On the Role of the General Transcription Factor Sp1 in the Activation and Repression of Diverse Mammalian Oxidative Phosphorylation Genes*

Ahmed Zaid,[1] Ronggui Li,[1] Katarina Luciakova,[2] Peter Barath,[2] Susana Nery,[1] and B. Dean Nelson[1,3]

To gain insight into the role of the general transcription factor, Sp1, in the expression of nuclear genes involved in mitochondrial biogenesis, we investigated Sp1 activation of the adenine nucleotide translocator 2, cytochrome c_1, F_1-ATPase β subunit, and the mitochondria transcription factor (mtTFA) promoters transfected into *Drosophila* cell lines. The numbers and organization of GC elements vary in the four promoters, but the magnitude of activation by coexpressed human Sp1 was similar. A feature common to the four promoters is the presence of multiple, proximal Sp1-activating elements that account for 50% or more of the transcription activation by Sp1. The distribution and function of individual distal Sp1 elements is less defined and appear to be more promoter-specific. Finally, data from transfected *Drosophila* cells provide the first direct proof for the involvement of Sp1 in the negative regulation of the ANT2 promoter and as a possible participant in repression of the β-subunit promoter. The role of Sp1 in both the positive and negative regulation of OXPHOS promoters is unique.

INTRODUCTION

Several nuclear transfactors, or putative transfactor binding-elements, have been implicated in the expression of specific genes encoding proteins of oxidative phosphorylation (OXPHOS) (Basu *et al.*, 1993; Breen *et al.*, 1996; Efiok *et al.*, 1994; Evans and Scarpulla, 1990; Haraguchi *et al.*, 1994; Li *et al.*, 1990; Seelan Sathiagana and Grossman, 1997; Suzuki *et al.*, 1990, 1991; Vander Zee *et al.*, 1994; Virbasius and Scarpulla, 1991). However, cognate binding sites for these factors are not present in all OXPHOS promoters and, therefore, they probably function in a promoter-specific context. In contrast, GC box binding sites for the Sp1 are found in most if not all OXPHOS promoters characterized to date. Since the level of transcriptional activation supported by Sp1 is strongly influenced by the numbers and organization of Sp1 binding elements within the promoter (Das *et al.*, 1995, Means and Farnham, 1990; O'Shea-Greenfield and Smale, 1992; Smale *et al.*, 1990) and since GC box organization varies widely in OXPHOS promoters (Nelson *et al.*, 1995), it was of interest to test the relative activity of Sp1 on individual OXPHOS promoters. In the present study, we analyzed the control exerted by Sp1 on the promoters of four functionally diverse OXPHOS genes using Sp1-deficient *Drosophila* cells as a model. These cells have been widely used to investigate the action of Sp1 (Courey and Tjian, 1988; Hagen *et al.*, 1994, 1995; Lin *et al.*, 1996) as they allow an assessment of Sp1 function in a background that is relatively uncomplicated by additional transfactors. A common feature that emerges for the OXPHOS promoters inves-

[1] Department of Biochemistry, Arrhenius Laboratories, Stockholm University, S-106 91 Stockholm, Sweden.
[2] Permanent address: Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia.
[3] Author to whom all correspondence should be sent. e-mail: buck@mail.biokemi.su.se.

tigated is the distribution of Sp1-activating sites into proximal and distal groupings, with the proximal elements having a more clearly defined and central role in determining the level of transcription. In addition, we provide data that implicates Sp1 directly in the negative regulation of two of the four promoters tested.

EXPERIMENTAL PROCEDURES

Preparation of Clones

CAT reporter constructs containing wild-type or mutated human ANT2, pCATANT2($-1238/+46$) (Li *et al.*, 1996a), and cytochrome c_1, pCATCC1($-1339/+128$) (Li *et al.*, 1996b), promoter fragments were described previously.

The human mtTFA promoter [nucleotides (nts.) -634 to $+92$, numbered relative to transcription start (Parisi and Clayton, 1991)] was amplified from human peripheral lymphocyte DNA by the polymerase chain reaction (PCR) using $5'$ and $3'$ primers that included *PstI* and *XbaI* sites, and was cloned directly into the polylinker of pCATbasic (Promega). The $-79/+92$ promoter fragment was prepared by cloning a *RsaI*/*XbaI* restriction fragment from fragment $-634/+92$ into pCATbasic. The $-39/+92$ promoter fragment was amplified by PCR, and cloned into a T vector pGEM (Promega) for sequencing. An *EcoRI*/*PstI* fragment containing nts. -39 to $+92$ was digested from pGEM and cloned into the polylinker of pCATbasic.

Preparation of the human F_1-ATPase β-subunit promoter region reporter plasmid pCATATPase($-593/+206$) was described previously (Li *et al.*, 1996. Numbering is relative to the transcription start site 1 reported in Ohta *et al.* (1988). The $-132/+206$ fragment was prepared by digesting the $-593/+206$ fragment with *StyI* and *EcoRI*, blunting the ends with T_4 DNA polymerase and ligating into the blunted *SalI* site in pCATbasic. The correct orientation was checked with *HindIII*/*SalI*. The $-40/+206$ β-subunit promoter fragment was removed with *AatII* and *EcoRI* and then cloned into pCATbasic, as described for the $-132/+206$ fragment.

Cell Culture and Transfection

Drosophila SL2 and mbn2 (Samakovlis *et al.*, 1992) cells were grown at 24°C on 60-mm Falcon plates in Schneider medium (SNE 115, Nord Cell)

containing 10% fetal bovine serum (GIBCO), 2 mM glutamine, 50 U of penicillin, and 50 μg of streptomycin/ml. Actively growing cells (5×10^6 cells) were transfected for 48 h using the calcium phosphate/DNA co-precipitation procedure as described (Di Nocera and Dawid, 1983). All cells were transfected with 5 μg of a CAT reporter plasmid bearing the promoter regions indicated in each figure. To study induction by Sp1, one-half of the cells were cotransfected with 2 μg of a human Sp1 expression vector, pPacSp1 (Courey and Tjian, 1988) and one-half were cotransfected with 2 μg of an empty control plasmid, pGEM. All cells were transfected with pPac-βGAL (a gift from Y. Yngstrom, Stockholm University) as a control of transfection efficiency. Transfections were carried out in triplicate for each experimental point. After 48 h, cells were collected and chloramphenicol acetyl transferase (CAT) and β-galactosidase activities were measured as previously described (Li *et al.*, 1996a). Fold activation was calculated as the ratio of CAT activities (corrected for transfection efficiencies) in cells transfected with or without the Sp1 expression vector.

DNase I Protection Assay

The DNase protection assay was performed as described in (Promega, 1991). Radiolabeled primers for the upper and lower strands were prepared by PCR using $5'$-end labeled oligonucleotides corresponding to nts. 2230–2208 (M13 primer), or nts. 2307–2285, of pCATbasic. Recombinant, purified human Sp1 was from Promega. One footprint unit or less was used in each assay.

RESULTS

Sp1-deficient *Drosophila* cell lines (Courey and Tjian, 1988; Hagen *et al.*, 1994, 1995; Lin *et al.*, 1996) were employed to assess the relative importance of Sp1 on the transcriptional activity of diverse human OXPHOS promoter regions. Transfection of long promoter regions of the human ANT2, cytochrome c_1, mtTFA, and F_1 β-subunit promoter regions show that all were activated 30- to 100-fold by Sp1 (Fig. 1).

To locate the Sp1-activating regions within each promoter, a series of deletion clones were constructed for the ANT2 (Fig. 2), cytochrome c_1 (Fig. 4), mtTFA (Fig. 5) and F_1-β subunit (Fig. 6). A common feature of these promoters is that the proximal region of each
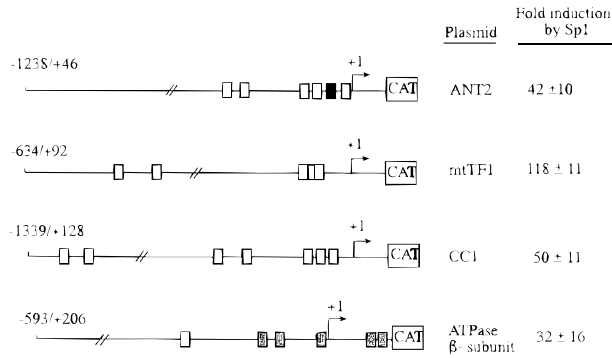
Fig. 1. Sp1 activation of diverse human OXPHOS promoters in transfected Drosophila cells. Reporter plasmids pCA-TANT2(−1238/+46), pCATCC1(−1339/+128), pCATmt-TFA(−634/+92), and pCATATPase β-subunit (−593/+206) were transfected into SL2 cells either in the presence or absence of the human Sp1 expression vector, pPacSp1, as described in the section on Experimental Procedures. All experimental points were run in triplicate and were corrected for transfection efficiency. Activation by Sp1 is expressed as the mean ± S.D. of three independent experiments in which different batches of plasmids were used. Putative or established Sp1-binding sites (open boxes), imperfect Sp1 sites (shaded boxes) protected by Sp1 in the DNase assay (see Fig. 7), and the TATA box (closed boxes) are indicated.
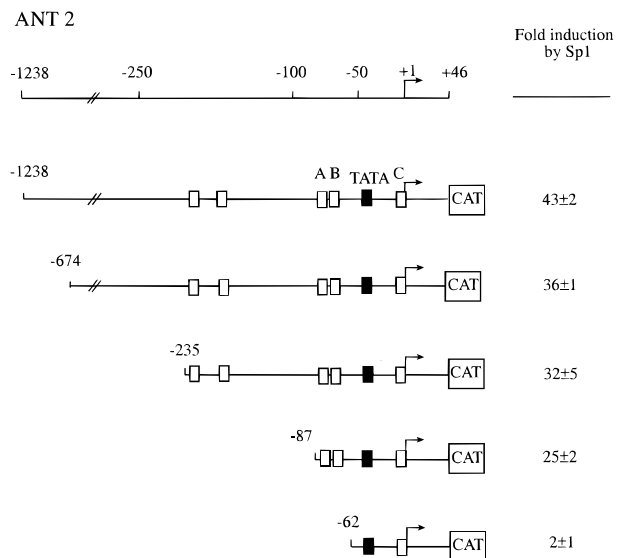

Fig. 2. Sp1 activation of the human ANT2 promoter through multiple sites. Deletion constructs of the human ANT2 promoter linked to the pCAT reporter plasmid were prepared as described in the Section on Experimental Procedures. All experimental points were run in triplicate and were corrected for transfection efficiency. Activation by Sp1 is given as the mean ± S.D of three independent experiments in which different batches of plasmids were used. The boxed elements are defined in Fig. 1.
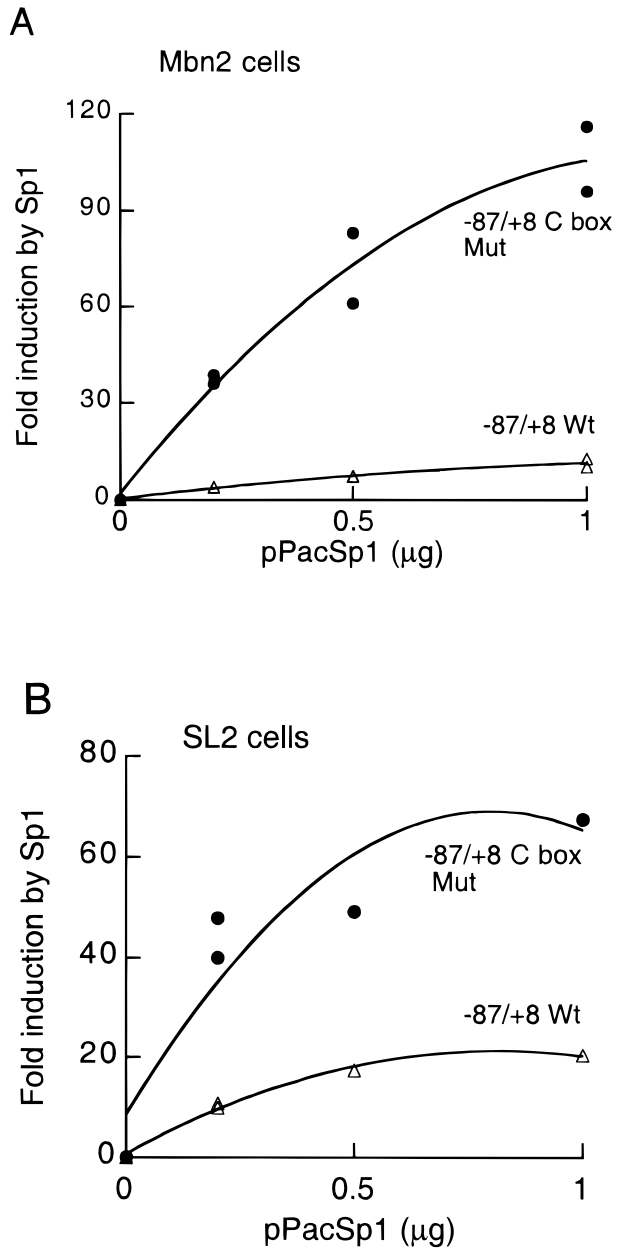

Fig. 3. Evidence that Sp1 is directly involved in suppression of the ANT2 promoter via the C box. *Drosophila* mbn2 cells (A) and SL2 cells (B) were transfected with reporter plasmids driven either from the wild-type −87/+8 region of the ANT2 promoter (−87/+8 Wt) or from the same region containing a mutated Sp1 C box element (−87/+8 Mut; CCGCCC to CCACAC)(Li *et al.*, 1996a). Increasing amounts of the Sp1 expression vector, pPacSp1, were cotransfected. Each data point represents the average of triplicate samples. The data points are collected from two or three separate experiments using different plasmid preparations for each experiment.
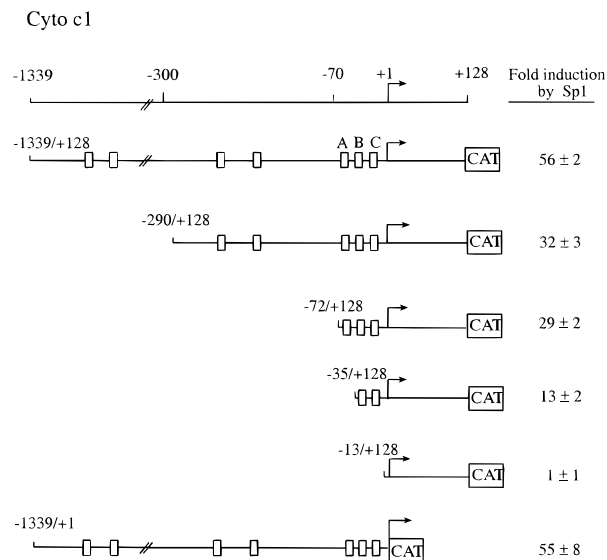
Cyto c1

Fig. 4. Sp1 activation of the human cytochrome c_1 promoter. SL2 cells were transfected with deletion constructs of the human cytochrome c_1 promoter linked to the pCAT reporter plasmid, together or in the absence of cotransfected pPacSp1 as in Fig. 2. The values for activation by Sp1 represent the mean $\pm$ S.D. of three independent experiments in which all experimental points were collected in triplicate. The boxed regions represent core Sp1-binding elements.

contains multiple GC elements that account for a large part of the Sp1-mediated activation. These proximal sites usually lie within 100 bp of the transcription start. Our assignments of proximal Sp1-activating sites confirm and extend data obtained on the four promoters
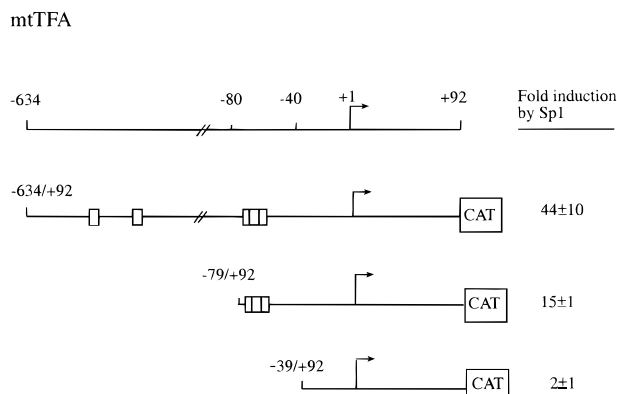
mtTFA

Fig. 5. Sp1 as an essential activator of the human mtTFA promoter. SL2 cells were transfected with deletion constructs of the human mtTFA promoter linked to the pCAT reporter plasmid, together or in the absence of cotransfected pPacSp1. The values for activation by Sp1 represent the mean $\pm$ S.D. of three independent experiments in which all experimental points were collected in triplicate. The boxed regions represent core GC boxes.
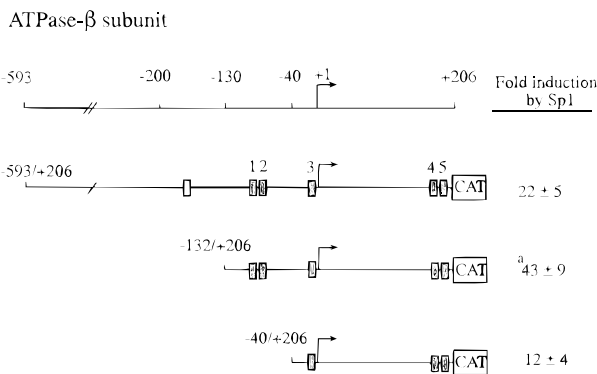
ATPase-β subunit

Fig. 6. Sp1 activation of the human F_1-ATPase β-subunit promoter. SL2 cells were transfected with deletion constructs of the human β-subunit promoter linked to the pCAT reporter plasmid, together or in the absence of cotransfected pPacSp1. The values for activation by Sp1 represent the mean $\pm$ S.D. of three independent experiments in which all experimental points were collected in triplicate. The shaded boxes represent imperfect GC boxes protected by Sp1 in the DNase I protection assay (Fig. 7). The open box represent core Sp1-binding elements.

using mammalian cells (Haraguchi *et al.*, 1994, Li *et al.*, 1996a, b; Villena *et al.*, 1994, Virbasius and Scarpulla, 1994).

In addition to multiple proximal Sp1 elements, the four promoters appear to contain varying numbers of distal Sp1-activating sites (upstream of -100). These are less well defined and may differ in their functions. For example, ANT2 (Fig. 2) and cytochrome c_1 (Fig. 4) promoters contain upstream Sp1 consensus elements (CCGCCC) that appear from deletion experiments to contribute little or nothing to Sp1-mediated transcription in the *Drosophila* system. Furthermore, the locations of active and inactive GC boxes can be widely spread. For instance, in cytochrome c_1, GC boxes close to the proximal promoter region (in fragment $-290/-73$) do not support Sp1 activation, whereas removal of more distal GC boxes ($-1339/-291$) is accompanied by loss of Sp1-mediated activation. With the exception of the latter sites ($-1339/-291$), all of the above mentioned GC elements in ANT2 and cytochrome c_1 have been shown to bind Sp1 in the DNase I protection assay (Li et al., unpublished data) and, therefore, represent the most probable site of Sp1 interaction on these promoters.

We previously showed that mutating the C box Sp1 element in the ANT2 promoter (see Fig. 2) leads to increased reporter gene expression in transfected mammalian cell lines (Li *et al.*, 1996a). These findings were interpreted to mean that Sp1 binding to the C box site acts as a suppressor, which is a unique function

for Sp1. However, the presence of endogenous Sp1 prevented a direct test of this conclusion. We therefore conducted these experiments in *Drosophila* cells cotransfected with a human Sp1 expression vector. Results with two *Drosophila* cell lines show that Sp1-mediated activation is increased several fold if the C box alone is mutated (Fig. 3) and that this activation is dependent upon the A and B box activating sites (Table 1), as previously reported for mammalian cells (Li *et al.*, 1996a). Thus, these experiments provide the first unequivocal proof that Sp1 is involved in repressing transcription via the C box.

The response of the β-subunit promoter to Sp1 differed somewhat from the other OXPHOS promoters tested. First, as observed in transfected mammalian cells (Haraguchi *et al.*, 1994; Villena et al., 1994), removal of nts. −593 to −133 containing the single-core Sp1 element (CCGCCC) increased, rather than decreased, promoter activity in SL2 cells (Fig. 6). Together, these data suggest the presence of negative regulatory elements in this region of the β-subunit promoter. Our findings in SL2 cells extend this observation considerably since they directly implicate Sp1 in the negative regulatory process and raise the possibility that repression could be mediated through the single conserved GC element present in this fragment. Sp1-dependent activation in the absence of the negative regulatory region appears to be supported via several Sp1-bindings sites that deviate slightly from the consensus sequence, as shown by DNase I protection (Fig. 7). Removal of the distal DNase I-protected GC boxes, including the strongly protected box 5 (Fig. 7), decreased Sp1-dependent activation by 70% (Fig. 6). The remaining strongly protected GC boxes (3, 4, and 5) still support a 12-fold activation by Sp1 (Fig. 6).

DISCUSSION

Our experiments show that Sp1 plays a central role in the transcriptional expression of several functionally diverse OXPHOS promoters (ANT2, cytochrome c_1, mtTFA, and the F_1-ATPase β subunit). Indeed, Sp1-activating sites seems to be the single, common feature shared by these promoters. The numbers and positions of the Sp1 sites vary in the four promoters, but appear to be distributed as clusters within the proximal region sites (within the first 100 nts.) and as more dispersed individual elements in the distal regions. Both groupings contribute to Sp1-mediated transcription, but the requirement for the
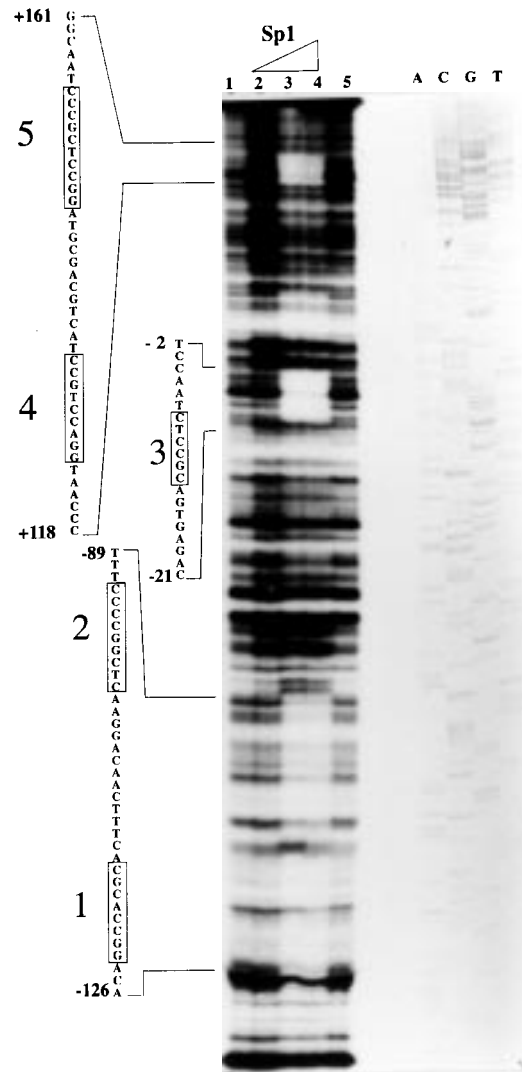

Fig. 7. DNase I footprint analysis of Sp1 binding to the F_1-ATPase β-subunit core promoter. DNase footprint analysis of the F_1-ATPase β-subunit promoter region (nt. −132 to nt. +206) was performed using increasing concentrations of purified human Sp1 (0.25, 1.0, and 1.5 footprinting units; lane 2–4, respectively). No protein was present in lanes 1 and 5. The sequence for the upper strand is shown. The complete sequence of the same region is shown on the right. The three protected regions contain five GC rich boxes (boxed sequences 1–5) that represent putative Sp1-binding sites. Nucleotide numbering is relative to transcription start site no. 1 (Ohta *et al.*, 1988).

proximal sites is a more clearly defined and consistent feature. Our findings, emphasizing a central role of the proximal elements, agree well with results obtained with the individual promoters when tested in mammalian cells (Carter *et al.*, 1992; Evans and Scarpulla, 1989; Haraguchi *et al.*, 1994; Li *et al.*, 1996a,b; Virbas-

ius and Scarpulla, 1994). Proximal promoter Sp1 elements (-100 to $+100$) are common in OXPHOS promoters (Nelson *et al.*, 1995) and most probably represent a general feature of these promoters. We propose that the Sp1 elements in the proximal core promoter may play a central role in determining the relative rates of transcription of the OXPHOS genes.

We also provide the first unequivocal proof that Sp1 acts as a repressor on the ANT2 promoter. Previous studies on mammalian cells lines (Li *et al.*, 1996a) suggested a repressor function for Sp1, but this could not be proved because of the presence of endogenous Sp1 and the possible participation of addition mammalian transfactors. Utilizing the *Drosophila* system, we now demonstrate that Sp1 alone enhances promoter activity when its binding to the mutated Sp1 C box element (Li *et al.*, 1996a) is eliminated. The detailed molecular mechanism by which Sp1 decreases promoter activity from the wild-type C box remains to be studied. However, our results suggest that Sp1 may act through a mechanism that is fundamentally different from those in which Sp1 activation is prevented by: (a) associating with a suppressor protein (Chen *et al.*, 1994; Mukhopadhyay *et al.*, 1997), or (b) through competition for Sp1-binding sites (Harrington *et al.*, 1993; Suzuki *et al.*, 1995).

Our results also provide insight into the negative acting upstream region of the β-subunit promoter. While others have shown that deletion of this region results in activation of the proximal β-subunit promoter in transfected mammalian cells (Haraguchi *et al.*, 1994; Villena *et al.*, 1994), our data show that this response is related to the action of Sp1 alone. Furthermore, our results raise the possibility that negative regulation involves the single conserved core Sp1 element found in the promoter. In support of this, a two- to threefold increase in β-subunit promoter activity has been observed upon mutating the Sp1 element (Hodny, unpublished). However, several possible models can be evoked to explain the mechanism by which Sp1 activation is decreased via distal sites and to choose between them requires an analysis of the possible contribution by the entire distal region, even that beyond nt -200.

Finally, our data show that the β-subunit promoter can be strongly activated by Sp1 acting through binding sites that deviate slightly from consensus Sp1 elements. Although the physiological relevance of these sites is unclear at the moment, it is possible that they participate in activating transcription from a second start site that has been mapped *in vivo* (Ohta *et al.*, 1988) and *in vitro* using the HeLa nuclear extract tran-

scription system (K. Luciakova and B. Nelson, unpublished). GC box 5 (Fig. 6) is located only 35 nts upstream of the second start site (Ohta *et al.*, 1988) and is the only apparent transfactor binding element between transcription start sites 1 and 2. Thus, it is possible that GC boxes 2 and 5 are utilized to initiate transcription from start site 1 and 2, respectively. This remains to be rigidly tested.

In summary, using Sp1-deficient *Drosophila* cells lines, we have been able to partially characterize the response of diverse OXPHOS promoter to the general transcription factor, Sp1, and to provide proof for the direct involvement of Sp1 in the unique role of a repressor molecule.

REFERENCES

Basu, A., Park, K., Atchison, M. L., Carter, R. S., and Avadhani, N. G. (1993). *J. Biol. Chem.* **268**, 4188–4196.

Breen, G. A. M., Vander Zee, C. A., and Jordan, E. M. (1996). *Gene Expression* **5**, 181–191.

Carter, R. S., Bhat, N. K., Basu, A., and Avadhani, N. G. (1992). *J. Biol. Chem.* **267**, 23418–23426.

Chen, L. I., Nishinaka, T., Kwan, K., Kitabayashi, I., Yokoyama, K., Fu, Y.-H. Courey, A. J. and Tjian, R. (1988). *Cell* **55**, 887–898.

Das, G., Hinkley, C. S., and Herr, W. (1995). *Nature* **374**, 657–660.

Di Nocera, P. P. and Dawid, I. B. (1983). *Proc. Natl. Acd. Sci. USA* **80**, 7095–7098.

Efiok, B. J. S., Chiorini, J. A. and Safer, B. (1994). *J. Biol. Chem.* **269**, 18921–18930.

Evans, M. J. and Scarpulla, R. C. (1989). *J. Biol. Chem.* **264**, 14361–14368.

Evans, M. J. and Scarpulla, R. C. (1990). *Genes Develop.* **4**, 1023–1034.

Hagen, G., Muller, S., Beato, M., and Suske, G. (1994). *EMBO J.* **13**, 3843–3851.

Hagen, G., Dennig, J., Preib, A., Beato, M., and Suske, G. (1995). *J. Biol. Chem.* **270**, 24989–24994.

Haraguchi, Y., Chung, A. B., Neill, S., and Wallace, D. C. (1994). *J. Biol. Chem.* **269**, 9330–9334.

Harrington, M. A., Konicek, B., Song, A., Xia, X.-L., and Fredericks, W. J. (1993). *J. Biol. Chem.* **268**, 21272–21275.

Li, K., Hodge, J. A., and Wallace, D. C. (1990). *J. Biol. Chem.* **265**, 20585–20588.

Li, R., Hodny, Z., Luciakova, K., Barath, P., and Nelson, B. D. (1996a). *J. Biol. Chem.* **271**, 18925–18930.

Li, R., Luciakova, K., and Nelson, B. D. (1996b). *Eur. J. Biochem* **241**, 649–656.

Lin, S.-Y., Black, A. R., Kostic, D., Pajovic, S., Hoover, C. N., and Azizkhan, J. C. (1996). *Mol. Cell. Biol.* **16**, 1668–1675.

Means, A. L. and Farnham, P. J. (1990). *Mol. Cell. Biol.* **10**, 653–661.

Mukhopadhyay, D., Knebelmann, B., Cohen, H. T., Ananth, S., and Sukhatme, V. P. (1997). *Mol. Cell. Biol.* **17**, 5629–5639.

Nelson, B. D., Luciakova, K., Li, R., Betina, S. (1995). *Biochim. Biophys. Acta* **1271**, 85–91.

O'Shea-Greenfield, A., and Smale, S. T. (1992). *J. Biol. Chem.* **267**, 1391–1402.

Ohta, S., Tomura, H., Matsuda, K., and Kagawa, Y. (1988). *J. Biol. Chem.* **263**, 11257–11262.

Parisi, M. A. and Clayton, D. A. (1991). *Science* **25**, 965–969.

Promega (1991). *Promega Protocol and Application Guide*, 2nd edn.

Samakovlis, C., Asling, B., Boman, H. G., Gateff, E., and Hultmark, D. (1992). *Biochem. Biophys. Res. Commun.* **188**, 1169–1175.

Seelan Sathiagana, R. and Grossman, L. I. (1997). *J. Biol. Chem.* **272**, 10175–10181.

Smale, S. T., Schmidt, M. C., Berk, A. J., and Baltimore, D. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 4509–4513.

Suzuki, H., Hosokawa, Y., Toda, H., Nishikimi, M., and Ozawa, T. (1990). *J. Biol. Chem.* **265**, 8159–8163.

Suzuki, H., Hosokawa, Y., Nishikimi, M., and Ozawa, T. (1991). *J. Biol. Chem.* **266**, 2333–2338.

Suzuki, M., Kuroda, C., Oda, E., Tsunoda, S., Nakamura, T., Nakajima, T., and Oda, K. (1995). *Mol. Cell. Biol.* **15**, 5423–5433.

Vander Zee, C. A., Jordan, E. M., and Breen, G. A. M. (1994). *J. Biol. Chem.* **269**, 6972–6977.

Villena, J. A., Martin, I., Vinas, O., Cormandi, B., Iglesias, R., Mampel, T., Giralt, M., and Villarroya, F. (1994). *J. Biol. Chem.* **269**, 32649–32654.

Virbasius, J. V. and Scarpulla, R. C. (1991). *Mol. Cell. Biol.* **11**, 5631–5638.

Virbasius, J. V. and Scarpulla, R. C. (1994). *Proc Natl. Acad. Sci. USA* **91**, 1309–1313.