fig. 6A,B) and GFP-SMNΔ7 (Fig. 6C,D) constructs generated more nuclear gems in GFP-SMNΔ7-transfected cells and large numbers of cytoplasmic aggregates in cells transfected with GFP-SMN+7 (Fig. 6A,B).

4. Discussion

SMA is caused by motor neuronal dysfunction and cell death associated with a critical reduction in SMN protein levels. The precise function of SMN has not been identified, but it is believed to play a part in the transport of spliceosome components between the cytoplasm and nucleus [24,25]. SMN and associated spliceosome components, such as hnRNP U, are concentrated in intranuclear gems, which may be involved in snRNP processing or transcriptosome assembly. Gems are significantly reduced in number in SMA patients and are virtually absent from cells of transgenic mice in which exon 7 has been removed by gene targeting [12]. SMA patients normally express low levels of SMN, but this has previously been thought to arise from the minority of SMN2-derived transcripts that retain exon 7 after splicing, although this has never been unequivocally demonstrated. Several studies have evaluated endogenous SMN expression in a variety of animal and human tissues, demonstrating that endogenous levels of SMNΔ7 are insufficient to: (a) form gems and (b) support motor neurone survival [17,19,22,23]. Here, however, we have overexpressed recombinant SMN molecules with or without the exon 7 region in COS cells, primary neurones, and fibroblasts from SMA type II patients in order to evaluate the effect of high levels of different SMN isoforms on subcellular expression patterns.

Our results demonstrate that recombinant SMN fused to GFP or c-myc epitopes can form cytoplasmic and nuclear aggregates which mirror the pattern of endogenous SMN. Colocalisation with p80-coilin in the nucleus confirmed that our recombinant SMN proteins formed structures consistent with gems. In concordance with others, an increase in SMN was associated with an increase in p80-coilin bodies, again indicating that the recombinant versions of SMN expressed in this study reproduce the behaviour of native protein [17]. Counter to expectation, we found that SMN lacking the exon 7 domain is capable of entering the nucleus and forming gems when expressed at high levels. SMA patients with more than two copies of SMN2 generally develop a milder phenotype [10] and the lethal phenotype of SMN knockout mice is rescued by multiple copies of human SMN2 [15]. While this may be due to higher levels of full-length SMN with increased SMN copy number, it may suggest that SMNΔ7 protein is also capable of ameliorating SMN deficiency. Frugier et al. [12] showed a lack of gems in mice expressing only SMNΔ7 in their motor neurones and suggested that this was due to an inherent defect in nuclear targeting. This may be the case, but these animals expressed SMN2 only at relatively low levels. In future, expression of varying levels of recombinant SMN using a less powerful or inducible promoter system may identify the minimum level of SMN required for gem formation.

Importantly, our observation of large numbers of recombinant gems in transfected nuclei from rat neurones and SMA patients demonstrates that normal expression patterns can be restored to cells where endogenous SMN levels are much reduced. Furthermore, we have provided evidence that recombinant SMNΔ7 associates with p80-coilin in a similar way to endogenous SMN. This shows that the trafficking of SMNΔ7 to the nucleus can occur and is not dependent on the presence of SMN1, as supported by others [26]. Consistent with our observations that intranuclear gems appear to accumulate more readily with high levels of SMNΔ7, it is possible that exon 7 may encode a nuclear export signal such that SMN+7 protein is in dynamic equilibrium between intranuclear gems and transport into the cytoplasm. SMNΔ7 on the other hand is capable of entering the nucleus but may be poorly exported into the cytoplasm.

While much attention has been given to the presence or absence of gems and their composition, many tissues expressing SMN do not have gems suggesting that SMN also has a significant function in the cytoplasm [17]. Moreover, in human motor neurone cell bodies, staining in perikarya and dendrites is a prominent feature [18]. Here we also observed recombinant SMN in dendritic processes of transfected neurones. Our observed differences in cytoplasmic localisation of the two isoforms may be consistent with differences in their ability to form oligomers via the exon 7 domain, a feature that may influence disease severity in SMA [27]. Poorer oligomerisation by recombinant SMNΔ7 may have led to fewer cytoplasmic aggregates (Fig. 4). More recently, the domain encoded by exon 2b has also been shown to mediate self-association, perhaps aiding SMN binding to SIP1 [28].

In summary, we have shown that both full-length and exon 7-deficient SMN proteins can form intranuclear gems when expressed at sufficient levels and that these molecules reproduce subcellular localisation patterns similar to endogenous SMN in neuronal cultures and cells from SMA patients. Our observations on the subcellular localisation of recombinant SMN support the value of exploring gene transfer as a therapeutic strategy in SMA.

Acknowledgements: The authors are very grateful to Professors Glenn Morris and Angus Lamond for supplying antibodies. We are also grateful to Dr Payam Mohaghegh and Professor Kay Davies for providing SMN cDNA as well as Steve Ackerley, Dr Andrew Grierson and Dr Chris Miller for help with cloning and neuronal cultures. This work was supported by the Special Trustees for Guy's and St Thomas' Hospital, London, and Families of SMA (USA).

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