doi:10.1006/jmbi.2000.4335 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2001) 306, 985–996

JMB

Sp1 and Sp3 Physically Interact and Co-operate with GABP for the Activation of the Utrophin Promoter

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The utrophin gene codes for a large cytoskeletal protein closely related to dystrophin which, in the absence of dystrophin, can functionally substitute it. Utrophin is transcribed by two independently regulated promoters about 50 kb apart. The upstream promoter is TATA-less and contains a functional GABP binding site which, in muscle, restricts the promoter activity to post-synaptic nuclei. Transient transfections analysis of mutant promoters in rhabdomyosarcoma cells showed that the upstream promoter contains three functional GC elements that are recognised by Sp1 and Sp3 factors *in vitro*. Co-transfections of the promoter with Sp1, Sp3 and GABP factors in *Drosophila* SL2 Schneider cells, which lack of endogenous Sp factors, demonstrated that both Sp1 and Sp3 are positive regulators of the utrophin promoter and that they activate transcription synergistically with GABP. Consistent with this result, we observed physical interaction of both Sp factors with the GABPα subunit *in vitro*. Functional domain interaction analysis of Sp1 and Sp3 revealed that both factors interact with GABPα through their DNA binding zinc finger domain. The modulation and correct interaction between Sp1, Sp3 and GABP in muscle cells may be critical for the regulation of the utrophin promoter, and provide new targets for therapies of Duchenne muscular dystrophy.

Keywords: utrophin; Sp1; GABP; muscle-transcription; protein-protein interaction

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Introduction

The utrophin gene (also named dystrophin-related gene) is an autosomal homologue of dystrophin (Love *et al*., 1989), which when mutated is responsible for Duchenne and Becker muscular dystrophies (DMD and BMD, respectively). Utrophin is transcribed in a large mRNA of 13 kb coding for a 395 kDa protein, with up to 73% of amino acid identity with dystrophin in important functional domains (Grady *et al*., 1997; Pearce *et al*., 1993). Functional substitution of utrophin with dystrophin in mice has demonstrated that a cure of DMD and BMD up-regulating the utrophin gene in patients is conceivable (Campbell & Crosbie, 1996; Deconinck *et al*., 1997; Grady *et al*., 1997; Rafael *et al*., 1998; Tinsley *et al*., 1996, 1998). Utrophin is expressed ubiquitously, although in adult skeletal muscle its expression is mainly restricted to neuromuscular junctions (Gramolini *et al*., 1997; Khurana *et al*., 1990; Schofield *et al*., 1993). Utrophin is transcribed by two independently regulated promoters (Burton *et al*., 1999; Dennis *et al*., 1996). The upstream promoter is a TATA-less promoter associated with a CpG island. It contains several GC sequences which are putative Sp factor binding sites, and a functional N box (Dennis *et al*., 1996). This promoter is also under the control of a downstream utrophin enhancer (DUE) localised at about 9 kb within the second intron (Galvagni & Oliviero, 2000). A second promoter is localised about 50 kb further downstream, which gives rise to a utrophin with a different N-terminal domain (Burton *et al*., 1999). Both promoters drive a wide distribution of utrophin transcripts with overlapping expression in most tissues. The upstream utrophin promoter is mostly expressed in skeletal muscle, while the intronic promoter is more active in heart muscle (Burton *et al*., 1999; Love *et al*., 1991).

Abbreviations used: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; DUE, downstream utrophin enhancer; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase.

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0022-2836/01/050985–12 $35.00/0

The upstream utrophin promoter in cultured muscle cells responds by two- to threefold induction to treatment with heregulin or by the transfection of the Ets-related GABP factor (Gramolini *et al*., 1999; Khurana *et al*., 1999). GABP has been reported to activate several viral and cellular promoters (Fromm & Burden, 1998; LaMarco *et al*., 1991; Thompson *et al*., 1991; Triezenberg *et al*., 1988; Virbasius *et al*., 1993; Watanabe *et al*., 1993). In response to heregulin, the GABPα protein level is increased and both α and β subunits are phosphorylated (Schaeffer *et al*., 1998). GABP activates transcription synergistically with several factors, including Sp1, and has been demonstrated to interact directly with ATF and HCF factors (Ding *et al*., 1999; Dittmer *et al*., 1994; Gégonne *et al*., 1993; Rosmarin *et al*., 1998; Sawada *et al*., 1999; Vogel & Kristie, 2000).

The Sp family of transcription factors is composed of four zinc finger proteins Sp1-4, which in addition to the conserved DNA-binding domain, contain a glutamine-rich activation domain at the N-terminal region. Sp1, Sp3 and Sp4 recognise the consensus GC box element with identical affinity (Hagen *et al*., 1992, 1994). Sp4 expression is most abundant in neuronal tissues (Supp *et al*., 1996), while Sp1 and Sp3 are both ubiquitously expressed (Dynan & Tjian, 1983a,b; Hagen *et al*., 1994; Kingsley & Winoto, 1992). Sp1-deficient embryos die after day 10 of embryonic development, while Sp3-deficient mice die at birth as a result of respiratory failure (Bouwman *et al*., 2000; Marin *et al*., 1997). Knockout mice phenotypes suggest that both factors have functional redundancy during early embryo development but exert distinct functions at later developmental stages. Sp1 binding to G + C-rich sequences are found in close proximity of transcriptional start sites and in enhancers (Pugh & Tjian, 1990). Accordingly, Sp1 has been shown to be associated both with general coactivators and with several promoter-specific transcription activators (Hoey *et al*., 1993; Kardassis *et al*., 1999; Lee *et al*., 1993; Rotheneder *et al*., 1999; Ryu *et al*., 1999; Seto *et al*., 1993). Sp3 has been shown to activate several promoters. It also seems to act as a repressor, since it also contains an inhibitory domain (Hagen *et al*., 1994; Liang *et al*., 1996; Majello *et al*., 1997; Udvadia *et al*., 1995; Zhao & Chang, 1997).

Here, report the identification and characterisation of functional GC sites present on the upstream utrophin promoter. Using *in vitro* binding experiments and transient transfections, we demonstrated that both Sp1 and Sp3 act as activators and co-operate with GABP to activate the utrophin promoter. We propose that the synergistic transcriptional activation observed is due to direct physical interaction of GABP with both Sp1 and Sp3, and mapped it to the α-subunit of GABP and the DNA-binding zinc finger domain of both Sp1 and Sp3 factors.

Results

Sp1 and Sp3 bind to the utrophin promoter

The utrophin promoter is a TATA-less promoter rich in GC elements (Dennis *et al*., 1996). To identify which GC boxes are functionally relevant for the promoter activity, we first performed a DNA footprinting assay with recombinant Sp1 using DNA fragments spaning the promoter region from −352/ + 47 as probes. Sp1 protection from DNase I revealed three main protected sites identified as S1 (−73/ − 27), S2 (−114/ − 96) and S3 (−151/ − 135) (Figures 1 and 2). Interestingly, not all the putative Sp1 sites on the promoter are recognised by Sp1 *in vitro*. The proximal S1 GC element is a tandem repeat of three non-canonical GC boxes,

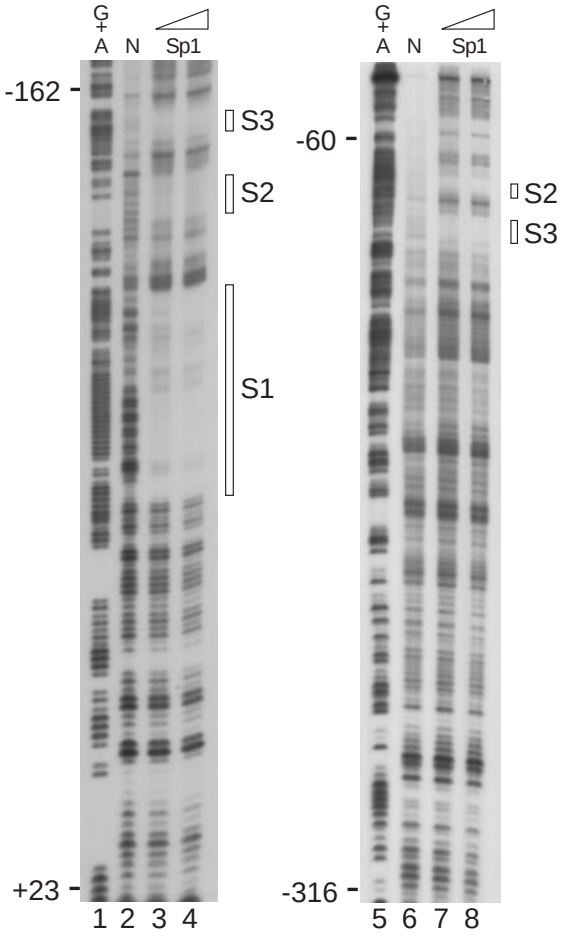

Figure 1. Sp1 binds the utrophin promoter. Purified Sp1 specifically protects three regions (S1, S2 and S3) from DNase I in footprint assay. Protected regions are indicated with open boxes. Lanes 1 and 5 contain Maxam and Gilbert G + A sequence reactions used as markers. Lane 2 and 6 contain DNase I digestion reactions of the naked probes, while lanes 3, 4, 7 and 8 are the DNase I digestion reactions of the probes in presence of either 1 or 2 μg of recombinant Sp1. Lanes 1-4 probe −238/ + 47 (sense strand); lanes 5-8 probe −352/ + 47 (antisense strand).

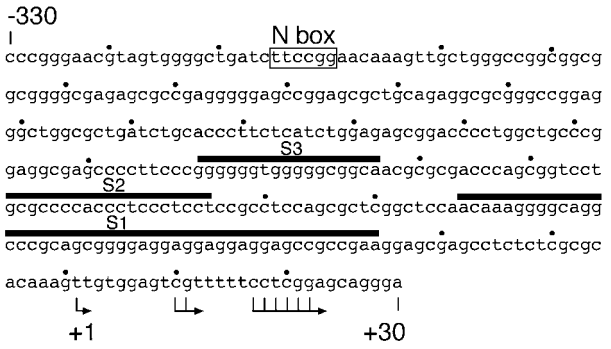

Figure 2. Schematic representation of utrophin promoter. The filled boxes identify the limits of the sequences protected by Sp1 in DNase I footprint assay (S1, S2 and S3) as shown in Figure 1. The N box core sequence recognised by GABP nuclear factor is boxed. The transcription start sites are indicated by arrows. The transcription initiation site located more upstream was used as reference for the numbering.

while the distal S2 and S3 elements are each composed of partially overlapping Sp1 consensus sites (Figure 2). To analyse nuclear factors binding to these elements on the utrophin promoter, we labelled the three probes containing the protected Sp1 sites and performed electrophoretic mobility shift assays (EMSA) with nuclear extracts from rhabdomyosarcoma RD cells. All probes formed similar complexes that were named C1-4 (Figure 3). In addition to the complexes common to all three probes, the DNA fragment S1 showed the formation of the slower migrating complex C5. Retarded complexes were specifically competed with an excess of unlabelled homologous oligonucleotides, while non-specific oligonucleotides did not affect the formation of the retarded bands (Figure 3(a)). All complexes were strongly reduced and supershifted with anti-Sp1 or anti-Sp3 antibodies (Figure 3(b)). Specifically, complexes C1-3 and C5 were inhibited and supershifted with anti-Sp3 antibodies, while the complex C4 was inhibited and supershifted with antibodies anti-Sp1. The presence of multiple bands containing Sp3 is not surprising, since Sp3 has been described to be expressed in at least three variants with different molecular sizes in several tissues (Kennett *et al*., 1997). The slower migrating band C5 that is formed with probe S1 (Figure 3(a) and (b), lanes 1) must contain more that one Sp3 factor, since this probe contains three protected tandem GC boxes (Figures 1 and 2). Retarded bands with slower mobility than C5 on the S1 probe, which could be observed with longer exposure, were reduced with both anti-Sp1 and anti-Sp3 antibodies (data not shown), suggesting the formation of higher molecular mass complexes.

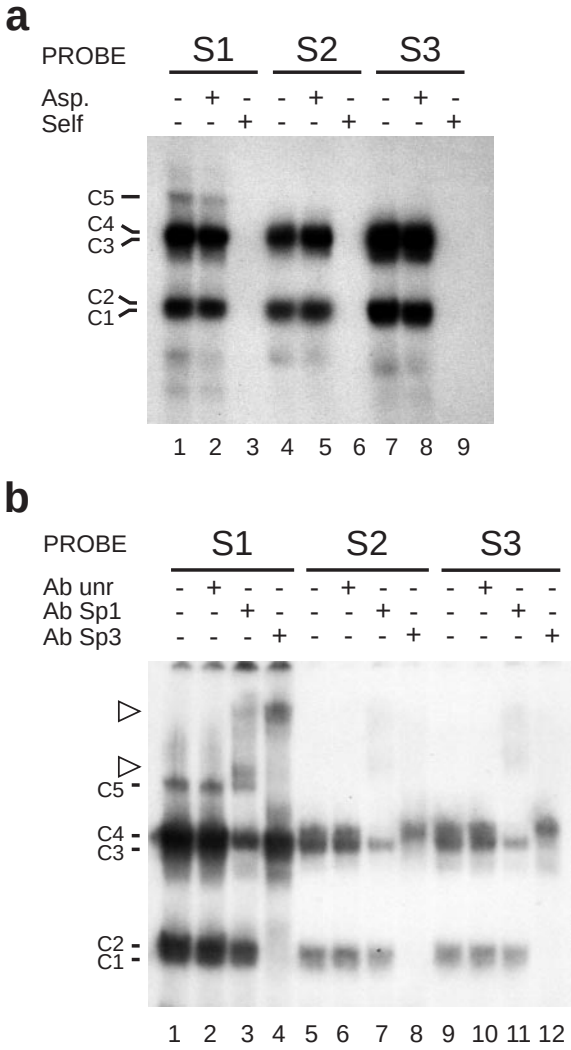

Figure 3. Nuclear Sp1 and Sp3 specifically bind the regions S1, S2 and S3 of the utrophin promoter. (a) Nuclear extracts obtained from RD rhabdomyosarcoma cells were incubated with the indicated DNA-labelled probes (S1, S2 and S3) covering the correspondent regions of the utrophin promoter protected by recombinant Sp1 in footprint assay (lanes 1, 4 and 7). Complexes formed are indicated as C1-5. The complexes were competed by incubation with 100-fold molar excess of self (S1, S2 and S3, respectively) unlabelled probe (lanes 3, 6 and 9), but not by incubation the same fold excess of a non-specific (Asp.) unlabeled probe (lanes 2, 5 and 8). (b) Specific complexes were supershifted (open arrows) by adding specific antibody for Sp1 (lanes 3, 7 and 11) or Sp3 (lanes 4, 8 and 12) to the reaction mixture. The unrelated (unr) antibody anti-SRF did not affect the complexes formation (lanes 2, 6 and 10).

Sp1 and Sp3 activate the utrophin promoter binding to GC boxes

To test the functional role of GC elements, we generated mutants corresponding to the S1, S2 or S3 sites. Wild-type and mutant utrophin promoters

in front of the CAT reporter gene were transfected into RD cells. As shown in Figure 4, the deletion of the proximal S1 site affected utrophin promoter activity by over 60 %, while mutations of either S2 or S3 sites affected utrophin promoter function in RD muscle cells by 30 and 20 %, respectively.

Direct activation mediated by Sp1 and Sp3 on the utrophin promoter was tested in *Drosophila* Schneider SL2 cells as these cells lack Sp-like activity. We therefore transfected the wild-type and mutant utrophin-CAT constructs in these cells along with a *Drosophila* expression vector carrying either the Sp1 ($pP_{ac}Sp1$) or Sp3 ($pP_{ac}USp3$) cDNA. Co-transfection of $pP_{ac}Sp1$ with the utrophin wild-type promoter enhanced the promoter activity in SL2 cells ca 25-fold (Figure 5). Thus, Sp1 factor is sufficient to activate the utrophin promoter in SL2 cells. By transfecting the promoter carrying the deletion of the proximal S1 binding site we observed a strong reduction of the utrophin promoter activity, while mutation of S2 affected the promoter activity in SL2 cells, but to a lesser extent. Co-transfection of the wild-type and mutant promoters together with Sp3 expression vector demonstrated that this factor is also an activator, albeit at weaker activity when compared with Sp1, since in the same conditions, Sp3 was activating the utrophin promoter of about five- to sixfold (Figure 5).

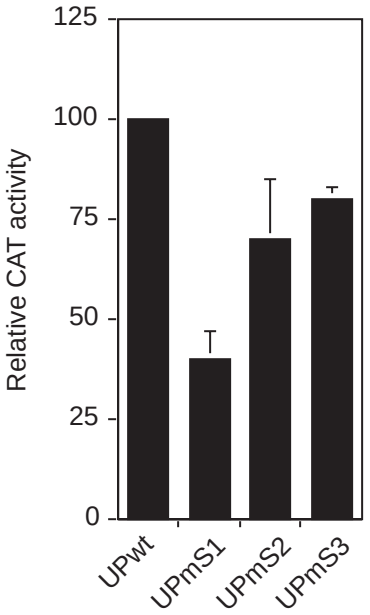

Figure 4. GC boxes are required for full promoter activity in RD rhabdomyosarcoma cells. Human RD rhabdomyosarcoma cells were transfected with 5 μg of reporter plasmid containing the utrophin promoter wild-type or mutants, respectively, in the S1, S2 or S3 elements. All transfections were performed including 0.75 μg of pRSVβgal as internal control using LipofectAMINE™ Reagent. The mean of three independent experiments is shown.

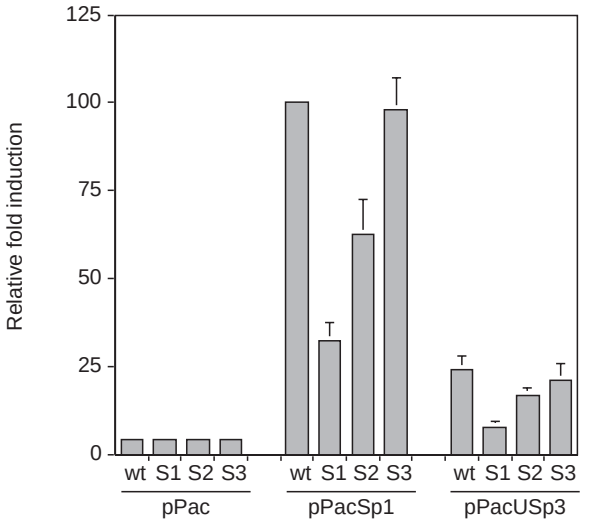

Figure 5. Sp1 and Sp3 independently activate the utrophin promoter. Co-transfections in *Drosophila* Schneider cells SL2 of the wild-type and mutant utrophin promoters together with the *Drosophila* empty expression vector (pP_{ac}), Sp1 expression vector (pP_{ac} Sp1) or Sp3 expression vector (pP_{ac} Sp3) as indicated. The experiments were carried out as described in the legend to Figure 4. The mean of three independent experiments is shown.

Sp1 and Sp3 activate the utrophin promoter in co-operation with GABP

The previous experiment demonstrated that both Sp1 and Sp3 activate the utrophin transcription in the heterologous SL2 cells. However, it was previously shown that Sp3 can act either as an activator or as a repressor in different promoter settings (Fandos *et al*., 1999; Hagen *et al*., 1994; Kennett *et al*., 1997; Liang *et al*., 1996; Majello *et al*., 1997; Udvadia *et al*., 1995; Zhao & Chang, 1997). In order to test the activity of these factors in muscle cells we transfected either Sp1 or Sp3 under the control of the CMV promoter in RD cells. Although in these cells we could not observe high levels of activation due to the presence of endogenous factors, by transfecting increasing amounts of either Sp1 or Sp3 expression vector we observed a low, but reproducible, increase of utrophin transcription with both factors (Figure 6). These results allow us to exclude the possibility that the utrophin promoter Sp3 acts as a negative regulator of Sp1 activation.

It has been previously shown that the utrophin promoter contains a functional N box recognised by GABP (Gramolini *et al*., 1999; Khurana *et al*., 1999). As Sp1 and GABP have been shown to co-operate in the transcriptional activation of several promoters and enhancers (Gégonne *et al*., 1993; Nuchprayon *et al*., 1999; Rosmarin *et al*., 1998), we analysed the the effect of Sp1 and Sp3 activation on a utrophin promoter mutant that has been mutated in the GABP binding site. As shown in

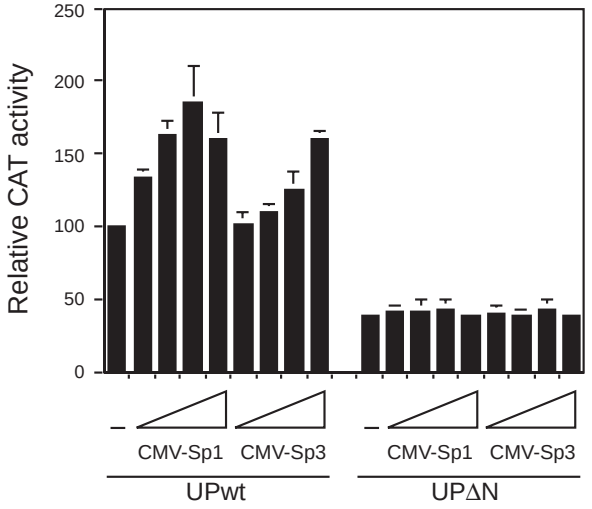

Figure 6. Sp1 and Sp3 activate the utrophin promoter in muscle cells and require the N box for the activation. RD cells were co-transfected with 1 μg of the reporter plasmid containing the wild-type utrophin promoter (UPwt) or the promoter lacking the N box (UPΔN) and increasing amounts (0.5, 1, 2 and 4 μg) of mammalian expression vectors carrying the cDNA of Sp1 or Sp3 as indicated. The amount of transfected plasmids was held constant by addition of pcDNA3. The mean of three independent experiments is shown.

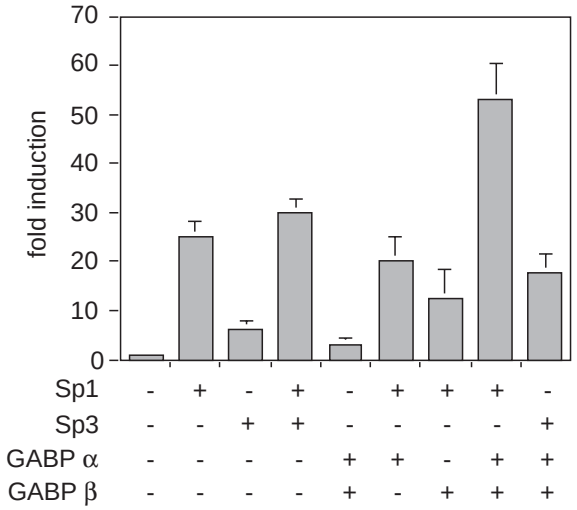

Figure 7. Sp1 and Sp3 co-operatively activate with GABP the utrophin promoter. Schneider cells were co-transfected with UPwt and pP_{ac} expression vectors carrying the cDNA of GABPα, GABPβ, Sp1 or Sp3 as indicated. Where needed, empty pP_{ac} vector was added to reach a total of 7.5 μg of expression vectors.

Figure 6, the utrophin promoter mutated in the N box shows a reduced transcription activity with respect to the wild-type promoter, and is no longer activated by Sp1 or Sp3. These results suggest that in RD cells, Sp1 and Sp3 activate the utrophin promoter in co-operation with GABP.

The synergistic activation between Sp factors and GABP was studied in greater detail in *Drosophila* Schneider cells by transient transfections of GABPα, GABPβ, Sp1 and Sp3, along with the utrophin promoter into SL2 cells. Although GABPα/β was revealed to be a poor activator in these cells, its co-transfection with Sp1 activated the promoter over 50-fold and showed a transcriptional effect that is significantly more than the sum of individual effects (Figure 7). Similar synergistic effect was obtained by co-transfecting GABPα/β with Sp3, although the maximal transcriptional activation was lower (Figure 7). No synergy was observed when co-transfecting GABPα or GABPβ separately with either Sp1 (Figure 7) or Sp3 (data not shown). Interestingly, Sp1 or Sp3 activated transcription in SL2 cells in the absence of GABP. This activation was also observed with a promoter deleted in the N box (data not shown), thus excluding the possibility that a GABP-like factor present in SL2 cells could synergise with these factors. Moreover, the co-transfection of both Sp1 and Sp3 showed a lower transcriptional activation in comparison with the sum of the activation obtained when transfecting the two factors independently (Figure 7). This result could be explained by the fact that these factors compete for the same sites, and that Sp3 is a weaker activator of this promoter. Taken together, the above results demonstrated that Sp1 or Sp3 can independently activate the utrophin promoter and synergise with GABP for activation.

Physical interaction between GABPα and Sp1 or Sp3

Synergistic activation of transcription in the heterologous system of *Drosophila* cells suggests direct interaction between GABP and Sp1 or Sp3 factors. We initially tested the putative interaction by incubating ^{35}S-labelled *in vitro* synthesized Sp1 factor with roughly equal amounts of either GST-GABPα, GST-GABPβ or GST bound to glutathione-sepharose beads (Figure 8(a)). The assays were carried in the presence of 50 μg/ml ethidium bromide to exclude the possibility that interactions were dependent on the DNA binding activities of the interacting factors (Lai & Herr, 1992). The labelled Sp1 factor was retained by GST-GABPα beads (10% of input), while it was not retained by the fusion protein GST-GABPβ or by the GST protein alone (Figure 8(b)).

To confirm the *in vivo* interaction, GABPα was co-transfected either with Sp1 or Sp3 expression vectors in SL2 cells. GABPα was immunoprecipitated with anti-tag antibodies and the pellet was revealed with specific anti-Sp1 or Sp3 antibodies. As shown in Figure 9, either Sp1 or Sp3 are co-immunoprecipitated with GABPα.

To map the Sp1 domain responsible for interaction with GABPα, a series of Sp1 deletion

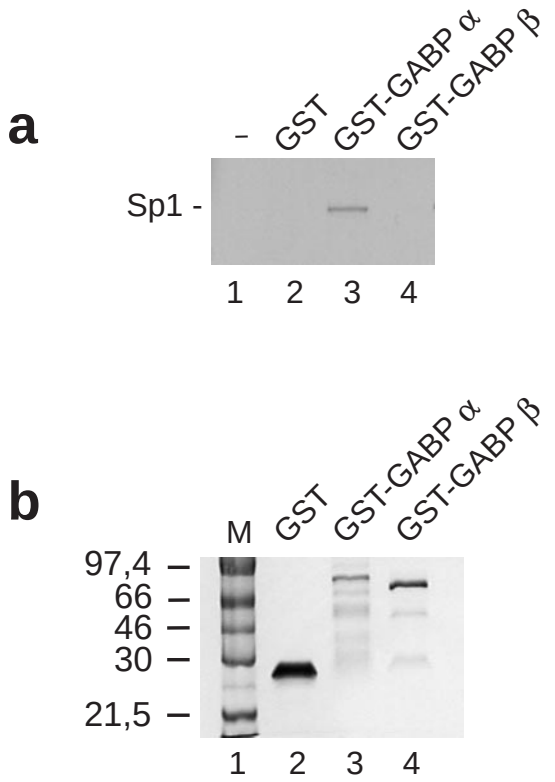

Figure 8. Sp1 and GABP can interact physically *via* GABPα subunit. (a) *In vitro* translated Sp1 protein was incubated with glutathione-Sepharose beads alone (lane 1) or in presence of GST (lane 2), GST-GABPα fusion protein (lane 3) or GST-GABPβ fusion protein (lane 4). The pull-down products were boiled in sample buffer, and the eluates were analysed by electrophoresis in 10% polyacrylamide gel. (b) Input *in vitro* translated polypeptides analysed on a 12% polyacrylamide gel.

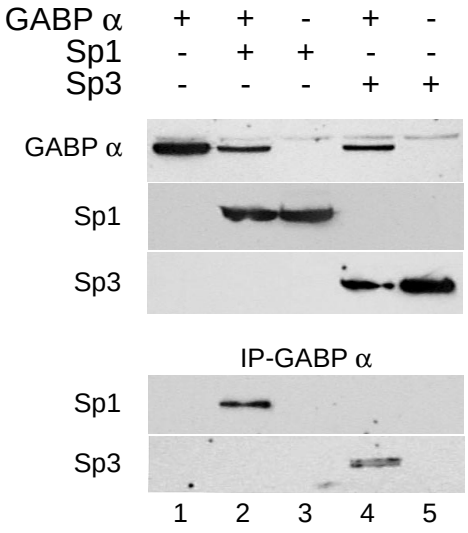

Figure 9. Sp1 and Sp3 co-immunoprecipitate with GABPα. SL2 cells were transfected with the expression vectors pP_{ac}GABPα, pP_{ac}Sp1 and pP_{ac}USp3 as indicated. pP_{ac}GABPα vector carried GABPα cDNA with engineered six consecutive histidine codons at the N-terminal end. An aliquot (10 µl) of total cell extract of each sample was analysed by immunoblotting using antibodies against GABPα, Sp1 or Sp3, to check the protein expression. Cell extracts were immunoprecipitated for GABPα (IP-GABPα) with histidine tag specific monoclonal antibody. Co-immunoprecipitated proteins were separated by SDS-10% PAGE and analysed by immunoblotting using antibodies against Sp1 or Sp3.

mutants (Figure 10(a)) were tested by GST pull-down assay. Roughly equal amounts of labelled Sp1 mutants (Figure 10(b)) were incubated with full-length GST-GABPα. Removal of the zinc finger domain (Sp1 ΔC566) led to the complete loss of the interaction with GST-GABPα (Figure 10(c), lane 2). By contrast, the zinc finger domain alone (Figure 10(c), lanes 3 and 4) was sufficient for direct interaction between these factors.

As with Sp1, both the full-length Sp3 and its DNA-binding domain alone were interacting with GST-GABPα in the *in vitro* binding assay (Figure 11).

Discussion

The upstream utrophin promoter is a typical TATA-less promoter rich in GC residues. It contains a functional N box, recognised by GABP, which has been demonstrated to confer a promoter response to heregulin (Gramolini *et al*., 1999; Khurana *et al*., 1999). We now report the identification and characterisation of three functionally distinct GC elements on the promoter: a proximal element composed of three tandem repeated GC boxes, which behaves as a basal promoter element, and two upstream GC boxes required for full promoter activation. All three GC elements of the utrophin promoter are recognised by the ubiquitous Sp1 and Sp3 factors. Sp1 was originally defined as a proximal promoter factor, required for basal promoter activity, which was thought to function only when located in close vicinity from the transcription start site. However, early experiments also showed that Sp1 can function from distant sites as a weak activator (Courey *et al*., 1989). In line with these observations, the utrophin GC elements, recognised by Sp1 as well as Sp3 factors, was shown to be necessary for utrophin promoter activity. Although a clear functional distinction between these elements cannot be made, the mutation of the proximal GC tandem repeats strongly impaired the promoter basal activity both in rhabdomyosarcoma and in Sp1/Sp3 transfected *Drosophila* SL2 cells, while mutations in the distal sites were less effective in the same conditions. Thus, the function of the proximal GC boxes cannot be entirely replaced by the upstream elements in spite of the fact that all three GC elements are

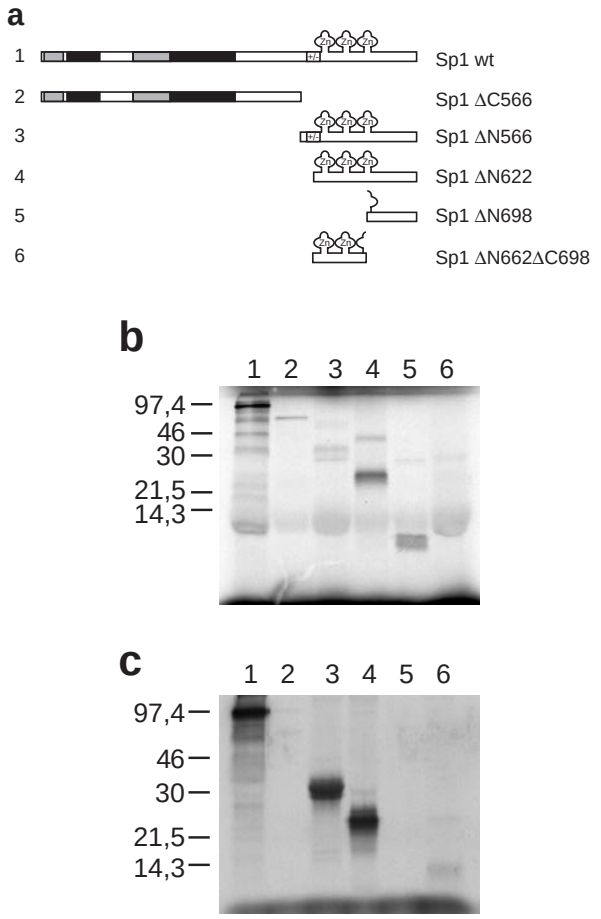

Figure 10. The interaction between Sp1 and GABPα requires the zinc finger domain of Sp1. (a) Schematic representation of the Sp1 proteins used in the GST pull-down assay. Sp1 wt indicates the 696 C-terminal amino acid residues of Sp1. For the purpose of this assay and SL2 transfection, this polypeptide is considered to be wild-type (Courey & Tjian, 1988). The DNA-binding zinc fingers, the serine-threonine-rich domains (gray boxes), the glutamine-rich domains (black boxes) and the high charge density region (+/−) are indicated. Sp1 ΔC566 is a Sp1 polypeptide deleted from the C-terminal. Sp1 ΔN566, Sp1 ΔN662, Sp1 ΔN698 are the N-terminal deletion polypeptides up to amino acid in the position 566, 622 and 698, respectively. (b) Input *in vitro* translated polypeptides analysed on a 15% polyacrylamide gel. The numbers correspond with the polypeptides shown in (a). (c) *In vitro* translated polypeptides were incubated with glutathion-Sepharose beads in presence of GST-GABPα fusion protein. The pull-down products were analysed by electrophoresis in 12% polyacrylamide gel. An over exposure is shown to reveal Sp1 ΔN662ΔC698 (lane 6) which contains only two methionine residues.

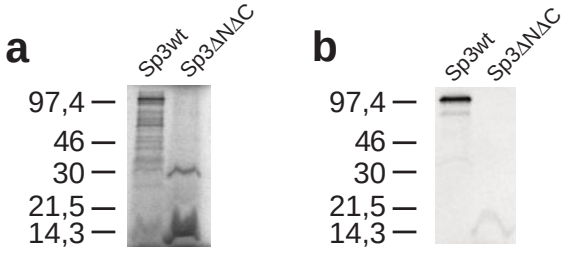

Figure 11. The GABPα physically interacts with Sp3 and requires the zinc fingers domain of Sp3. (a) Input *in vitro* translated polypeptides analysed on a 15% polyacrylamide gel. The mutant Sp3ΔNΔC spans from amino acid in the position 545 to amino acid in the position 624, and corresponds to Sp1 mutant ?N662?C698. (b) *In vitro* translated polypeptides were incubated with glutathione-Sepharose beads in presence of GST-GABPα fusion protein. The pull-down products were analysed by electrophoresis in 12% polyacrylamide gel.

recognised by the same factors, suggesting that Sp1 and Sp3 play a specific role in the promoter architecture. This could be explained by the fact that the proximal GC element binds more than one Sp1/Sp3 molecule. In fact, this element, in contrast to the upstream utrophin GC elements, shows an extended footprint, and gel retardation experiments revealed the formation of slower migrating complexes that are supershifted with anti-Sp1/Sp3 antibodies.

Knockout experiments showed distinct functions of Sp1 and Sp3 during development (Bouwman *et al*., 2000; Marin *et al*., 1997). This could be due to the fact that Sp3 can act as an activator or a repressor in different promoter contexts (Fandos *et al*., 1999; Hagen *et al*., 1994; Liang *et al*., 1996; Majello *et al*., 1997; Udvadia *et al*., 1995; Zhao & Chang, 1997). On the utrophin promoter, transient transfection experiments revealed that both Sp1 and Sp3 behaved as activators, with Sp1 being more active than Sp3 in both *Drosophila* and muscle RD cells.

It has been previously demonstrated that GABP activates the utrophin promoter (Gramolini *et al*., 1999; Khurana *et al*., 1999). Here, we demonstrated that GABP synergises with both Sp1 and Sp3 for utrophin promoter activation, and that both Sp1 and Sp3 directly interact with GABP *in vitro* and *in vivo*. Mapping the Sp1 and Sp3 interaction domains revealed that both Sp factors interact *via* their zinc finger DNA-binding domain.

The GABP factor is composed of one Ets-related GABPα subunit and an ankyrin repeat-containing GABPβ subunit (Batchelor *et al*., 1998; LaMarco *et al*., 1991; Thompson *et al*., 1991; Watanabe *et al*., 1993). GST pull-down experiments revealed that only the GABPα subunit interacts with both Sp1 and Sp3 factors. This result was confirmed by immunoprecipitation experiments in which GABPα co-immunoprecipitated with either Sp1 or Sp3. Thus, our experiments suggest that the synergy between GABP and Sp1/Sp3 is due to direct interaction between the zinc finger domain of either Sp1 or Sp3 and the GABPα subunit. Although we observe direct interaction only with GABPα, in order to activate transcription synergistically both

GABPα/β subunits are required. This result is in line with the observations that the β-subunit is necessary both for DNA binding of the α-subunit and for transactivation of the heteromeric GABP factor (Batchelor *et al*., 1998; Guneja *et al*., 1995, 1996; Thompson *et al*., 1991). Thus, the direct interaction between Sp1/Sp3 and GABPα subunit is able to recruit to the utrophin promoter the active GABPα/β complex.

Our experiments also suggest that post-translational modifications are not required for interaction between GABP and Sp1/Sp3, since we observed direct binding of these factors synthesised *in vitro*. Interestingly, it has been previously demonstrated that stimulation of muscle cells with heregulin increases GABPα protein levels beside inducing the phosphorylation of both α and β-subunits (Altiok *et al*., 1997; Fromm & Burden, 1998; Schaeffer *et al*., 1998). Moreover, using transient transfection experiments it was also demonstrated that the co-transfection of GABP cDNAs, along with the utrophin promoter in muscle cells, resulted in the activation of the promoter transcription to levels comparable to those obtained with cell treatment with heregulin (Gramolini *et al*., 1999; Khurana *et al*., 1999). Thus, the increase of GABP *per se* is sufficient to induce utrophin up-regulation, suggesting that GABPα is present in limiting amounts in muscle cells. Small increase of GABPα can, when interacting with GABPβ, synergise with either Sp1 or Sp3 and increase utrophin transcription.

In addition, in post-synaptic nuclei it has been shown that Sp1 is phosphorylated in response to synaptic-specific stimuli suggesting that, upon phosphorylation, Sp1 may increase its binding to DNA *via* protein-protein interactions, facilitating the formation of Sp1 multimers and/or increasing its interaction with other proteins (Alroy *et al*., 1999). Thus, it is possible that Sp1 phosphorylation may allow a fine modulation of the utrophin promoter in response to extracellular stimuli. Further studies will address the role of Sp and GABP phosphorylations with respect to their interaction. This could be relevant in view of the possible regulation of the utrophin transcription by pharmacological means in DMD and BMD patients.

Materials and Methods

Protein preparation and footprinting

Recombinant Sp1 was expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion protein and purified as previously described (Kadonaga *et al*., 1987) with some modification. Briefly, the insoluble fraction of sonicated *E. coli* culture containing Sp1 was resuspended in 1.3 ml of buffer A (40 mM Tris-HCl (pH 7.7), 20% (w/v) sucrose, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 4 M urea) and incubated one hour at 25 °C. The solution was clarified by centrifugation at 20,000 ***g*** at 4 °C. The supernatant was dialysed against buffer B (20 mM Tris-HCl (pH 7.7), 20% (v/v) glycerol, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 μM $ZnSO_4$, 1 mM dithiothreitol a, 0.2 mM phenylmethylsulfonyl fluoride, 1 M urea) at 4 °C for four hours. Subsequently, two further dialyses were performed in buffer B without urea at 4 °C for three hours and overnight.

The DNA probes for DNase I footprinting was obtained from UPwt plasmid (see below) after digestion with the enzymes *Bst*EI (antisense strand) or *Eag*I (sense strand) and labelled by the Klenow fill-in reaction in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP. The labelled DNA fragments obtained were purified with QIAquick columns (Qiagen Inc., Chatsworth, CA) and digested with *Eag*I and *Pst*I, respectively. The probes were purified on 5% polyacrilamide gel (1× TBE) and ≈20,000 c.p.m. were used for each reaction. Protein-DNA complexes were formed in 50 μl of footprinting buffer (10 mM Tris-HCl (pH 8), 50 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, 10% glycerol, 0.04 mg/ml poly(dI-dC), 1 mg/ml bovine serum albumin) for 20 minutes at room temperature. One microlitre of DNase I (1 unit/μl) in footprinting buffer containing 5 mM $CaCl_2$ was added, and incubation was continued for one minute at room temperature. DNase I digestion was stopped by the addition of stop buffer (192 mM sodium acetate, 32 mM EDTA, 0.14% (w/v) SDS, 64 μg/ml sonicated calf thymus DNA). Samples were then extracted with one volume of phenol-chloroform, precipitated in ethanol and resuspended in 5 μl of sequencing loading buffer (US Biochemicals). After denaturation at 95 °C for four minutes, the samples were subjected to urea 6% gel electrophoresis and autoradiographed.

Nuclear extracts and gel mobility shift assay

Nuclear extracts were obtained as described (Schreiber *et al*., 1989). Probes and competitors for gel mobility shift assay were obtained by annealing of the following oligonucleotides: H65 (TTTCCAACAAAGGGCAGGCCCGCAGCGGGGAGGAGGAGGAGGAGCCG) and H66 (TTTCGGCTCCTCCTCCTCCTCCCCGCTGC GGGCCTGCCCCTTTGTTGG) for probe S1; H67 (TT TCTGCGCCCCACCCTCCCTCCTCCGCCT) and H68 (TTTAGGCGGAGGGAGGGTGGGGCGCAG) for probe S2; H69 (TTTGGGGGGTGGGGGCGGCAACGCGCGA) and H70 (TTTTCGCGCGTTGCCGCCCCCACCCCCC) for probe S3; GATA-F (CGAAATCCGATACTAGATATGAGGT) and GATA-R (CGACCTCATATCTAGTATCGGATTT) for the aspecific competitor. The probes were labelled by Klenow fill-in reaction in presence of [α-^{32}P]dATP and [α-^{32}P]dCTP.

Binding reactions (20 μl) contained 10 μg of nuclear extracts and 2 μg of poly(dI-dC) in 10 mM Tris (pH 7.9), 5 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 4% (v/v) Ficoll. Complexes were allowed to form for 20 minutes on ice and resolved on 5% (39:1) acrylamide-bisacrylamide gels in 0.5% Tris borate-EDTA. Where indicated, 0.5μl of sc-59 X anti-Sp1, sc-644 X anti-Sp3 or sc-335 X anti-SRF polyclonal antibodies (Santa Cruz Biotechnology, Inc.) were added in the reaction mixture.

Plasmid construction

The CAT reporter construct UPwt has been described (Galvagni & Oliviero, 2000). The promoter mutants UPmS1, UPmS2 and UPmS3 were produced by PCR using the G5 (GGATAAGCTTGGAACAGGCTCTATAACAGATG) and G6 (GGATTCTAGA CAATCGGCTTC TGGAGCCAGAGGCT) as external primers, H63 (GA

GATCTAGATTTGTTGGAGCCGAGCGCT) and H64 (GAGATCTAGACCGAAGGAGCGAGCCTCTC), H62 (GAGATCTAGACCAGCGCTCGGCTCCAACAA) and H51 (GAGATCTAGAGCGCAGGACCGCTGGGTCGC), H49 (GAGATCTAGAACCCCCCGGGAAGGGGCTCG) and H48 (GAGATCTAGAGGCAACGCGCGACCCAG CGG) as internal primers for UPmS1, UPmS2 and UPmS3, respectively. The promoter mutant UPΔN was produced by *Bst*EII-*Pst*I digestion of UPwt followed by Klenow fill-in reaction and ligation. Mammalian Sp1/Sp3 expression vectors (CMV-Sp1 and CMV-Sp3) were obtained by cloning the full-length coding sequences of the human proteins in pcDNA3 vector (Invitrogen) under the control of the cytomegalovirus promoter. The Sp1 insert was obtained by PCR amplification of the Sp1 cDNA using the primers H143 (GAGAGAATTCGCCGC-CACCATGACAG GTGAGCTTGACCTCACAGCC) and H144 (GAGAGACTCGAGTCAGAAGCCATTGCCACT-GATATTAATGGACTG), while Sp3 was amplified with primers H145 (GAGAGAATTCGCCGCCACCATGATT CCAAGTGCTGCTACTTCAAGTGGG) and H146 (GAG AGACTCGAGTCACTCCATTGTCTCATTTCCAGAAA CTGTGAC).

For *in vitro* synthesis of radiolabelled proteins, the cDNAs were cloned in-frame in the plasmid pCITE-2a (Novagene). pCITE-Sp1 was created by insertion of the *Eco*RI-*Sal*I-digested Sp1 cDNA from pGEX-Sp1 in pCITE-2a. pCITE-Sp3 was obtained from pPAC-Sp3 by *Bam*HI-*Xho*I digestion and subcloning. The pCITE-Sp1 and Sp3 mutant plasmids encoding various shorter forms of Sp1 and Sp3 were constructed using available restriction sites or polymerase chain reactions.

The sequences of all reporter constructs were confirmed by automated dideoxynucleotide sequencing (Applied Biosystems, Inc., model 377).

Cell culture, transfection, and CAT assay

Human RD rhabdomyosarcoma cells (ATCC CCL-136) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). All recombinant plasmids were purified using the Qiagen plasmid kit (Qiagen Inc., Chatsworth, CA). For each transfection experiment, cells were seeded at 4×10^5 cells/60 mm dish and transfected 18 hours later with an equal molar amount of reporter plasmid (maximum 5 μg) and 0.75 μg of pRSVβgal as transfection efficiency control, using LipofectAMINE™ Reagent and following the manufacturer's indications. Four hours later, serum-free DNA-containing medium was replaced by fresh growth medium and the cells were harvested after 24-48 hours. Cell extracts and CAT assays were performed as described (Galvagni *et al*., 1997).

Drosophila melanogaster Schneider line 2 (SL2) cells were grown at 25°C in Schneider's *Drosophila* medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. Cells were plated onto 35 mm dishes in 3 ml of medium at a density of 1×10^6 cells/ml 16 hours prior to transfection. Transfections of the SL2 cells were performed using the standard calcium phosphate method. Typically, a transfection experiment included 2.5 μg of reporter plasmid, 0.5 μg of phsp82LacZ as transfection efficiency control (Chin *et al*., 1998) and 1 μg of each pP_{ac} *Drosophila* expression vectors (Sun *et al*., 1995). The amount of expression vectors was normalized with the empty pP_{ac} vector. After addiction of DNA, cells were incubated at 25°C and harvested 48 hours later.

GST pull-down assay

Recombinant GABPα and -β were expressed in *E. coli* growing at 30°C as GST fusion proteins, purified by glutathione affinity chromatography and eluted from the column by adding 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.8). *In vitro* expression of radiolabelled proteins was performed in reticulocyte extracts (TNT™ Coupled Reticulocyte Lysate System; Promega) in the presence of [^{35}S]methionine. GST pull-down assays were performed as described (Seto *et al*., 1993). Labelled proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and gels were dried and exposed to X-ray films.

Immunoprecipitation

SL2 cells were plated at 2×10^7 cells per 75 mm flask and transfected 24 hours later with 8 μg of expression vectors pP_{ac}GABP α, pP_{ac}Sp1 and pP_{ac}USp3 as indicated in Figure 9. The total amount of DNA was held constant (16 μg) by addition of empty pP_{ac} expression vector. pP_{ac}GABP α vector carried the murine cDNA of GABPα engineered with six consecutive histidine codons at the N-terminal end of the open reading frame (Sun *et al*., 1995).

After 48 hours, the cells were collected, washed with phosphate-buffered saline and lysed for one hour in 1 ml of lysis buffer containing 25 mM Hepes (pH 7.9), 200 mM KCl, 0.1% (v/v) Nonidet P-40, 10 mM NaF, 0.1 mM $NaVO_4$, 5 mM dithiothreitol and protease inhibitors (complete™, EDTA-free, Roche). Cell debris was removed and 250 μl of the resulting lysate was incubated with mouse anti-histidine tag monoclonal antibody sc-8036 (Santa Cruz biotechnology, Inc.) for one hour on ice. Immune complexes were collected with recombinant G-agarose beads (Roche) by rocking at 4°C for one hour, and the immunoprecipitates washed, resolved by SDS-PAGE and examined by immunoblotting experiment using antibodies against GABPα (kindly provided by Dr Steven McKnight), Sp1 or Sp3.

Acknowledgements

We thank Dr Michael Chin (phsp82LacZ), Dr Barbara Graves (pP_{ac}GABPα and β), Drs Hiroshi Handa and Alan Rosmarin (pGEX-GABPα and β), Dr Guntram Suske (pP_{ac}USp3), Dr Robert Tjian (pP_{ac}Sp1), Dr Yosef Yarden (pGEX-Sp1), and Steven McKnight (anti-GABPα antobodies) for providing the indicated reagents. We also thank Beatrice Grandi for her excellent technical help. This work was supported by Telethon project N 970, by Italian PNR ''Chromatin dynamics and gene expression'' and by EEC program BMH4-CT98-3505.

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Edited by M. Yaniv

(Received 22 August 2000; received in revised form 31 October 2000; accepted 15 November 2000)