Different Sp1 family members differentially affect transcription from the human elongation factor 1 A-1 gene promoter

Søren J. NIELSEN^{1,2}, Morten PRÆSTEGAARD¹, Helle F. JØRGENSEN and Brian F. C. CLARK³

Department of Biostructural Chemistry, Institute of Molecular and Structural Biology, University of Aarhus, Gustav Wieds Vej 10C, Forskerparken, Århus, Denmark

The GC box is an important transcriptional regulatory element present in the promoters of many mammalian genes. In the present study we examine the effect of known GC-box-binding proteins on the promoter of the human elongation factor 1 A-1 (hEF1A-1) gene in human HeLa cells and *Drosophila* SL2 cells. In HeLa cells co-transfection with the GC-box-binding protein BTEB resulted in a 4–10-fold increase in hEF1A-1 promoter activity. This stimulation was dependent on a single GC box located between positions –69 and –50 of the promoter. Little or no effect was observed of other GC-box-binding proteins including Sp1, Sp3, Sp4 and BTEB2. In SL2 cells stimulation by

Sp1 and Sp3 through the single GC box of the proximal promoter led to 13-fold and 21-fold increases respectively in promoter activity. Inclusion of further upstream sequences resulted in high levels of expression when Sp1 or Sp3 was co-transfected with the reporter plasmid. In this setting Sp1 stimulated transcription by 750-fold, whereas Sp3 was even more potent, yielding a 1150-fold stimulation. Mobility-shift assays performed with the promoter-proximal GC box demonstrated the binding of Sp1, Sp3 and Sp4 to this sequence. To our knowledge, the present study represents the first comparison of all known GC-box-binding proteins on a natural promoter.

INTRODUCTION

Transcriptional initiation in eukaryotes requires the ordered assembly at the promoter of a large number of protein factors into a functional preinitiation complex [1,2]. This process is regulated by a diverse group of gene-specific activator and repressor proteins, many of which have been cloned and characterized [3,4]. The first mammalian gene-specific transcription factor to be cloned was the activator Sp1 [5]. Sp1 contains three zinc-fingers of the Cys2-His2 type, and these mediate the binding of Sp1 to a number of GC-rich sequences collectively referred to as GC boxes [5,6]. Functional analysis of Sp1 in Sp1-negative *Drosophila* SL2 cells demonstrated that Sp1 might stimulate transcription by multiple distinct mechanisms. Apart from activation from a single Sp1-binding site, Sp1 stimulates transcription in a synergistic manner when multiple Sp1 sites are present in either a juxtaposed or a separate organization [7,8]. In addition, the co-expression of a non-DNA-binding mutant of Sp1 did not interfere with transcriptional activation; rather, it superactivated transcription possibly by forming more potent higher-order complexes with DNA-bound wild-type Sp1 [8]. Two molecules of DNA-bound Sp1 also possess the ability to multimerize, resulting in looping out of the intervening DNA [9,10]. Biochemical dissection of Sp1 led to the delineation of four separate activation domains that contribute differentially to the processes of simple activation, synergistic activation and superactivation [7,11].

Although some variations exist, Sp1 can be detected in all tissues [12]. GC boxes are found in a large number of mammalian promoters, suggesting that Sp1 might be a crucial factor for the expression of many genes [6]. This has been tested recently by generating mice in which both Sp1 alleles have been disrupted [13]. This mutation has adverse effects on mouse development:

homozygous mutant mice die very early during embryogenesis, demonstrating that Sp1 is indeed a fundamentally important cellular factor. However, of a number of putative Sp1 target genes tested, only two showed decreased expression in the absence of Sp1. This suggests the existence of several, at least partly, redundant GC-box-binding factors. Indeed, a number of factors capable of binding to Sp1-binding sites including Sp3, Sp4, BTEB and BTEB2 have been identified [14–17]. Sp3 and BTEB possess both activating and repressing activity, whereas Sp4 and BTEB2 are genuine activators [16–19]. In addition, other zinc-finger transcription factors such as Zif268 bind to GC-rich sequences, but these factors have distinct sequence requirements [20].

Eukaryotic protein synthesis elongation factor 1 A (eEF1A, formerly abbreviated EF-1 Alpha) delivers aminoacyl-tRNA to the ribosomal A site in a GTP-dependent manner [21]. More recent observations suggest that eEF1A might have cellular roles beyond its participation in protein synthesis. eEF1A binds to and structurally modifies both microtubules and actin filaments [22,23]. Moreover, it activates phosphatidylinositol 4-kinase and interacts with calmodulin in a Ca²⁺-dependent manner [24,25]. Circumstantial evidence for a role for eEF1A during mitotic spindle assembly has been reported [26,27].

Mammals contain two different eEF1A isoforms, eEF1A-1 and eEF1A-2 [28–33]. In humans, hEF1A-1 is expressed at high levels in all tissues examined except for skeletal muscle, with intermediate levels of expression being detected in brain and heart. In contrast, hEF1A-2 is most abundant in skeletal muscle, lower levels being present in heart and brain. All other tissues examined show no hEF1A-2 expression [32]. An almost identical expression pattern is observed in rats [34]. hEF1A-1 expression might be regulated at both the transcriptional and the translational levels. Owing to a polypyrimidine motif located at the

Abbreviations used: CAT, chloramphenicol acetyltransferase; eEF1A, eukaryotic elongation factor 1 A; hEF1A-1, human elongation factor 1 A-1; tk, thymidine kinase.

¹ The first two authors contributed equally to this work.

² Present address: Wellcome/CRC Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QE, U.K.

³ To whom correspondence should be addressed (e-mail clark@biobase.dk).

extreme 5' end of the mRNA, hEF1A-1 is translationally repressed in serum-starved cells [35,36]. hEF1A-1 mRNA is hyperabundant in a variety of human tumours compared with normal tissue [37]. Similarly, cultured cells express higher amounts of eEF1A-1 mRNA than the corresponding tissues [38]. This is indicative of a requirement for high eEF1A-1 expression during rapid growth. Consistent with this a high, deregulated level of eEF1A-1 expression leads to increased transformation susceptibility in a mouse cell line [39].

The regulatory regions of the human hEF1A-1 gene are not yet fully characterized. An enhancer located in the first intron of the gene together with two elements (termed EFP1 and EFP2 by their discoverers) in the vicinity of the TATA box might be important determinants for the regulation of hEF1A-1 transcription [40,41]. In addition we have shown that further elements located upstream of the EFP1 site contribute to the overall level of transcription from this promoter (S. J. Nielsen, M. Præstegaard, H. F. Jørgensen and B. F. C. Clark, unpublished work). The identity of the factors binding to the EFP1 site is not known, whereas the EFP2 element was previously shown to bind Sp1 in band-shift and footprinting experiments [41]. However, it was not established whether this interaction is required for transcriptional activity mediated by this element. Furthermore the putative interaction of other members of the Sp1 family with the EFP2 element was not investigated. Here we demonstrate that the remaining factors binding to this element are variants of Sp1 and Sp3. In addition we investigate the effect of individual GC-box-binding proteins on the hEF1A-1 promoter in both human HeLa cells and *Drosophila* SL2 cells.

MATERIALS AND METHODS

Plasmids

For the generation of reporter plasmids containing the 5' end of the human hEF1A-1 gene, a 2.4 kb *EcoRI*/*PstI* fragment was excised from pEFq1 [42] (kindly provided by S. Nagata) and inserted into the *EcoRI* and *PstI* sites of pBluescript KS- (Stratagene) to generate pEF-ep. Subsequently, all sequences upstream of the TATA box of pEF-ep were amplified with the primer 5'-TACGGTTCTCCCCACC-3' and the universal M13 reverse primer and inserted into the *SmaI* site of pBluescript KS-. From there it was subcloned into pBLCAT5 (in which CAT stands for chloramphenicol acetyltransferase) [43] (vectors from the pBLCAT series were kindly provided by G. Schütz) by using *Bam*HI and *Sal*I, thus generating pEFCAT5. From pEFCAT5, nested deletions were generated by the *Exo*III/*S1* procedure [44] with the Double Stranded Nested Deletion Kit from Pharmacia. To generate variants of these lacking the cryptic Sp1 site of the thymidine kinase (*tk*) promoter [45], sequences between the unique *Bam*HI and *Rsr*II sites were excised to generate pEFA1090CATSp1-, pEFA119CATSp1- and pEFA119EFP2*CATSp1- (see Figure 2 for diagrams of these plasmids). The mutations in the EFP1 and EFP2 elements were introduced with the U.S.E. Mutagenesis Kit from Pharmacia. The primer 5'-GTCTGGATCTCGATGCATGCA-TGCCTGCAGG-3' was used for restriction site selection and the mutagenic oligonucleotides were EFP1* (5'-GGTGGCGC-GGGGTTTTCTGGGTTTGTGATGTCGTGTTACTGG-3') and EFP2* (5'-CGTGTACTGGCTCATACTTTTTCCCGAG-GG).

pPac0 and pPacSp1 were supplied by Dr. R. Tjian [7]; Dr. G. Suske provided us with pPacUSp3, pPacSp4 and pCMVSp4 [18,19,46]. pCMVSp1/flu and pCMVSp3/flu were from Dr. J. Horowitz [47,48], and pRSVBTEB and pCMVBTEB2 were supplied by Dr. Fujii-Kuriyama [16,17].

Cell culture, transfections and reporter assays

Drosophila Schneider SL2 cells were purchased from ATCC. They were cultured in Schneider's *Drosophila* medium supplemented with 10% (v/v) heat-inactivated fetal calf serum in atmospheric CO₂ at ambient temperature. HeLa cells and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum in a humidified air/CO₂ (19:1) atmosphere at 37 °C. SL2 cells were transfected by a slight modification of the procedure of Di Nocera and Dawid [49]. In brief, the day before transfections, cells were seeded in six-well trays at a density of 3×10^5 cells/ml. Each well received 0.7 µg of reporter plasmid together with the specified amount of expression plasmid. The total amount of plasmid was kept constant at 2.7 µg per well, with the balance being made up with the empty expression plasmid pPac0. After transfection, the cells were incubated for 44–48 h at room temperature before being harvested by pipetting. Cells were processed for CAT assays by the phase-extraction method as described [50]. CAT activities were normalized to protein content in the samples.

HeLa cells were transfected by the calcium phosphate coprecipitation method as described [51]. Each well of a six-well plate received 1.4 µg of reporter plasmid together with an equal amount of the specified expression plasmid or empty CMV vector. For normalization of transfection efficiency we included 0.7 µg of pCMV-βGal (in which βGal stands for β-galactosidase) in each transfection. CAT assays were performed as above. All transfections were performed in triplicate and were repeated at least twice with nearly identical results.

Nuclear extracts and mobility-shift assays

Nuclear extracts were prepared by a slight modification of the method of Dignam [52] as described previously [53]. For gel mobility-shift (band-shift) assays, 3.5 µg of nuclear extract was incubated for 15 min at 37 °C in 20 mM Hepes (pH 7.9)/1.5 mM MgCl₂/50 mM KCl/0.5 mM dithiothreitol/0.1 µg/µl poly(dI-dC)·(dI-dC) [designated poly(dI-dC)]/4% (w/v) Ficoll/4 µg/µl BSA, together with the appropriate antibodies or competitor oligonucleotides. Subsequently 10000 c.p.m. of T4 polynucleotide kinase-end-labelled EFP2 double-stranded oligonucleotide was added and the reaction was left for 30 min at 37 °C before loading on a 4% (w/v) polyacrylamide gel containing 1 × Tris/glycine/EDTA buffer. The gel was run for 90 min at 130 V before being dried and exposed to X-ray film (Fuji). The sequences of the double-stranded EFP2 oligonucleotides are 5'-CT-AGATGTACTGGCTCCGCCTTTTCCCGG-3' and 5'-GATCCCGGGAAAAAGCGGAGCCAGTACAT-3', whereas the double-stranded EFP1 oligonucleotides are 5'-AGC-TTGGCGCGGGGTAAACTGGGAAAGTCG-3' and 5'-TC-GACGACTTTCCAGTTTACCCCGCGCCA-3'. The polyclonal Sp1 antiserum was kindly provided by J. Horowitz; the Sp3 antibody (sc-644 X) was purchased from Santa Cruz Biotechnology.

RESULTS

Proteins binding to the EFP2 element

By using transcription *in vitro* it has been demonstrated that the human hEF1A-1 promoter has very strong transcriptional activity [42]. Part of this activity was ascribed to the Sp1-binding EFP2 element located between -69 and -50 relative to the transcriptional start site ([41], and S. J. Nielsen, M. Præstegaard, H. F. Jørgensen and B. F. C. Clark, unpublished work). Members of the Sp1 family bind to GC boxes in DNA through three zinc-fingers of the Cys2-His2 type. All the residues involved in

Figure 1 Sp1 and Sp3 bind the EFP2 element

(A) Competition band shift analysis with a 32 P-end-labelled EFP2 oligonucleotide incubated with HeLa cell nuclear extracts (lanes 2–5). Unlabelled competitor oligos were in 100-fold excess (lanes 3–5). Lane 1, probe without extract; lane 2, extract only; lane 3, 1 pmol of EFP1; lane 4, 1 pmol of EFP2; lane 5, 1 pmol of Sp1. (B) Immunodepletion band shift with antibodies directed against Sp1 and Sp3. HeLa or HEK 293 nuclear extracts were incubated with a 32 P-end-labelled EFP2 oligonucleotide and treated with Sp1- and Sp3-specific antibodies. Lane 1, probe without extract; lanes 5–9, HeLa cell nuclear extract; lanes 10–14, HEK 293 cell nuclear extract; lanes 2, 7, 9, 12 and 14, anti-Sp1 antibody; lanes 3, 8, 9, 13 and 14, anti-Sp3 antibody; lanes 4, 6 and 11, unrelated serum. Sp1- and Sp3-containing complexes are indicated with arrows. Sp1/Sp3 denotes a possible contribution from both proteins to this band. An asterisk marks non-specific complexes.

the zinc-finger fold and in the determination of target site specificity are highly conserved between Sp1, Sp3, Sp4, BTEB and BTEB2. The EFP2 element can be considered a divergent Sp1 element as it contains multiple substitutions in the GC box consensus. However, it has not been established how these substitutions affect regulation by Sp1 family members. We therefore decided to investigate the effect of known Sp1 family members on the hEF1A-1 promoter. First we examined the nature of endogenous HeLa and HEK 293 proteins binding to the EFP2 element by using band-shift analyses with an end-labelled oligonucleotide encompassing the EFP2 element and surrounding sequences from the human hEF1A-1 promoter. The specificity of the assay was established in competition experiments, as shown in Figure 1(A). Incubation of the EFP2 probe in the absence of competitor DNA resulted in three retarded bands (Figure 1A, lane 2). These complexes were not affected by the inclusion of a molar excess of an oligonucleotide spanning the neighbouring, unrelated EFP1 site (Figure 1A, lane 3). In contrast, an excess of unlabelled EFP2 element completely abolished complex formation and similar results were obtained with an oligonucleotide containing a consensus Sp1 site (Figure 1A, lanes 4 and 5).

The only known GC-box-binding factors present in HeLa cells are Sp1 and Sp3. To identify the factors binding to the EFP2 element we therefore employed antibodies directed against these two proteins in the band-shift analysis. As expected [41], the slowest-migrating band originated from Sp1 (Figure 1B, lanes 5–7). Our analysis also demonstrates that the fastest-migrating band was caused by Sp3 (compare lanes 5 and 8). The band that showed intermediate mobility seemed to be affected by antisera directed against both Sp1 and Sp3 (Figure 1B, lanes 7 and 8). Accordingly, this band is labelled Sp1/Sp3 in the Figure. We

cannot rule out the possibility that some cross-reactivity occurred with the antisera used in this study. However, on the basis of a different study [46] we tentatively assign this band to an alternative form of Sp3 binding to the EFP2 oligonucleotide. The inclusion of antisera against both proteins completely abolished specific complex formation with the EFP2 oligonucleotide (Figure 1B, lane 9). Similar results were also obtained for a different human cell line HEK 293 (Figure 1B, lanes 10–14). Furthermore, ectopically expressed Sp1, Sp3 and Sp4 bound the EFP2 element when nuclear extracts from transfected *Drosophila* SL2 cells were used in band-shift assays (results not shown). On the basis of this analysis we conclude that Sp1 and Sp3 proteins present in HeLa cells and at least ectopically expressed Sp4 are able to bind the EFP2 element within the human hEF1A1 promoter.

Effect of Sp1 family members in HeLa cells

To determine the effect of the different GC-box-binding factors on hEF1A-1 promoter activity we co-transfected expression vectors for these proteins into HeLa cells with the promoter constructs illustrated in Figure 2(A). First we examined the effect on pEFA1090CATSp1–, which contains all of the regulatory elements of the region upstream of the hEF1A-1 TATA box fused to the *tk* promoter. Although this construct contains one verified and several putative Sp1-binding sites, co-expression of Sp1 had no discernible effect on promoter activity (Figure 2B). A similar picture was observed with Sp3 or Sp4 co-expression. We ascribe this to the high endogenous levels of Sp1 and Sp3 in HeLa cells. As a consequence of this, co-transfection experiments with Sp1 expression vectors have generally proved difficult, presumably because most Sp1-binding elements are saturated by endogenous proteins. However, co-transfection of a BTEB

Figure 2 Co-transfections with GC-box-binding factors in HeLa cells

(A) Schematic illustration of promoter constructs. hEF1A-1 promoter fragments were fused to the CAT gene (CAT) through the *tk* TATA box (TK-TATA). The locations of the EFP1 and EFP2 promoter elements are indicated. *EFP2** denotes a mutated EFP2 site incapable of binding Sp1 and other factors. (B) Co-transfections with a full-length hEF1A-1 promoter (pEFΔ1090CATSp1—). (C) Co-transfections with a minimal hEF1A-1 promoter (pEFΔ119CATSp1—). In (B) and (C) CAT activities have been normalized to β -galactosidase activity and are represented relative to transfections without expression of GC-box-binding proteins, arbitrarily set to 1. Expression vectors (pCMVSp1/flu, pCMVSp3/flu, pCMVSp4, pRSVBTEB, pCMVBTEB2) were included as specified in the Figure. Each transfection was performed in triplicate.

expression vector led to a 4-fold stimulation of reporter gene transcription, whereas BTEB2 had a more modest 2-fold stimulatory effect. To investigate whether the Sp1-binding EFP2 element also mediates activation by BTEB, we performed a similar series of experiments with the shortened reporter plasmid pEFΔ119CATSp1— (Figure 2C). This construct, containing only 90 bp of hEF1A-1 promoter sequence, was activated 10-fold by BTEB, whereas the other expression vectors had no impact on reporter gene transcription. Activation by BTEB was dependent on the integrity of the EFP2 element, as no activation was observed with a variant of pEFΔ119CATSp1— in which the EFP2 sequence had been mutated (pEFΔ119EFP2*CATSp1—; Figure 3).

Activation of the EF1A-1 promoter in *Drosophila* cells

Because Sp1, Sp3 and Sp4 had no effect on hEF1A-1 promoter activity in HeLa cells, we decided to re-evaluate the importance of these factors for hEF1A-1 expression in a cell line devoid of GC-box-binding activity. For this purpose the *Drosophila* Schneider SL2 cell line is often used as it is highly responsive to exogenous Sp1 [7,8]. As demonstrated in Figure 4(A), co-transfection of increasing amounts of pPacSp1 with

Figure 3 Effect of mutation of the EFP1 and EFP2 elements on BTEB activation potential

hEF1A-1 promoter constructs mutated in the EFP1 element (pEFΔ119EFP1*CATSp1—) or in the EFP2 element (pEFΔ119EFP2*CATSp1—) as well as a non-mutated construct (pEFΔ119CATSp1—) were co-transfected with or without pRSV-BTEB as specified. CAT activities have been normalized to β -galactosidase activity and are represented relative to transfections without the inclusion of pRSV-BTEB, arbitrarily set to 1. All transfections were done in triplicate.

Figure 4 Co-transfections in *Drosophila* Schneider SL2 cells with full-length hEF1A-1 promoter

Full-length hEF1A-1 promoter (pEFΔ1090CATSp1—) was transfected in conjunction with increasing amounts of pPac vectors expressing Sp1, Sp3 and Sp4. The amount of expression vector per well in six-well trays is specified. All transfections were done in triplicate and CAT activities were measured relative to protein content. Transfections without expression vector were arbitrarily set to 1. (A) Co-expression with pPacSp1; (B) co-expression with pPacSp3; (C) co-expression with pPacSp4.

Figure 5 Co-transfections in *Drosophila* Schneider SL2 cells with a minimal hEF1A-1 promoter

A minimal hEF1A-1 promoter (pEFA119CATSp1-) was transfected in conjunction with increasing amounts of pPac vectors expressing Sp1, Sp3 and Sp4. The amount of expression vector per well in six-well trays is specified. All transfections were done in triplicate and CAT activities were measured relative to protein content. For each reporter plasmid, transfections without expression vector were arbitrarily set to 1. (A) Co-expression with pPacSp1; (B) co-expression with pPacSp3; (C) co-expression with pPacSp4.

pEFA1090CATSp1- led to a huge activation of reporter gene expression: a maximal stimulation of 750-fold over the activity observed with pPAC0 alone occurred with 150 ng of pPacSp1. Further addition of Sp1 to 500 ng of pPacSp1 caused a small decrease in activity to approx. 400-fold stimulation, suggesting that activation by Sp1 is saturable. Although slightly higher amounts of Sp3 were required for a similar level of stimulation, no decrease in activity could be observed at high concentrations of Sp3, indicating that at high concentrations Sp3 is a more potent activator of pEFA1090CATSp1- than Sp1 (Figure 4B). At the highest concentration of Sp3 tested (500 ng of pPacUSp3), stimulation of reporter gene activity was as high as 1150-fold. In contrast, co-expression of Sp4 did not have any significant stimulatory effect in this system (Figure 4C; note the different scales). The lack of stimulation by Sp4 was not caused by poor expression of this factor as reported earlier [18], because we detected comparable expressions of all three Sp1 family members in our system (results not shown).

As with HeLa cells, we also tested whether the single Sp1-binding EFP2 element located in pEFA119CATSp1- was sufficient to mediate transcriptional activation by the Sp proteins. Sp1 stimulated transcription from pEFA119CATSp1- by a mere 13-fold (Figure 5A). This stimulation was observed with

Figure 6 Level of Sp1-mediated activation depends on promoter length and presence of the EFP2 element

A full-length (pEFA1090CATSp1-) , a minimal (pEFA119CATSp1-) and an EFP2-mutated (pEFA119EFP2*CATSp1-) hEF1A-1 promoter were co-transfected with or without 150 ng of pPacSp1 expression vector as specified. Transfections were performed in triplicate and CAT activities were normalized to protein content. For each reporter plasmid, transfections without Sp1 expression vector were arbitrarily assigned the value 1.

500 ng of pPACSp1; no saturation of activity was observed. Similarly to the situation observed for Sp1, activation by Sp3 was much more modest with pEFA119CATSp1- than that observed earlier for pEFA1090CATSp1-. Increasing amounts of pPacUSp3 led to increased transcription from pEFA119CATSp1-, with a maximal stimulation of 21-fold observed at 500 ng of pPacUSp3 (Figure 5B). We also observed a slight (3-fold) increase in reporter activity at 2 µg of pPacSp4 (Figure 5C). Taken together, these results suggest that the EFP2 site mediates transcriptional activation by Sp1 and Sp3 factors on its own. However, for full stimulation of the hEF1A-1 promoter by Sp1 and Sp3 sequences upstream of position -119 are also required. To prove formally that the activation of transcription from pEFA119CATSp1- operated through the EFP2 element we compared the effect of transfecting 150 ng pPACSp1 with pEFA1090CATSp1-, pEFA119CATSp1- or pEFA119EFP2*CATSp1-. As expected, Sp1 had a strong (almost 400-fold) stimulatory effect on reporter activity derived from pEFA1090CATSp1- (Figure 6). A more modest 30-fold stimulation was observed in the case of pEFA119CATSp1-, whereas pEFA119EFP2*CATSp1- was virtually unaffected by Sp1 co-expression. This indicates that the EFP2 site mediates the transcriptional activation of pEFA119CATSp1- by Sp1. In addition it illustrates the significant difference in response to Sp1 between pEFA1090CATSp1- and pEFA119CATSp1- within a single experiment.

DISCUSSION

The GC box is a widely occurring promoter element targeted by the transcriptional activator Sp1 [6]. In addition to Sp1, a number of other transcriptional regulators are capable of binding the GC box, including Sp3, Sp4, BTEB and BTEB2 [14-17]. A sequence alignment of these five transcription factors shows that the ability of these proteins to bind to identical sequences is reflected in the conservation of critical residues in their DNA-binding domains.

A construct containing all of the upstream elements required for optimal transcription from the human hEF1A-1 promoter (pEFA1090CATSp1-) was activated 4-fold and 2-fold by BTEB and BTEB2 respectively. We could not detect any effect of either

of the Sp proteins on pEFA1090CATSp1–, although clearly Sp1, Sp3 and Sp4 are able to bind to the EFP2 element present within this construct [41], and the present study). The lack of activation by Sp1 and Sp4 might be explained by the high endogenous levels of Sp1 in HeLa cells. It is entirely possible that GC box(es) in the hEF1A-1 promoter are already saturated for activation in the absence of Sp protein co-expression. In contrast, we were slightly puzzled by the observation that Sp3 had no effect on promoter activity, as this protein has previously been characterized as a repressor of Sp1-mediated activation [46,55]. However, more recently the stereotypical view of Sp3 as a static repressor has been challenged by a number of reports demonstrating that Sp3 might also under some circumstances function as an activator [56–58]. It was suggested that Sp3 contains distinct domains for repression and activation of transcription, and that the outcome of Sp3 binding might depend on cellular context and/or promoter architecture [19,59].

We also tested the effect of the various GC-box-binding factors on a shorter version of the hEF1A-1 promoter containing only a single GC-box (pEFA119CATSp1–). In this context the only effect observed concerned a 10-fold activation by BTEB. Furthermore mutation of this GC box (the EFP2 site [41]) led to a complete ablation of BTEB-mediated activation, indicating that BTEB activates transcription of the hEF1A-1 promoter through the EFP2 site. This was an unexpected result because BTEB was originally characterized as a factor that would activate transcription from multiple binding sites but repress transcription when only a single binding site was present [16]. Our results question this and place BTEB as a more general activator of transcription. In addition, BTEB might be a more potent activator of transcription than Sp1 because it stimulates reporter gene expression from the hEF1A-1 promoter even in a high-Sp1 background such as HeLa cells (where exogenous Sp1 is inactive), suggesting that the displacement of Sp1 from promoter sites by BTEB leads to increased levels of transcription. It is not known whether the differential response to Sp1 and BTEB is a general feature of their activation domains [glutamine-rich (Sp1) compared with acidic (BTEB)] or whether this is an effect particular to the hEF1A-1 promoter.

Our results from SL2 cells, which are devoid of GC-box-binding activity, demonstrate that the Sp factors might also activate transcription from the hEF1A-1 promoter. In these experiments Sp4 performed as a relatively poor activator. In contrast both Sp1 and Sp3 were very potent activators of the hEF1A-1 promoter in the *Drosophila* cell line. The stimulatory effect was much greater on the full-length promoter than on the 90 bp promoter fragment harboured in pEFA119CATSp1–. As with BTEB, activation of pEFA119CATSp1– by Sp1 was dependent on the EFP2 element, because a mutation of this element abolished stimulation. Furthermore this promoter element was shown to bind Sp1 and Sp3 present in HeLa cell nuclear extracts, and Sp1, Sp3 and Sp4 when these factors were expressed ectopically in SL2 cells.

We were intrigued by the large difference in stimulation manifested with pEFA1090CATSp1– compared with pEFA119CATSp1–. Several possible explanations for this observation can be envisaged. Sp1 is able to activate transcription synergistically when multiple GC boxes are present [8]. However, this requires the D domain of Sp1, which is not conserved between Sp1 and Sp3 [11,15]. In addition it is now thought that Sp3 activates transcription from a single binding site, whereas the binding of Sp3 to multiple sites leads to repression, most probably through improved exposure of its repression domain [19,59]. The human hEF1A-1 promoter contains multiple putative Sp1- and Sp3-binding sites. However, so far we have been able to detect

stable interaction only with Sp-factors to the EFP2 site in the proximal promoter region.

Alternatively, Sp1 and Sp3 could interact favourably with distinct factors bound to more upstream regions to activate transcription synergistically from the full-length promoter. A number of different transcriptional regulators are known to interact with Sp1, including NF- κ B [55], c/EBP β [60], YY1 [61] and GATA-1 [62]. Activation partners for Sp3 are less well characterized; however, it is worth noting that in the context of the HIV-1 long terminal repeat (LTR), Sp1, but not Sp3, interacts favourably with NF- κ B [55]. Closer examination of the region between –1090 and –119 should reveal the mechanism by which the full-length promoter is activated to high levels by Sp1 and Sp3. It has previously been reported that enhancer sites upstream of the EFP1 and EFP2 elements confer no significant additional activity on the hEF1A-1 promoter [41]. However, we consistently observe a 2–3-fold additional activation from upstream enhancer sites in transfections in HeLa and HEK 293 cells (S. J. Nielsen, M. Præstegaard, H. F. Jørgensen and B. F. C. Clark, unpublished work). This is probably due to differences in promoter constructs, because our reporter constructs contain only transcriptional regulatory signals, whereas the constructs used in the earlier study did not distinguish between transcriptional and translational regulation.

The human hEF1A-1 mRNA is expressed at high levels in all tissues examined except for skeletal muscle and, to a smaller extent, brain and heart [32]. In most of these tissues the only GC-box-binding factors present are Sp1 and Sp3. In some contexts Sp3 functions as an inhibitor. However, given the intimate involvement of hEF1A-1 in one of the most fundamental cellular processes (i.e. protein synthesis) it might be advantageous for the cell not to subject the expression of hEF1A-1 to the control of a balance between positive (Sp1) and negative (Sp3) regulators. Instead, by ensuring that both Sp1 and Sp3 functions as activators of the hEF1A-1 promoter, a uniformly high level of hEF1A-1 expression can be achieved throughout most tissues. This might be physiologically important because Sp1 expression varies between tissues [12]. It is not known whether other GC-box-binding proteins contribute to hEF1A-1 promoter activity *in vivo* because these proteins, including Sp4, BTEB and BTEB2, are expressed in only a limited range of tissues [15,17,63]. However, our studies suggest that especially BTEB might have the potential to be a regulator of hEF1A-1 expression. In addition, the recent report concerning the ablation of Sp1 activity in mice suggests that other GC-box-binding factors might have physiologically relevant roles because the expression of a number of putative Sp1 target genes was unaffected by the mutation [13]. Further studies in cell lines with different compositions of GC-box-binding proteins should help to define the role of individual factors in the expression of hEF1A-1 and other genes.

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