Identification of a novel cyclic AMP response element (CRE-II) and the role of CREB-1 in the cAMP-induced expression of the survival motor neuron (SMN) gene

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Abbreviations: CRE, cAMP response element; IVGF, In vivo genomic footprinting; STAT1, Signal transducer and activator of transcription1.
ABSTRACT

Spinal muscular atrophy (SMA), an autosomal recessive disorder is caused by loss of the SMN1 (survival motor neuron) gene while retaining the SMN2 gene. SMN1 produces a majority of full length SMN transcript, whereas SMN2 generates mostly an isoform lacking exon7. Here, we demonstrate a novel cAMP Response Element, CRE-II, in the SMN promoter that interacts with the CREB (cAMP Response Element Binding) family of proteins. In vitro DNase I protection analysis and in vivo genomic footprinting of the SMN promoter using the brain and liver nuclei from SMN2 transgenic mice revealed footprinting at the CRE-II site. Site-directed mutation of the CRE-II element caused a marked reduction in the SMN promoter activity as revealed by transient transfection assay. Activation of the cAMP pathway by dibutyryl cAMP (0.5mM) alone or in combination with forskolin (20µM) caused 2 to 5-fold increase in the SMN promoter activity, but had no effect on the CRE-II mutated promoter. Electrophoretic mobility shift assay and UV-induced DNA-protein cross-linking experiment confirmed that CREB1 binds specifically to the CRE-II site. Transient overexpression of CREB1 protein resulted in 4-fold increase of the SMN promoter activity. Intraperitoneal injection of epinephrine in mice expressing two copies of the human SMN2 gene resulted in a two-fold increase in full length SMN transcript in the liver. Combined treatment with dibutyryl cAMP and forskolin significantly increased the level of both the full length and exon 7-deleted SMN (exonΔ7SMN) transcript in primary hepatocytes from mice expressing two copies of human SMN2 gene. Similar treatments of type I SMA mouse and human fibroblasts as well as HeLa cells resulted in augmented level of SMN transcript. These findings suggest that the CRE-II site in SMN promoter positively regulates the expression of SMN gene, and treatment with cAMP elevating agents increase expression of both the full length and exonΔ7SMN transcript.
INTRODUCTION

Proximal spinal muscular atrophy (SMA) is a common autosomal recessive disorder characterized by loss of motor neurons in the spinal cord (1). SMA occurs with a frequency of 1 in 10,000 live births with a carrier frequency of 1/50 (2,3) and is the leading genetic cause of infant mortality (4). Based on age of onset and severity of the disease, SMA patients are often classified as type I, II or III (5). All three forms of SMA are caused by loss or mutation of the telomeric survival motor neuron gene (SMN1) but the centromeric survival motor neuron gene (SMN2) is retained (6-10). The SMN1 and SMN2 gene differ functionally by a single nucleotide change in exon 7 that does not alter an encoded amino acid but does alter the activity of an exon splice enhancer (11-13). Thus SMN1 produces a majority of full length SMN transcript, whereas SMN2 generates mostly an isoforms lacking exon7. The protein product of the Δ7 transcript is thought to be unstable and rapidly degraded (14,15). SMA patients who lack SMN1 but carry varying copies of SMN2 do not produce sufficient SMN for motor neuron survival. There is a tight correlation between clinical severity of SMA, SMN2 copy number and the SMN protein level (2,16,17).

The 38kDa SMN protein is ubiquitously expressed (16-18) and often localizes in the nuclei as dot like structures termed gems (18,19). SMN is important in snRNP biogenesis (20-24) and has been shown to bind a series of other protein partners (25,26) However, it is not understood which of SMNs function is critical specifically for motor neurons. Consistent with SMNs housekeeping functions, Smn knockout mice are embryonic lethal (27). An animal model of SMA was created by introducing the SMN2 gene into Smn<sup>−/−</sup> mice (28,29). Introduction of one or two copies of SMN2 gene in Smn<sup>−/−</sup> mice exhibit a type I SMA phenotype whereas 8-16 copies of SMN2 completely ameliorates the disease phenotype (29). The presence of SMN2 in all SMA patients, the ability of more copies of SMN2 to modify the SMA
phenotype and the rescue of SMA mice by multiple copies of SMN2 makes it an attractive therapeutic candidate. Molecules capable of inducing SMN2 expression (30,31) or altering the splicing of SMN2 such that more full length SMN transcript is produced have been identified (32-37). At the present time there is limited information on the mode of action of these compounds as well as the protein complexes that interact with the SMN promoter. In this study we identified two CRE sites in the SMN promoter, demonstrated that the CREB-1 protein binds to the CRE II site and showed that cAMP stimulating agents activate SMN expression.

MATERIALS AND METHODS

Cell culture and treatment

Cultures of mouse motor neuron cells EHMN (embryonic hybrid motor neuron) (38) and HeLa cells were maintained in Dulbecco’s modified Eagle medium DMEM) containing 10% heat inactivated fetal calf serum (Atlas Biologicals, CO). Human SMA type fibroblasts 3813 and mouse SMA fibroblasts were grown in DMEM medium containing 10% fetal calf serum and 2mM glutamine. All the cell cultures contained penicillin/streptomycin and were incubated at 37°C in a 5% CO2 humidified atmosphere. For all the experiments, the cells were plated the day preceding treatment with cAMP elevators, bt2cAMP and forskolin and harvested at indicated time.
**Preparation of nuclear extract**

The nuclei were isolated from HeLa and EHMN cells and nuclear extracts were prepared in buffer containing 0.35 M KCl following the protocol of (39). The protein concentration in the nuclear extracts was measured with Bio-Rad reagent according to Bradford’s method using bovine serum albumin as standard.

**In vivo genomic foot printing**

*In vivo* genomic foot printing of the human SMN promoter was performed as described (40,41). The human *SMN* promoter was amplified by ligation-mediated PCR (LM-PCR) according to the procedure of Mueller and Wold (42). Briefly, intact nuclei isolated (43) from the brain and liver of *Smn<sup>−/−</sup>* mice with eight copies of the human *SMN2* gene (29) were exposed to limited dimethyl sulfate treatment (1 µl/ml, 2 min at room temperature) in phosphate buffered saline pH 7.4. The genomic DNA was isolated from the cells, purified and subjected to piperidine cleavage (10%) at 90°C for 30 min. The purified cleaved DNA (2 µg) was then subjected to LM-PCR (Ligation-Mediated) to amplify *SMN* promoters. The following primers were used to amplify the region between +210 to +283 of the *SMN* promoter:

SMN/5′-1: 5′-AACACAGTGAAATGAAAGGATTGAG-3′

SMN/5′-2: 5′-GATAACCACTCGTAGAAAGCGTGAG-3′

SMN/5′-3: 5′-CCACTCGTAGAAAGCGTGAGAAGTTACTAC-3′

The annealing temperature for this set of primers were 58.8°C, 60.6°C and 63.5°C respectively.

The following primers were used to amplify the region between -312 to -443 of the *SMN* promoter:

SMN/3′-1: 5′-TGTGTGTAGATATTTATCCCCCTC-3′
SMN/3′-2: 5′-TATCCCCCTCCCCCTTG-3′
SMN/3′-3: 5′-CCCCCTCCCCCTTGGAAGAAG-3′

The annealing temperature for the 3′-primers were 57.6°C, 60°C and 66.6°C, respectively.

**In vitro** DNase I foot printing Analysis

In order to generate the labeled probe for *in vitro* DNase I foot printing; the plasmid p750 (44) was digested with Hind III. To label the lower strand the Hind III fragment was end labeled with (γ-32p) ATP. To label the upper strand the Hind III fragment was filled in using klenow in the presence of (α-32p)dGTP. The 32p- labeled p750 linear DNA was digested with Pst I and the probes were gel purified for DNase I footprinting assays. To perform the binding reaction 25 - 75µg of HeLa nuclear extract was added to 40µl of the reaction buffer (48mM Hepes, pH 7.9, 240mM KCl, 2mM DTT, 48% glycerol and 20mM MgCl₂) on ice. The binding was initiated by the addition of 1µl probe containing approximately 20,000 cpm and was incubated at room temperature for 40 min. For competition experiments unlabeled Hind III/Pst I fragment at the concentrations of 50X and 100X were added to the reaction mixture prior to addition of the probe. The DNA protein complexes were then subjected to DNase I digestion at room temperature for 2 min with optimum amount of DNase I to generate a ladder both in the presence and absence of binding protein. The DNase I digestion was terminated by the addition of 50µl stop buffer containing 100mM Tris pH 8.0, 600mM NaCl, 50mM EDTA, 1% SDS, and proteinase K (0.4mg/ml). Samples were then incubated at 37°C for 30 min for proteinase K digestion, phenol-extracted, and ethanol precipitated. Labeled coding and non-coding strands were chemically sequenced (45) to generate combined purine (A+G) ladder, which were separated alongside the DNase I treated samples on a 6% sequencing gel. Gels were dried and exposed to X-ray film at –80°C.
Overexpression of CREB and Western Blot Analysis

For Western blot analysis of CREB, whole cell extracts from cells overexpressing CREB-1 protein were resolved by SDS-PAGE and transferred to ECL membrane (Amersham Biosciences). The membrane was blocked in 0.05% TBST (0.05% Tween-20 in Tris buffered saline, pH 7.5) containing 5% milk, followed by incubation with human anti-mouse CREB/ATF-1 IgG (1:500 dilution) (Santa Cruz Biotechnology, Inc.) in the blocking buffer for 1 h at room temperature. After incubation with anti-mouse IgG-peroxidase conjugate (1:5000 dilution) overexpression of CREB was confirmed with ECL-TM Western Blot detection reagents (Amersham Biosciences) following the manufacturer's protocol.

Electrophoretic mobility shift assay

Nuclear extracts used for the DNA binding activities of CREB family of proteins were prepared as described (39). A typical binding reaction contained 5µg of HeLa or 10µg of EHMN nuclear extract, 0.1pmole labeled DNA, 2µg E.coli DNA and 5X Ficoll binding buffer (50mM Tris-HCL pH 7.5, 5mM EDTA, 20% Ficoll, 5mMDTT, 375mM KCl) in a final volume of 20µl. The binding reaction was initiated by the addition of 1µl of the reaction buffer containing approximately 50,000 cpm of end-labeled double stranded oligonucleotide and incubated at room temperature for 30 min. ATF-1 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) or excess double stranded oligonucleotides CRE-II wild type (5′-GGCGGCGGAAGTCGTTCACTCTTAAAGAAGG-3′), mutated CRE-II (5′-GGCGGCGGAAGTGCTGTCACTCTTAAAGAAGG-3′) and the CREB consensus (5′-AGAGATTGGCCTGACGTCAGAGAGCTAG-3′) were added to the reaction buffer 30 min prior to the addition of the labeled oligonucleotide as indicated. Samples were then chilled on ice, and the entire volume was loaded onto a 5% polyacrylamide gel containing 0.5X TBE and electrophoresed at 4°C.
UV crosslinking and SDS-PAGE

UV-crosslinking and identification of the DNA binding protein was performed according to the published protocol (46,47). For this purpose the CRE-II oligonucleotide (5′-GGCGGCGGAAGTCGTCACTCTTAAGAAGG-3′) was annealed with a 10bp oligo (5′-CCTTCTTAAG-3′) and internally labeled using klenow (fill in reaction) in presence of α-32p-dCTP. The labeled probe was purified on a sephadex G-50 spin column to remove unincorporated nucleotides. Binding reactions were performed as described for EMSA using EHMN nuclear extracts and 0.05 pmole labeled oligonucleotide in a final volume of 80µl (4X reactions). The entire reaction mixture was separated on a 5% acrylamide gel in 0.5X TBE. The wet gel was exposed to a short wave UV light from a distance of 2-3cm at 4°C for 30 min. The gel was then exposed overnight to X-ray film to locate the complexes. The region of the gel containing the desired complexes were excised, and eluted overnight at room temperature in the elution buffer (0.5mM ammonium acetate, 5mmDTT, 1mM EDTA pH 8.0, 0.1% SDS). The eluted proteins were precipitated with two volumes of ethanol, washed with 70% ethanol and were separated by SDS-PAGE. The labeled proteins were visualized by autoradiogram.

Site-directed mutagenesis

Site-directed mutation at CRE-II site was introduced into the p750 by overlap extension PCR (48,49). Plasmid p750 (44) contains –450bp to +300bp of the SMN2 gene in pGL3-basic vector (Promega). The primers used for mutagenesis are as follows: mut CRE-II oligo-F: 5′-GGCGGGAAGTCGTGTCTCTTAAGAAGG-3′; mut CRE-II oligo-R: 5′-CCTTCTTAAGAGACACGACTTCCGCC-3′; SstI primer: 5′-TCGCTTGAGCTCTGGAGGTCGAGGCTG-3′ and NcoI primer: 5′-
TTACCCATGGAGGCTTTACC AACAGTACCG-3’. Two sets of PCR reactions were run using the mut CRE-II oligo-R / SstI primer, and mut CRE-II oligo-F / NcoI primer pairs. The second PCR reaction was carried out using the gel-purified PCR products from the first set of PCR as templates and SstI primer/NcoI primer. The condition for the PCR was 94°C for 5 min, and then 30 cycles of 94°C for 1 min, 64°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The final PCR product was gel purified, blunt-ended with pfu polymerase, digested with Sst I and Nco I and cloned back into pGL3-Basic (Promega) vector to produce p750mCRE. Mutation was confirmed by sequence analysis.

**Transient transfection assay**

EHMN or HeLa cells were seeded onto 6 well plates 24h prior to transfection. Cells were transfected with 0.150µg (for EHMN cells) and 0.500µg (for HeLa cells) p750 DNA or p750mCRE, using 2µl LipofectAMINE (Life Technologies, Inc.) according to manufacturer’s protocol. For overexpression studies, 2µg of pSG-RSVCREB plasmid (a gift from Dr. Tsonwin Hai, Ohio State University) or the corresponding empty vector DNA was transfected along with 0.5µg p750. Cells were harvested 48hr after transfection in lysis buffer and luciferase activity was assayed using the Dual luciferase assay kit (Promega, Madison, WI). Normally each transfection assay cocktail consists of 2.5µg DNA along with the reporter plasmid RLTK (renilla luciferase gene with thymidine kinase promoter, Promega, Madison, WI) used as an internal control. To see the effect of cAMP on the p750 promoter, HeLa cells were transfected with p750 in 100mm dishes, cells were split after 6hrs of transfection and seeded in to 6 well plates. After 24hrs of transfection cells were treated with 0.5mM bt2cAMP or 20µM forskolin as
indicated. Cells were then harvested after 24hrs of treatment and assayed for luciferase activity as described previously.

**In vivo treatment of animals and hepatocytes:**

*Smn*+/− mice expressing two copies of human *SMN2* gene (29) received intraperitoneal injection of epinephrine (2mg/kg body weight) every 2 hours for six hours and were sacrificed 2 hours after the last injection. The mice were sacrificed by cervical dislocation and the livers were snap frozen in liquid nitrogen for RNA isolation.

Primary mouse hepatocytes were isolated as described by Matsuda et.al. (50), washed and resuspended in Dulbecco’s modified Eagle medium (DMEM) containing 5% fetal bovine serum and 100units/ml penicillin G sodium and 100µg/ml streptomycin sulfate. Cell viability determined by trypan blue dye exclusion, was found to be 85-90%. The cells were plated in the above medium at a density of 1.5X10⁶ on 60mm dishes coated with rat tail type I collagen (Sigma). After incubation for 14-16 hours, fresh medium was added to the cells and was either left untreated or treated for 8 h with 20mM forskolin alone or in combination with 0.5mM dibutyryl cAMP.

**RT-PCR and Semi-quantitative RT-PCR analysis of SMN transcripts**

Total RNA was isolated from untreated and treated HeLa cells, mouse , human fibroblasts, primary hepatocytes and liver using Guanidine isothiocyanate method (51). First strand cDNA was synthesized from 3µg total RNA using RT-PCR kit (Perkin-Elmer). One tenth of the reaction mixture was used for the amplification of *SMN* gene. To amplify the different splice variants of SMN transcripts, a multiplex PCR was performed as described previously (8,32) where different splice variants of *SMN* gene were
amplified along with *HPRT* (hypoxanthine phosphoribosyl-transferase) gene as an internal control. PCR primers used for amplification of exon 4-8 of the *SMN* gene (4 forward, 5′-GTGAGAACTCCAGTCTCCTGG-3′ and 8 reverse 5′-CTACAACACCCTTCTCAG-3′), yielding four possible RT-PCR products (derived from the full-length SMN transcripts and isoforms lacking exons 5 and/or 7). Primers selected for amplification of HPRT (forward, 5′-TGTAATGACCAGTCAACAG-3′ and reverse 5′-ATTGACTGCTTCTTACTTCTT 3′) generated a product that is similar in size to (but distinguishably different from) the full length SMN transcript (32). The forward primers of mouse and human HPRT and SMN were end-labeled with (γ $^{32}$p)-ATP. cDNA was amplified by PCR in a 25µl reaction mixture containing 0.5mM dNTP, 1 U *Taq* polymerase, 30ng of each SMN primer, 7.5ng of each HPRT primer, 2.5mM MgCl$_2$ in 1X PCR buffer. Cycling conditions consisted of an initial denaturation step at 95°C for 4 min, followed by 22 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 1 min, with a final extension step at 72°C for 8 min. Ten microlitres of the resulting PCR products was combined with 5µl loading dye (95% formamide, 10mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) and was electrophoresed on a 6% denaturing polyacrylamide gel. The gel was dried and exposed to hyperfilm (Amersham) or to a phosphorimager screen. Quantitative analysis of the band intensity was performed using ImageQuant software (Molecular Dynamics) and the ratio of SMN transcript to that of HPRT was presented as a bar diagram.
RESULTS

In vivo genomic footprinting studies demonstrate occupancy of a CRE/ATF site on the human SMN2 promoter

Transient transfection studies have identified a 750bp segment spanning from -450 to +300bp with respect to transcription start site on both human SMN1 and SMN2 gene that demonstrated maximal transcriptional activity (44). There was minimal difference in the sequence between the SMN1 and SMN2 promoters that is reflected in the comparable promoter activity (12,44,52,53). Since the SMN2 gene remains active in SMA patients, we selected this promoter for further study. Analysis of the sequence spanning the 750bp region revealed cis-elements for several transcription factors, including two putative CREB/ATF binding sites, CRE-I (5′ TGACGACA3′) and CRE-II (5′AGTCGTCA3′) (Fig. 1A and B). To identify the critical cis elements involved in the expression of this promoter in the chromatin context, we performed in vivo genomic footprinting using brain and liver nuclei from Smn⁻⁻ SMN2 mice that have eight copies of the human SMN2 gene (29). We used these mice to amplify the footprinting signal because they express multiple copies of the SMN2 gene. The region between +210 and +283bp revealed footprinting at the CRE-II site and the adjacent Sp1 site (Fig. 2A). The results showed that in the brain and the liver, the G- and A-residues (indicated by stars) spanning the CRE-II site of the SMN2 promoter were rendered hypersensitive to dimethyl sulfate and one A-residue (denoted by an arrow) was protected compared to the A/G-ladder of the naked DNA. A G residue adjacent to the CRE-II element was also found to be hypersensitive in control brain and liver nuclei. The A/G-ladder of the naked DNA was generated by LM-PCR of purified genomic DNA from the brain or liver of the transgenic mice. Two G-residues at the Sp1 site of SMN2 gene were hypersensitive and three G-residues
were protected in both tissues compared to the naked DNA. This observation implicates that Sp1 interacts with its cognate binding site in the brain and liver in which the SMN2 promoter is active. These footprints of the SMN2 promoter were observed on the lower strand. The lack of appropriate LM-PCR primers prevented analysis of the same region on the complimentary strand. The minimal SMN2 promoter also harbors a second CREB binding site (CRE-I) located 400bp upstream of the transcription start site (Fig.1A). We designed another set of LM-PCR primers that could read the upper strand of the promoter spanning the CRE-I site. No footprint at this element was observed either in the brain or the liver nuclei (Fig.2B). This set of data indicates the involvement of CRE-II site in the SMN promoter activity.

**DNase I footprinting reveals protection of the CRE-II site located in the proximal promoter of SMN gene in HeLa nuclear extract**

To establish whether the CREB family of proteins could bind to the CRE-II site in the SMN2 promoter, we performed *in vitro* DNase I footprinting using transcriptionally active HeLa nuclear extracts, as HeLa cells express SMN2 protein at a relatively high level (15,18). For this purpose, $^{32}$P–labeled DNA fragments encompassing the CRE-II site (Fig.1A) were incubated with increasing amounts of HeLa nuclear extract in presence of poly(dI-dC) and then subjected to limited DNase I digestion. Separation of the resulting DNA fragments revealed protection of the CRE-II site on both the coding and non-coding strands (Fig.3A, lanes 3-5 & Fig.3B, lanes 3-6). DNase I digestion of the reaction mixture preincubated with 50 to 100-fold molar excess of the unlabeled probe was able to compete out the specific footprinting at the CRE-II site (Figs.3A, lanes 6, 7 & B, lanes 7, 8). The characteristic
footprinting at the Sp1 site as observed by IVGF analysis was also detected adjacent to the CREB/ATF binding site on both the strands where the nucleotides encompassing the cis-element were hypersensitive to DNase I digestion and competed away with excess cold probe (Figs.3A&B). To identify other regulatory elements within the 750bp region of SMN2 promoter, two other end-labeled probes that encompass the non-coding and coding strands spanning the CRE-I site were generated. DNase I footprinting of either probe in presence of HeLa nuclear extract did not reveal any specific footprinting (data not shown). These results demonstrate in vitro binding of transcription factors to the CRE/ATF site located in Exon1 but not the promoter region of SMN gene that corroborated the IVGF data obtained with brain and liver nuclei.

EMSA and UV cross-linking studies show that CREB-1 proteins interact with CRE-II element of the SMN promoter

The in vivo and in vitro footprinting studies implicate that the CRE-II site plays an important role in regulating SMN promoter activity. To characterize the transcription factor binding to the CRE-II site, we initially studied the interaction of this cis-element with nuclear extracts from a mouse motor neuron (EHMN) cell line by electrophoretic mobility shift assay (EMSA). Like HeLa cells the spinal motor neuron hybrid EHMN cells show high levels of SMN gene expression and intense staining of the protein in the cytoplasm (unpublished observation). There is very little difference between the CRE consensus sequence and CRE-II sequence of the SMN2 gene (Fig.1B). Incubation of the $^{32}$P-labeled double stranded CRE-II oligo with the EHMN nuclear extracts resulted in formation of three distinct DNA/protein complexes (C1, C2 and C3) (Fig. 4A). To determine the specificity of these DNA/protein
complexes, we included 200-fold excess of unlabeled CRE consensus oligo (CRE-C), CRE-II oligo (CRE-II) or mutant CRE-II oligo (mCRE-II) in the reaction mixture. All three complexes were competed out with the consensus as well as the CRE-II oligo, whereas the mutant CRE-II (Fig. 1B) and a non-specific oligo (STAT1 binding element) competed out only the complex C3 (Fig 4A, compare lane 1 with lanes 2-5). This data suggested that C1 and C2 are the two specific complexes formed in EHMN nuclear extracts with the CRE-II oligo and both complexes must belong to the CREB/ATF family of proteins. As both the mutant CRE-II oligonucleotide and the non-specific oligonucleotide disrupted the C3 complex formation, it is most likely that the C3 is a non-specific complex. The CREB/ATF family of proteins comprises several isoforms coded by at least three highly related genes CREB, CREM and ATF-1, which share extensive sequence homology (54). Incubation of the EHMN nuclear extract with a monoclonal antibody cross-reacting with CREB-1, ATF-1 and CREM-1 before addition of the $^{32}$P-labeled oligo resulted in supershift of the C1 and C2 complex, but not C3 (Fig. 4A, compare lanes 1 and 6). In addition, C1 and C2 complexes were not shifted when an unrelated antibody was used (Fig. 4A, lane 7) or with antibodies against other ATF proteins (data not shown). This observation further substantiated that the complexes C1 and C2 belong to CREB family of proteins. Similar complex formation of CRE-II oligo with HeLa nuclear extract was also observed (data not shown). Based on these data, we conclude that the transcription factor interacting with CRE-II element consists entirely of a homodimer or heterodimer comprised of CREB-1, ATF-1 and/or CREM-1.

The CREB/ATF family of transcriptional activators consists of multiple protein species that recognize nearly identical binding sites (55,56). Since the complexes C1 and C2 were supershifted with the antibody that recognizes all the three factors (CREB-1, ATF-1 and CREM-1) we made an attempt to further characterize the protein components of these complexes by UV-induced DNA-protein cross-
linking. Analysis of complex 1 showed that it consists of two closely migrating DNA binding polypeptides of approximate molecular masses of 75 and 80 kDa (Fig. 4B, lane 1). That these polypeptides specifically bind to the CRE-II element was confirmed by the lack of cross-linking of these polypeptides to \(^{32}\)P-labeled oligo in presence of 100–fold molar excess of unlabeled CRE-II oligo (Fig.4B, lanes 1 and 3). Binding of the 80kDa polypeptide to the CRE-II element was disrupted in presence of the mutant CRE-II (Fig.4B, lane 5), indicating that the 75kDa polypeptide of C1 complex is the only protein that specifically binds to the CRE-II site of \(SMN\) promoter. The exact molecular mass of this protein was estimated to be 43kDa after correcting for the probe mass of 32kDa. Since the major cross-linked polypeptide of 43kDa is identical in mass to the CREB-1 protein (57) it is evident that CREB-1 homodimer interacts with CRE-II site in \(SMN\) minimal promoter. The other complex C2 detected by EMSA consisted predominantly of the 80kDa component and proportionately less of the 75kDa component. Since the 80kDa band from complex C2 was also competed out with mutant CRE-II (Fig.4B, lanes 2,4 & 6), we conclude that CREB-1 is the only component of the C2 complex. Although CREB-1 is the only DNA binding protein of both C1 and C2 complexes, we still observed a difference in their mobility by EMSA (Fig.4 A). This can be explained by the assumption that CREB-1 forms multimers of lower (C2) as well as higher order (C1) under \textit{in vitro} binding conditions that resulted in the observed difference in the mobility by EMSA. This set of data implicates that CREB-1, but not the other ATF family members, is the predominant protein responsible for CRE-II-binding activity.

**Transcriptional activation of \(SMN\) gene by cAMP requires CRE-II**

To study the functional importance of the interaction between CREB-1 protein and the CREB-binding site (CRE-II), we performed site-directed mutagenesis of the CRE-II site of the plasmid p750. The
plasmid p750 harbors the 750bp SMN2 promoter region in pGL3-basic vector (44). The ’TG’ in the CRE-II (5′-TGACGAC-3′) was replaced by ’AC’ (5′ACACGAC3′) in the 750 bp promoter of SMN2 gene by multiple rounds of PCR and then cloned into the pGL3-basic vector (Promega). The mutation at the CRE-II site was confirmed by sequencing. We also confirmed that the mutated CRE-II element disrupted the binding of the protein complex factor by EMSA (Fig. 4A, lanes 1 and 4). To determine the effect of this mutation on transcriptional activity of SMN promoter, the p750mCRE (CRE-II site mutated p750) and the wild type p750 were transiently transfected into EHMN and HeLa cells. The cells were harvested 48 hours post-transfection and the luciferase activity was measured in the whole cell extracts. The promoter activity expressed as ratio of SMN2 promoter driven firefly luciferase activity to that of the internal control (pRL-TK) showed 35% and 50% inhibition upon mutation of the CRE-II site (Fig. 5A) in EHMN and HeLa cells, respectively. This data suggests that the CRE-II site is essential for the upregulation of SMN promoter that may also involve cAMP signaling pathway.

The cAMP responsive element-binding protein (CREB) is a transcription factor that plays a key role in the development of different neuronal cells and is activated by a variety of signaling molecule (58). It is known that the members of CREB/ATF family of transcription factors are activated by phosphorylation in response to changes in cAMP levels (59). To determine whether the binding of CREB-I to the CRE-II is influenced by cAMP, we investigated the activity of the 750bp promoter in response to cAMP elevator, dibutyryl cAMP (bt2cAMP) and to the protein kinase A activator (PKA), forskolin. A dose response-curve of SMN promoter to bt2cAMP (0.1mM to 1mM) and forskolin (10µM to 100µM) was performed in order to determine the optimum time and concentration required for the maximal effect (data not shown). Next HeLa cells were transiently transfected with p750 and treated with various concentrations of bt2cAMP and forskolin 24 hours post transfection. Cells were harvested 24hrs after treatment and assayed for luciferase activity. The promoter activity was expressed as p750 activity/µg.
protein. Comparison of the activity of p750 showed activation of SMN minimal promoter when bt2cAMP or forskolin was added to the transiently transfected cells. A maximum of 3-fold activation of the p750 was observed in presence 0.5mM bt2cAMP and a 2-fold activation was observed upon treatment with 20μM forskolin for 24 hours (Fig. 5B). The effect of both agents added together on SMN promoter was additive (5-fold activation). Similar effect of forskolin and bt2cAMP on p750 was also observed when transfected into EHMN cells (data not shown). These results suggest that SMN promoter is cAMP-responsive and the CRE-II site behaves as an inducible element.

The p750 plasmid contains a second CRE site (CRE-I). Although in vitro DNase I or in vivo genomic footprinting did not demonstrate occupancy of this upstream CRE-I site, we investigated whether this element plays any role in regulating SMN promoter activity following transient transfection. The CRE-I site is still intact in the plasmid p750mCRE where the CRE-II site is mutated. Treatment of p750mCRE with bt2cAMP and/or forskolin should alter the promoter activity if the CRE-I site contributes to its regulation. To test this possibility, HeLa cells transfected with p750 or p750mCRE were treated with bt2cAMP for 12hrs and 24hrs. The activity of p750mCRE was not altered in presence of bt2cAMP, whereas p750 showed 2 and 3.5 fold increase after treatment with the cAMP analog for 12 and 24hrs respectively (Fig 5C). This data further reinforces the conclusion that CRE-II and not CRE-I is the cAMP-responsive element by which forskolin and cAMP activate the SMN promoter.

**CREB-1 overexpression upregulates SMN promoter activity**

Next we explored the effect of CREB-1 protein overexpression on the SMN promoter activity. HeLa cells were cotransfected with p750 and either a CREB-1 expression vector (pSGRSV-CREB) or the empty vector. Overexpression of CREB-1 protein was verified by Western blot analysis of the whole
cell extracts prepared from the transfected cells using CREB/ATF antibody (Fig.6A). A 5-6 fold increase in the expression of a ~43kDa protein was observed in HeLa cell extracts transfected with pSGRSV-CREB compared to cells transfected with the empty vector (Fig. 6A, lane 2). The effect of CREB-1 on the activity of SMN2 promoter was analyzed by determining the promoter activity in presence and absence of CREB1. The promoter activity expressed as the ratio of SMN promoter driven firefly luciferase activity to that of the internal control (pRL-TK) increased 4- fold in presence of CREB-1 relative to the basal promoter activity (Fig 6B), whereas transfection of the empty vector had no effect. The finding that the active CREB stimulates SMN promoter suggests that the CREB-1 mediates the cAMP dependent upregulation of SMN gene expression and further substantiates our conclusion that the DNA binding activity of CREB-1 plays an important role in this process.

**Dibutyryl cAMP upregulates endogenous SMN gene expression**

Next we sought to determine the effect of cAMP enhancing compounds on the expression of endogenous SMN gene. The SMN1 gene produces mostly full length SMN whereas the SMN2 gene produces four RNA isoforms: full length SMN, exon 7 deleted SMN, exon 5 deleted SMN and exon 5 and 7 deleted SMN transcripts (8,32,60). To analyze the effect of cAMP on the expression of different SMN isoforms, we performed semiquantitative multiplex PCR. The multiplex reaction yields four SMN PCR products including full length SMN transcript, isoform lacking exon 7 (exonΔ7), exon 5 (exonΔ5), or both (exonΔ5, 7) and one HPRT (hypoxanthine phosphoribosyl-transferase) PCR-product as internal control.
Primary hepatocytes isolated from \textit{Smn}^{+/-} mice expressing two copies of human \textit{SMN2} gene were treated with forskolin alone or a combination of forskolin and dibutyryl cAMP for 8 hours. The expression of different SMN transcript was analyzed by multiplex PCR and expressed as the ratio of SMN transcript to HPRT. The result revealed 2.5 fold and 3-fold increase in the full length SMN transcript in presence of forskolin alone and 4-5 fold increase in combination with bt2cAMP (Fig.7A&B). The increase in the exon\textit{\Delta7} transcript ranged between 2-fold and 3-fold (Fig.7A&B). To determine the effect of cAMP elevating agent on \textit{SMN} transcription in the intact liver, transgenic mice with two copies of human SMN2 were either left untreated or injected i.p. with epinephrine (see Methods for details). Analysis by multiplex PCR of the total RNA isolated from the livers (60) revealed two-fold increase in the full length SMN transcript in the epinephrine- treated mice compared to the control animals (Fig. 7C ).

We have also treated human fibroblasts from type 1 SMA patients (\textit{SMN1}^{-/-};\textit{SMN2}), mouse fibroblasts derived from SMA mice (\textit{Smn}^{-/-}; \textit{SMN2}) and HeLa cells with bt2cAMP and/or forskolin for 8 hours and cDNA synthesized from the total RNA was subjected to multiplex PCR as described. The results showed that treatment of SMA mouse fibroblasts with forskolin and bt2cAMP resulted in a 5–fold increase of both full length and exon\textit{\Delta7SMN} mRNA (Fig, 8A, lane 1 and 3). Forskolin alone increased the level of the full length and exon\textit{\Delta7SMN} mRNA 3-fold and 1.5-fold respectively (Fig.8A, lanes 1 and 2). On the other hand, treatment with bt2cAMP showed 4- and 3.4- fold increase in the level of full length and exon\textit{\Delta7SMN} mRNA, respectively (Fig, 8A, lane 1 and 4). The fold-increase is represented as a ratio of SMN transcript to that of the HPRT (Fig. 8B). Combined treatment of HeLa cells with forskolin and bt2cAMP showed an 8-fold increase in the full length SMN message and a 7-fold increase in the exon\textit{\Delta7SMN} message level (Fig. 8C lanes 1 and 3and Fig.8D). The increase in full length SMN and exon\textit{\Delta7SMN} mRNA was also observed upon treatment of human SMA fibroblast (3813) with
forskolin and bt2cAMP (data not shown). These data revealed a consistent increase in full length as well as exon 7-deleted SMN transcripts in whole animals, primary hepatocytes derived from the mouse liver as well as cells in culture when exposed to cAMP elevating agents.

DISCUSSION

Loss or mutation of the SMN1 gene causes spinal Muscular Atrophy (SMA). However, the SMN2 gene is always retained in SMA patients and does produce some SMN protein, but not sufficient levels for the survival of motor neurons (7,9,60-62). The severity of SMA correlates with the expression level of SMN protein, and large amounts of SMN protein from the SMN2 gene can correct the SMA phenotype in mice (16,17,29). Hence, upregulation of SMN2 is an attractive strategy for the treatment of SMA. In the present study we identified a cAMP Response Element (CRE-II) that interacts with CREB-1 protein and is located downstream of the transcription start site. The present studies showed that the CRE site when present in the 5′-untranslated region can still confer inducibility to the SMN gene. This CRE II site is oriented in the reverse direction and is adjacent to a Sp1 site. Footprinting showed that both the CRE II and Sp1 sites were occupied in an active promoter. It is possible that there is cooperative interaction between the CREB1 and Sp1 proteins in the upregulation of SMN gene expression.

The cAMP transcription factors belong to a multigene family with several isoforms that may function as transcriptional activators or repressors (55). We have demonstrated that a CREB1 homodimer binds to the CRE II site on the SMN promoter and upregulates its expression. The common motif shared by all the family members is a basic-domain-leucine-zipper (bZip) (55) at the carboxyl terminal end that
promotes dimer formation. Although the three members of the CREB family CREB-1, CREM-1 and ATF-1 can heterodimerize, the formation of homodimer is favored in vivo (63). This family of transcription factors is a component of intricate intracellular signaling pathway that is important for regulating biological functions ranging from spermatogenesis to circadian rhythms and memory (64). Here we show for the first time its involvement in upregulating spinal motor neuron gene.

The CREB protein is activated by phosphorylation at serine 133, which is mediated by protein kinase A in addition to other kinases (65). The CRE-II site conferred cAMP inducibility to the SMN gene. The present study has shown that cAMP analogue dibutyryl-cAMP and the PKA activator forskolin can activate SMN2 gene expression in primary hepatocytes as well as in cells in culture. Intraperitoneal injection of cAMP elevating agent, epinephrine (66) in SMN transgenic mice also resulted in increased level of SMN transcript in the liver. It has been shown previously that treatment of Schwann cells with forskolin causes an increase in coiled (Cajal) bodies. Coiled bodies and SMN usually colocalize with each other and an increase in SMN expression results in additional coiled bodies (15,19). This may indirectly indicate that gems (SMN nuclear deposits) and coiled bodies can be increased by forskolin treatment, which would be in agreement with our observation of the SMN promoter being inducible with forskolin. Cerebellar granule cells as well as motor neurons depend on activation of the N-methyl-D-aspartate (NMDA) receptor in order to survive and attain the fully differentiated state. Activation of the NMDA receptor in cerebellar granule cells results in an increase in SMN expression (30). NMDA receptor activation was also shown to activate the SMN promoter in the EHMN cell line. Recently, a link between NMDA receptor activation and increased levels of phosphorylated CREB during maturation of neurons has been established (58). Our study indicates that NMDA mediated upregulation of gem number and concurrent maturation of spinal motor neuron is mediated through newly identified CRE-II
Based on these observations, we propose a signal transduction pathway that involves initial activation of NMDA receptor. This may lead to CREB activation, which, in turn, activates SMN expression through the CRE-II site and ultimately spinal motor neuron maturation.

Previously, compounds have been identified which alter the incorporation of exon 7 in the SMN transcript from the SMN2 gene (32,33,35). In the present study, the compounds such as forskolin and dibutyryl cAMP were shown to increase the expression of SMN2 gene. Interferon β and γ have been shown to bind to an ISRE element in the SMN promoter and activate SMN expression (31). In some cases the activation results in a greater increase in the exonΔ7SMN isoform. The present study showed elevation of both the full length and exonΔ7SMN by forskolin and bt2cAMP. The availability of compounds that allow further enhancement of SMN expression through a combination of promoter activation and alteration in SMN2 splicing may allow sufficient levels of SMN expression in the patient.

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**FIGURES and LEGENDS**

**Fig. 1A.** Schematic diagram of 750 bp *SMN2* promoter region depicting relevant cis-elements. Arrows indicate the positions of selected restriction sites. The Sst I/Sty-I, Sty I/Pst I and Pst I/Hind III probes represent the fragments of the *SMN2* promoter that were subsequently used for DNase I footprinting studies. **B.** Sequence of the CRE consensus, CRE-I, II elements and the mutant CRE-II of *SMN2* promoter.

![Schematic diagram of 750 bp SMN2 promoter region](image)

**B**

- **CRE-consensus**: TGACGTCA
- **CRE-I**: TGACGACA
- **CRE-II**: TGACGACT
- **mut CRE-II**: ACACGACT

**Fig. 1**
**Fig. 2.** *In vivo* genomic footprinting demonstrates involvement of CRE-II site in *SMN2* gene expression. Intact nuclei isolated from brain and liver cells of *Smn*−/− mice expressing eight copies of human *SMN2* gene were exposed to limited dimethylsulfate treatment and genomic DNA was isolated. The DNA was then subjected to piperidine treatment followed by LM-PCR amplification of the *SMN2* promoter. The LM-PCR products were separated on 6% sequencing gel and exposed to X-ray film. N is naked DNA where DNA was treated with DMS and piperidine after isolation, and C is DNA isolated from control cells treated in vivo with DMS. Stars and arrows indicate hypersensitive indicate protected G-residues respectively. **A.** Lower strand spanning from +210 to +283 bp. **B.** Upper strand spanning from –312 to –443 bp of the *SMN2* promoter.
Fig. 3. DNase I footprinting reveals protection of CRE-II site in SMN proximal promoter. A. A radio labeled probe was generated by Pst I/Hind III digestion of human SMN gene promoter spanning the region between +150 to +300bp. The upper strand of the DNA fragment was labeled with (γ-32P)ATP and T4 polynucleotide kinase. HeLa nuclear extract was allowed to interact with the labeled probe and subjected to limited DNase I digestion. For competition assay unlabeled Pst I/Hind III fragment was added to the reaction prior to addition of the labeled probe. Lane 1: free probe, lane 2: DNA ladder, no extract; lanes 3-5: 25, 50 and 75 µg of HeLa nuclear extract, respectively; lanes 6&7: 75µg HeLa nuclear extract in presence of 50 and 100 fold excess of unlabeled Pst I/Hind III fragment respectively. B. Probe corresponding to the non-coding strand of the Pst I/Hind III fragment was labeled using Klenow and (α-32P)dGTP. Lane 1: free probe, lane 2: DNA ladder, no extract; lanes 3-6: 25, 50, 75 and 100 µg of HeLa nuclear extract; lanes 7&8: 100µg HeLa nuclear extract in presence of 50 and 100 fold excess of unlabeled Pst I/Hind III fragment, respectively. A/G lane: A+G ladder of the probe.
Fig. 4. EMSA and UV crosslinking study shows binding of CREB-1 protein to the CRE-II site of the SMN proximal promoter. A. DNA mobility shift and supershift assays were performed using (32P)-ATP end labeled CRE-II oligo. One nanogram of 32P-CRE-II and 10µg of EHMN nuclear extract (NE) was used in each reaction. Lane 1: NE only; lane 2: 200X CRE-II oligo; lane 3: 200X CRE consensus oligo (CRE-C); lane 4: 200X mutant CRE-II oligo (mCRE-II), lane 5, 200X non-specific oligo(N.S.), lane 6, anti CREB/ATF-1 antibody (monoclonal); lane 7, anti STAT1 antibody. B. EHMN NE and 32P-labeled CRE-II oligonucleotide were allowed to form complex in EMSA binding buffer, separated on 5% acrylamide gel, and the protein –DNA was cross-linked under UV light. The C1 and C2 complexes were recovered from the gel, eluted and ran on 10% SDS-PAGE. Lanes 1, 3 and 5: Complex C2 recovered from control reaction, reaction mixtures containing excess CRE-II and mCRE-II oligo, respectively. Lanes 2, 4 and 6: Complex C1, recovered from control reaction, reaction mixtures containing excess CRE-II and mCRE-II oligo respectively.
Fig. 5A. Transient Transfection assay demonstrates importance of CRE-II site in SMN promoter activity. EHMN and HeLa cells were plated at 1X10^5 cells/well in 6-well dishes and transfected with either 150ng and 500ng respectively of p750 (wild) or p750mCRE (mut) and 40ng pRLTK (internal control) plasmid DNA. Cell extracts were prepared in 1X lysis buffer (Promega), 48hr post tranfection and luciferase activity was measured using dual luciferase assay kit. The promoter activity is presented as the ratio of p750/pRLTK activity.  

B. Both bt2cAMP and forskolin upregulates SMN promoter activity: HeLa cells were transiently transfected with 4µg of p750 plasmid and were treated with different concentrations of bt2cAMP and/or forskolin 24hr post-transfection as indicated. Cells were harvested 24hrs after the respective treatment and measured for luciferase activity. The promoter activity is presented as the p750 activity per µg protein.
Fig.5C. Mutation at the CRE-II sequence abolishes cAMP responsiveness of the promoter. HeLa cells were transfected with plasmid p750 or p750mCRE. The cells were treated with 0.5mM bt2cAMP 24hr post-transfection and harvested after 12 and 24 hrs of treatment. Luciferase activity was measured as described and values are expressed as per μg protein. All the data are representative of three independent experiments±S.E.
Fig. 6. Transient overexpression of CREB-1 protein upregulates SMN promoter activity. A. HeLa cells were transiently transfected with empty vector (lane 1) or CREB-1 overexpression vector (pSGRSV-CREB) (lane 2). Whole cell extract prepared 48 hour post-transfection was subjected to Western blot analysis with anti CREB/ATF-1 antibody. B. HeLa cells were co-transfected with plasmids p750, internal control pRLTK and either pSGRSV-CREB (CREB) or the corresponding empty vector (E.V.). Cells were harvested 48 hr post-transfection, and luciferase activity measured using Dual luciferase assay kit. The promoter activity was expressed as a ratio of p750 to pRLTK activity. The data is representative of three independent experiments ± S.E.
cAMP elevating agents stimulates expression of SMN transcripts in mouse primary hepatocytes as well as in mouse liver. A. Primary hepatocytes isolated from Smn+/− mice expressing two copies of human SMN2 gene were treated bt2cAMP and/or forskolin for 8 hours. Total RNA isolated from untreated and treated cells were subjected to multiplex PCR and the products are separated on a sequencing gel. The experiment was done with hepatocytes isolated from two different mice. B. For quantitation of the mouse HPRT transcript and different splice variants of SMN transcripts the dried gel was exposed to storage phosphor screen (Molecular Dynamics) for different length of time and analyzed using ImageQuant software. The ratio of SMN transcript to HPRT transcript was calculated and data expressed as fold increase in SMN transcript compared to the untreated control taken as one. The increase in SMN full length transcript is 4.5±0.7 fold in presence of forskolin and bt2cAMP compared to the untreated control.
Fig. 7.C. Human SMN2 transgenic mice (in triplicate) were injected i.p with epinephrine every two hours for six hours and sacrificed two hours after the last injection. Total RNA isolated from the liver was analyzed for SMN gene transcription by multiplex PCR as mentioned above. After quantitation of the PCR product the data is presented as ratio of SMN transcript to that of HPRT for individual mouse (1,2,3). The level of SMN full length transcript in untreated mice is 9.6±2.0 and in epinephrine treated mice is 18±4.3.

![Graph showing ratio of SMN to mHPRT transcript](http://www.jbc.org/)

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**Fig.7**
Fig. 8. Forskolin and bt2cAMP upregulates expression of both the full length and exon7 deleted SMN transcript. A. Mouse type I SMA fibroblasts were treated with bt2cAMP and/or forskolin for 8 hours. Total RNA isolated from untreated and treated cells were subjected to multiplex PCR and the products are separated on a sequencing gel. B. Alteration in the transcript level of different SMN isoforms were quantitated using ImageQuant software, and represented as a ratio of SMN to HPRT (internal control) transcript level. C. HeLa cells were subjected to similar treatment as described for mouse fibroblasts, and analyzed by multiplex PCR. D. Bar diagram representing the effect of bt2cAMP and/or forskolin on different SMN isoforms in HeLa cells.
Identification of a novel cyclic AMP response element (CRE-II) and the role of CREB-1 in the cAMP-induced expression of the survival motor neuron (SMN) gene
Sarmila Majumder, Saradhadevi Varadharaj, Kalpana Ghoshal, Umrao Monani, Arthur H.M. Burghes and Samson T. Jacob

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