

**THE ROLE OF SP2, SP3 AND SP4 IN THE TRASCRIPTIONAL
REGULATION OF THE PROMOTER OF NUCLEAR
ENCODED MITOCHONDRIAL GENES**

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Received 4/7/2012– Accepted 24/4/2012

ABSTRACT

The GC-box is an important transcriptional regulatory element present in the promoters of many mammalian genes, and is found in most, if not all, oxidative phosphorylation (OXPHOS) promoters. In the present study we examine the effects of three Sp1 family members (Sp2, Sp3, and Sp4) on the adenine nucleotide translocase 2, cytochrome c1, F1-ATPase β -subunit, and the mitochondria transcription factor (mtTFA) promoters in *Drosophila* SL2 cell line. Sp3, like Sp1, strongly activates transcription all four promoters. SP4 stimulates, moderately, but Sp2 had no effect. In addition, Sp3 can, like Sp1, inhibit transcription from the proximal promoter of the *ANT2* gene through binding to the Cbox GC element. By contrast, Sp4 and Sp2 do not repress promoter activity. Furthermore, since Sp4 and Sp2 bind to the Cbox repression element on the *ANT2* promoter, but do not repress transcription, inhibition of transcription cannot be explained by steric hindrance of pre-initiation complex assembly. These data suggest that different Sp1 family members differentially affect transcription from the OXPHOS promoters.

Key words: Promoter, Transcriptional regulation, Mitochondrion, Sp1, Sp family.

INTRODUCTION

Three Sp1-related genes (Sp2, Sp3, and Sp4) have been cloned based on their homology with the Sp1 DNA-binding domain (Hagen *et al.*, 1992; Kingsley and Winoto, 1992). Sp1, Sp3, and Sp4 are the best characterized members of this subfamily, and each has been shown to

regulate transcription of a constellation of genes including those governing cell cycle control, oncogenesis, and differentiation (Zhao and Meng, 2005; Suske *et al.*, 2005; Chu and Ferro, 2005; Kaczynski *et al.*, 2003; Black *et al.*, 2001; Phillipsen and Suske, 1999; Suske, 1999) The DNA binding domain of Sp1, Sp3 and Sp4 are highly conserved, and bacterially expressed subfragments of the protein showed that they can bind to GC (GGGCGG) and GT (GGTGTGG) elements with identical affinities (Hagen *et al.*, 1992). In contrast, Sp2 seems to have a different DNA binding specificity, it has higher affinity for a GT box (Kingsley and Winoto, 1992). Sp1, Sp3, Sp4 proteins have two glutamine- and serine/threonine-rich amino acid stretches in the N-terminal part of the molecule. Sp1 and Sp3 are ubiquitously expressed in many mammalian cell lines, whereas Sp4 expression appears to be expressed predominately in brain, testis, epithelial tissue, and developing teeth. Sp2 is expressed in various cell lines, but its tissues expression is still unknown (Suske 2005 *et al.*; Phillipsen and Suske, 1999). In contrast to Sp1, Sp4 is not able to act synergistically through adjacent binding sites (Hagen *et al.*, 1995). However, Sp4-mediated activation is strongly enhanced (superactivated) by a non-DNA binding mutant of Sp1, suggesting that Sp1 can interact directly with Sp4 and that their activation domains are functionally related (Suske 2005 *et al.*; Hagen *et al.*, 1995). Based on the data shown above, it appears that different Sp family members might have different functions *in vivo*.

Functional analysis of Sp3 and Sp4 in mammalian cell lines and *Drosophila* SL2 cells lacking endogenous Sp factors demonstrated that Sp4, and Sp1, are transcriptional activators, whereas Sp3 represses Sp1-mediated activation by competitive binding to the same site (Hagen *et al.*, 1994, Suske 2005 *et al.*). However, Sp3 is a bifunctional protein containing independent modular repressor and activator domains (Lania *et al.*, 1997). Others have also reported that Sp3 can work as an activator under certain circumstances (Liang *et al.*, 1996; Netzker *et al.*, 1997; Prowse *et al.*, 1997). These results raise a question about which factors determine the role of these transcription factors on certain promoter.

With the aim of understanding the nucleo-mitochondrial interactions in mammalian cells, experiments were performed to identify nuclear transcription factors that regulate the expression of nuclear-encoded mitochondrial respiratory genes. Several nuclear transcription factors

and *cis*-acting promoter elements have been implicated in the expression of specific genes encoding OXPHOS proteins (Blattler *et al.*, 2012; Zanotto *et al.* 2008, Van Waveren & Moraes 2008, Zaid *et al.*, 1999). However, not all OXPHOS promoters which have been characterized to date have cognate binding sites for these factors. By contrast, most, if not all, OXPHOS promoters contain GC box elements. We previously tested the role of Sp1 on the expression of four nuclear genes involved in mitochondrial biogenesis (Zaid *et al.*, 1999). In the present paper, we extend our analysis to include Sp2, Sp3, and Sp4.

MATERIALS AND METHODS

The CAT reporter constructs containing human *ANT2*, pCATANT2 (-1238/+46) (Li *et al.*, 1996a), human cytochrome c1, pCATCC1 (-1339/+128) (Li *et al.* ., 1996b), human mtTFA (-634/+92) and F1-ATPase β -subunit promoter fragments (Zaid *et al.*, 1999) were described previously. The human *ANT2* reporter constructs, pANT-87/+8, containing wild type (wt) or a mutated (mut) C box Sp1-element were also prepared as described (Li *et al.*, 1996a). Expression plasmids pPacSp2, pPacSP3, were a gift from Long-Sheng Chang, Ohio State University, USA. pPacSp4 was a gift from G.Suske from Marburg University in Germany.

Growth and transfection of *Drosophila* SL2 and mbn2 (Samakovlis *et al.*, 1992) cells which lacks the endogenous Sp factors were done at the Research Unit of the Department of Biochemistry and Molecular Biology at the Faculty of Medicine, Tripoli university as earlier described (Zaid *et al.*, 1999). CAT (chloramphenicol acetyltransferase) and β -galactosidase activities were measured following the manufactures protocols (Promega). Activation was calculated as the ratio of CAT activities (corrected for transfection efficiencies) in cells transfected with or without the Sp2, Sp3, or Sp4 expression plasmids. All values represent the mean \pm SD (standard deviation) of 3 experiments in which each experimental point was collected in triplicate.

RESULTS

To determine the effects of Sp2, Sp3, and Sp4 on OXPHOS promoter activities, we co-transfected expression vectors for these proteins into

the Sp-deficient SL2 cell line. As demonstrated in Table 1, co-transfection 50 ng of pPacSp3 led to a 30-100 fold activation of reporter gene expression, which is similar to that obtained with Sp1 (Zaid et al., 1999). In contrast, co-transfection of 50 ng of Sp2 expression vector did not stimulate any of the four promoters, which is consistent with the fact that Sp2 lacks a glutamine rich A transactivation domain found in Sp1. Sp2 does, however, retain a glutamine rich domain which is homologous with the Sp1 B transactivation domain, but with a lower percentage of glutamine residues (Kingsley and Winoto., 1992). Sp4 has a moderate stimulatory effect in this system (Table 1). The lack of strong stimulation by Sp4 might be caused by poor expression of this factor as reported earlier (Hagen G., et al 1995). In keeping with their results, we used 4 µg of the pPacSp4 expression vector and 50 ng of the Sp2 and Sp3 vectors. Together, the data suggest that Sp3 and Sp4, but not Sp2, can activate transcription of all OXPHOS promoters.

We previously found that mutating the Cbox Sp1 element in the *ANT2* promoter increased reporter gene expression in transfected mammalian (Li *et al.*, 1996a) and *Drosophila* cell lines (Zaid *et al.*, 1999). These data suggested that Sp1 binding to the Cbox site acts as a repressor. To test the roles of Sp2, Sp3 and Sp4 in repression, we co-transfected *Drosophila* cells with Sp2 or Sp3 or Sp4 expression vectors and a reporter plasmid (pANT-87/+8) containing either a wild type (wt) or mutated (mut) Cbox (Fig 1). Interestingly, Sp3-mediated activation of reporter gene expression is increased several fold if the Cbox is mutated. By contrast, mutating the Cbox has no significant effect of promoter activity supported by Sp4 (Fig 1). These experiments suggest that Sp3 alone shares the ability of Sp1 to repress *ANT2* promoter activation when bound to Cbox. In addition, it appears that different members of the Sp1 family have different effects on the *ANT2* proximal promoter. As expected, co-transfecting Sp2 has no effect on promoter activity.

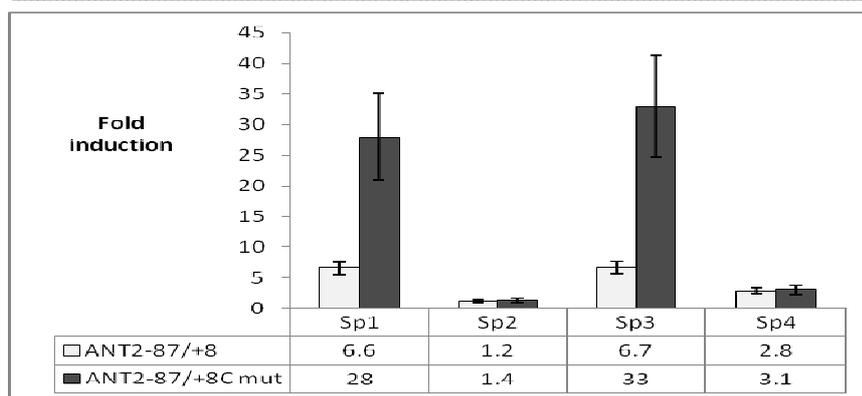


Figure 1. Sp1 and Sp3, but not Sp2 and Sp4, are directly involved in repression of the *ANT2* promoter via the C box. *Drosophila* SL2 cells were transfected with 50 ng of Sp1, Sp2, and Sp3, and 4 µg of Sp4 expression constructs. These expression vectors were transfected with 5 µg reporter plasmids driven either from the wild type-87+8 region of the *ANT2* promoter (-87+8wt) or from the same region containing a mutated Sp1 Cbox element (-87+8mut). Each data point represents the average of triplicate samples. The data are collected from three separate experiments using different plasmid preparations for each experiment.

Table 1. Sp2, Sp3, and Sp3 activation of diverse human OXPHOS promoters in transfected *Drosophila* cells. 5 µg of each reported plasmids pCATANT2(-1238/+46), pCATcc1(-1339/+128), pCATATPase β-subunit (-593/+206), pCATmtTFA(-634/+92) were transfected in SL2 cells either in the present or absence of human pPacSp2 (50 ng), pPacSp3 (50 ng), and pPacSp4 (4 µg) expression vectors. All experimental points were run in triplicate and were corrected for transfection efficiency. Activation by the three factors is expressed in the mean ± S.D. of three independent experiments in which different DNA batches of plasmids were used.

Promotor	Fold activation		
	Sp2	Sp3	Sp4
<i>ANT2</i>	1.4 ± 0.4	93 ± 52	6.9 ± 0.6
Cytochrome c1	1.6 ± 0.2	43 ± 13	4.5 ± 0.5
ATPase β-subunit	1.4 ± 0.7	29 ± 7	5.5 ± 0.5
mtTFA	0.7 ± 0.3	125 ± 50	8.5 ± 0.5

DISCUSSION

We showed previously that Sp1 plays a central role in the expression of several functionally diverse OXPHOS promoters (Zaid *et al.* 1999) In this study; we used the SL2 *Drosophila* cell line to dissect the possible roles of Sp2, Sp3 and Sp4 on the transcription regulation of OXPHOS promoters. Our results suggest that some of the Sp-family member factors might play a general important role in the transcription regulation of OXPHOS promoters (Table 1). These data also support the idea that the expression of OXPHOS subunits are transcriptionally controlled not by a single factor, but by a group of transcription factors which are promoter specific. Interestingly, since the proteins Sp2 and Sp4, are expressed in only limited range of tissues, and Sp3 can work as an activator or repressor depending on promoter context or cell type, this bring us to an interesting suggestion to study the role of Sp3 on the OXPHOS in different cell lines, to see if Sp3 can act as a repressor of certain OXPHOS promoters in certain cell line. Our data here support the possibility that different members of Sp family might have physiological role on the transcription of OXPHOS promoters, especially that inactivation of Sp1 in mouse did not have a dramatic effect at cellular level, and the function of Sp1 appears to be essential for differentiated cells after day 10 of development (Marin *et al.*, 1997). Inactivation of Sp2, Sp3 and Sp4 in mouse has exhibited different phenotypes, which also suggest different roles of these factors on different genes *in vivo* (Baur *et al.*, 2010, Bouwman *et al.*, 2000 and Supp *et al.*, 1996).

The data in Table 1 show that Sp3 work as a strong activator compared with Sp4, although both proteins have similar activation domains to Sp1. One possibility is that strong activation by Sp4 required a certain promoter structure, which not excised in the studied promoter. Another possibility is that Sp4 cannot act synergistically through adjacent binding sites similar to Sp1 (Hagen *et al.*, 1995).

In an attempt to understand the mechanism by which Sp1 represses transcription of the *ANT2* promoter (Luciakova *et al* 2008; Zaid 2001 *et al*; Zaid *et al.*, 1999; Li *et al.*, 1996a;). We compared the effects of Sp2, Sp3 and Sp4. We found that only Sp3 share the ability of Sp1 to represses the *ANT2* proximal promoter by binding to the Cbox (Zaid *et al* 2001; Zaid *et al.*, 1999). Sp1 protein has 4 domains A, B, C and D, only D domain is required for the formation of contacts between

Sp1 complexes on separate Sp1 binding elements on the same promoter (Pascal and Tjian, 1991). We showed earlier that Sp1 D transactivation domain is important for repression mediated by Sp1 on the Cbox of the *ANT2* proximal promoter (Zaid *et al.*, 2001). The observed Sp3 repression via the *ANT2* Cbox might be explained if Sp3 bound on the AB and C boxes could, like Sp1, build multimeric complexes. If so, Sp3 should have a domain similar to the Sp1 D transactivation domain. One possible mechanism that Sp3 might use to repress the *ANT2* proximal promoter via the Cbox is by using its repressing domain. Earlier report showed that Sp3 carries a unique repressing domain at the 5' of the zinc finger binding domain and can compete for the same site of Sp1 (Yu *et al.* 2003; Majello *et al.*, 1997). Transfection experiments with Sp3 deletion mutants might help to clarify the role of Sp3 the *ANT2* Cbox, and might identify which of Sp3 domain is involved in the repression of *ANT2* promoter.

Lack of repression by Sp4 compared with Sp1 (Fig 1) might be explained by findings that, in contrast to Sp1, Sp4 is not able to act synergistically through adjacent binding sites (Hagen *et al.*, 1995). We have shown earlier that in the *ANT2* promoter, interactions between the Sp1 AB and C boxes are crucial for repression, and that these interactions are most properly mediated through the D domain (Zaid, A *et al.*, 2001). However, sequence comparison of the C-terminal regions of Sp1 and Sp4 shows no significant homologies (Hagen *et al.*, 1995), suggesting that the Sp1 D domain and the corresponding domain are not functionally similar. Alternatively, Sp4 might not be able to target histone deacetylase to chromatin as Sp1 does (Doetzlhofer *et al.*, 1999), to date; there is no data in the literature which showed any interaction of Sp4 and histone deacetylases. In addition, there are data which showed Sp4 can compete for the same binding site as Sp1 (Hagen *et al.*, 1995), and since Sp4 expression is limited to a certain tissue, Sp4 might be involved in *ANT2* repression by competing with Sp1 in these tissues. This also suggests that Sp1/Sp4 ratio might be important in transcription regulation of the *ANT2* promoter. A final possible mechanism for the differential effect of different Sp family members on the *ANT2* promoter is that Sp1, Sp3 and Sp4 can interact favorably with distinct factors in the transcription machinery. Sp4 is less well characterized than Sp1, and more studies are required on the interaction of various Sp family members with other cellular factors. In addition it was shown that Sp4 can also act negatively to regulate

expression, most probably by competing with Sp1 (Ahlgren *et al*; 1999; Kwon *et al*; 1999; Yamaguchi *et al*; 1999). Recently, new data from Sp2 knockout mice show major role mouse development and autonomous proliferation of mouse embryonic fibroblasts (Brun., *et al* 2010). The Comparison of the Sp2 knockout phenotype with the phenotypes of Sp1, Sp3 and Sp4 knockout strains shows that, despite their structural similarity and evolutionary relationship, all four glutamine-rich members of the Sp family of transcription factors have distinct non-redundant functions in vivo. The recent data which show a new strategy for treatment of metastatic prostate cancer by the modulation of the activity of Sp family (Malek *et al* 2012), indicate the importance of understanding the mechanism of action of different Sp family members.

ACKNOWLEDGEMENTS

This study was funded by the Libyan National Agency for Scientific Research (NASR). The Authors thanks Dr. B. Dean Nelson from Stockholm University, Sweden for suggestions and comments on the manuscript. The Authors thanks the lab assistants at the research unit of the department of biochemistry at the Faculty of Medicine at Tripoli university where the experiments were conducted.

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