

Analysis of the *Xenopus laevis* CCAAT-enhancer binding protein α gene promoter demonstrates species-specific differences in the mechanisms for both auto-activation and regulation by Sp1

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Received as resubmission November 1, 2000; Accepted November 17, 2000

DDBJ/EMBL/GenBank accession no. AJ250304

ABSTRACT

Transcription factors belonging to the CCAAT-enhancer binding protein (C/EBP) family have been implicated in the regulation of gene expression during differentiation, development and disease. Autoregulation is relatively common in the modulation of C/EBP gene expression and the murine and human C/EBP α genes have been shown to be auto-activated by different mechanisms. In the light of this finding, it is essential that auto-regulation of C/EBP α genes from a wider range of different species be investigated in order to gauge the degree of commonality, or otherwise, that may exist. We report here studies that investigate the regulation of the *Xenopus laevis* C/EBP α gene (xC/EBP α). The $-1131/+41$ promoter region was capable of directing high levels of expression in both the human hepatoma Hep3B and the *Xenopus* kidney epithelial A6 cell lines, and was auto-activated by expression vectors specifying for xC/EBP α or xC/EBP β . Deletion analysis showed that the $-321/+41$ sequence was sufficient for both the constitutive promoter activity and auto-activation and electrophoretic mobility shift assays identified the interaction of C/EBPs and Sp1 to this region. Although deletion of either the C/EBP or the Sp1 site drastically reduced the xC/EBP α promoter activity, multimers of only the C/EBP site could confer autoregulation to a heterologous SV40 promoter. These results indicate that, in contrast to the human promoter and in common with the murine gene, the xC/EBP α promoter was subject to direct autoregulation. In addition, we demonstrate a novel species-specific action of Sp1 in the regulation of C/EBP α expression, with the factor able to repress the murine promoter but activate the *Xenopus* gene.

INTRODUCTION

CCAAT-enhancer binding protein- α (C/EBP α) belongs to a family of transcription factors that all contain a highly conserved C-terminal basic region-leucine zipper (bZIP) domain that consists of a basic region, involved in DNA recognition, and an adjacent helical structure, the leucine zipper, which mediates subunit dimerisation (1,2). In contrast, the N-termini of the proteins, which carry the regulatory and the *trans*-activation domains, are quite divergent. Six distinct C/EBP isoforms have been identified to date (C/EBP α to ζ) with the majority of these able to recognise similar DNA sequences, at least *in vitro*, activate gene transcription *in vivo* and form heterodimers in intrafamilial combinations (1,2). Additionally, in the case of C/EBP α and C/EBP β , polypeptides of different sizes and *trans*-activating capabilities can be produced from the same mRNA by alternative use of in-frame AUG codons because of a leaky ribosome scanning mechanism (3,4). For example, two polypeptides of 42 kDa (p42) and 30 kDa (p30) can be produced from the C/EBP α mRNA, with the latter having a lower *trans*-activation potential than the former (4). Thus, the number of C/EBP proteins that may be present in any given tissue/cell may be much higher than the number of C/EBP-encoding genes.

C/EBP α shows a restricted tissue expression pattern and *trans*-activates the promoters of several hepatocyte- and adipocyte-specific genes that are important for energy metabolism (1,2,5–7). The gene plays a pivotal role in adipocyte differentiation; for example, ectopic expression of C/EBP α in adipoblasts inhibits cell proliferation and activates genes characteristic for differentiated fat cells (5–9) and, conversely, introduction of antisense C/EBP α RNA into pre-adipocytes blocks adipogenesis (10). In support of the importance of C/EBP α , *in vivo*, the targeted inactivation of the gene in mice results in a failure of the liver and adipose tissue to synthesise glycogen and store lipids, respectively (11). Additionally, these mice have disturbed hepatic architecture with acinar formation, resembling proliferative or pseudoglandular hepatocellular carcinoma (11–13), and also display an early block in the maturation of granulocytes because of a loss of granulocyte

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colony stimulating factor (G-CSF) signal-directed maturation (13). An important role for C/EBP α , and other family members, in development has also been suggested by limited studies on *Drosophila* and *Xenopus laevis* homologues (14–16).

Studies that have examined the *cis*-acting elements and the *trans*-acting factors that are involved in the transcriptional regulation of the C/EBP α gene have, however, been rather limited. The proximal promoter region of the mouse C/EBP α gene has been shown to contain consensus recognition sequences for several transcription factors, including nuclear factor-1 (NF-1), upstream stimulatory factor (USF), Sp1, Myc/Max, C/EBP and activator-protein 2 α (AP-2 α) (7,17–21). AP-2 α [C/EBP α undifferentiated protein (CUP)] and Sp1 have been shown to repress C/EBP α expression (7,19–21). Additionally, both C/EBP α and C/EBP β can directly auto-activate the promoter by interacting with the C/EBP-recognition sequence (7,17,18). Such direct autoregulation has also been identified for the rat promoter (22). In contrast, autoregulation of the human gene occurs indirectly through C/EBP α -mediated stimulation of USF binding to the proximal promoter region (23). It is, however, currently unclear whether such a novel indirect mode of autoregulation is unique to the human C/EBP α gene or may also be applicable to other species. Similarly, it is unclear whether the repressive action of Sp1 is a general phenomenon or specific to the murine promoter.

We have previously studied the regulation of C/EBP gene expression from several species including *X.laevis*, a model system widely used to study embryonic development (16,24–27). In the present paper, we report the organisation of the 5' flanking region of the *X.laevis* C/EBP α gene (xC/EBP α) and investigate the importance of the regulatory elements within this region, particularly in relation to species-specific differences.

MATERIALS AND METHODS

Materials

The human hepatoma Hep3B and the *X.laevis* kidney epithelial A6 cell lines were obtained from the European Collection of Animal Cell Cultures and the American Collection of Animal Cell Cultures, respectively. All the cell culture reagents were purchased from Gibco BRL. The Sp1 antiserum was obtained from Santa Cruz.

Cloning and sequencing

A *X.laevis* genomic library (Stratagene) was screened under conditions of high stringency with a radiolabelled 579 bp *PstI/PstI* fragment from the xC/EBP α cDNA insert that contained sequences corresponding to the N-terminus of the gene (15,16). The cDNA fragment was radiolabelled with [α -³²P]dCTP by the random priming technique using the Megaprime DNA labelling kit (Amersham). The filters were prehybridised for 6 h at 42°C in 50% (v/v) formamide, 6 \times SSPE, 5 \times Denhardt's, 0.1% (w/v) SDS and 10 mg/ml yeast tRNA. Hybridisation with the denatured radiolabelled probe was carried out, overnight, at 42°C in the prehybridisation buffer. The filters were then washed three times (30 min each) at 65°C in 0.1 \times SSC, 0.1% (w/v) SDS and exposed to X-ray film. From 500 000 p.f.u. screened, a single positive clone was isolated that survived the tertiary round of screening. The coding and the putative promoter regions present in the isolated genomic clone were

identified by Southern blot analysis of restriction endonuclease digested recombinant phage DNA probed with radiolabelled probes derived from the xC/EBP α cDNA insert that corresponded to the N- and C-termini of the protein, respectively. The analysis indicated that ~1.1 kb of the putative promoter region was contained in a 1.5 kb fragment that could be obtained from digestion of recombinant phage DNA with *PstI*. This 1.5 kb fragment, along with two subfragments obtained by its digestion with *HindIII* and *EcoRV* (0.6 kb) and *XbaI* and *SalI* (1.1 kb), were subcloned into pUC18 (28) and sequenced using the Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit with 7-deaza-dGTP (Amersham). The sequence of both strands of the entire 1.5 kb fragment was determined using a combination of M13 universal and reverse primers and specific primers designed against the derived sequence. Electrophoresis was carried out using the LI-COR automated DNA Sequencer and the data analysed with the Base Image IR Software Package (MWG-Biotech). In some cases, manual sequencing was performed using the dideoxy chain-termination method and the Sequenase Version 2.0 sequencing kit (US Biochemical).

Preparation of manipulated xC/EBP α promoter-reporter constructs

PCR-based approaches were used to prepare the various xC/EBP α promoter-reporter constructs. The PCR reactions were performed using either Vent DNA polymerase or the ExpandTM system (Boehringer Mannheim), in order to minimise PCR-generated mutations, and this was confirmed by sequencing of all the products following subcloning into the pGEM-T vector (28). To produce a promoter-luciferase construct with the entire cloned putative xC/EBP α promoter region, recombinant pUC18 containing the 1.5 kb *PstI* fragment (see above) was used as a template for PCR with the M13 universal primer and an oligonucleotide designed against the +41 to +22 region of the xC/EBP α gene (designated as xalpha). The product was phosphorylated using polynucleotide kinase and ATP, and subcloned into dephosphorylated pGL2-Basic vector (28) that had been digested previously with *XhoI* followed by 'filling in' of the protruding ends using Klenow Polymerase and dNTPs. This construct was designated as -1131/+41. Three further deletions were produced similarly using a common xalpha primer and oligonucleotides designed against the -634 to -615, -321 to -302 and -121 to -108 regions, respectively. These DNA constructs were designated as -634/+41, -321/+41 and -121/+41, respectively.

Internal deletions of the C/EBP and the Sp1 binding sites were produced by the overlap-extension method (29) using recombinant pUC18 containing the -321/+41 promoter fragment. The oligonucleotide combinations used were 5'-CAGCGGAGTG-CAATGTAACG-3' and 5'-GCGTTACATTGCACTCCGCT-3' (delC/EBP) and 5'-GCAATAGAGAAGGCCTCCAG-3' and 5'-CTGGAGGCCTTCTCTATT-3' (delSP1). The fragments were subcloned into the pGL2-Basic vector (28) and verified by sequence analysis.

For the preparation of the pC/EBP \times 4 and pSp1 \times 4 DNA constructs, containing four copies of the C/EBP or Sp1 sites from the xC/EBP α promoter linked to a heterologous SV40 promoter in the pGL2-promoter vector (28), the following oligonucleotides were synthesised: 5'-CCGAGTGTTC-AGACAAAGTGTTCAGACAAAGTGTTCAGACAA-

AGTGTTCAGACAA-3' and 5'-TCGATTGTCTGGAA-ACACTTTGTCTGGAAACACTTTGTCTGGAAACACTTTGTCTGGAAACACT-3' for pC/EBP \times 4, and 5'-CCGG-AAGCAGGGGCGTGGCCAAGCAGGGGCGTGGCCAAG-CAGGGGCGTGGCCAAGCAGGGGCGTGGCC-3' and 5'-TCG-AGGCCACGCCCTGCTTGGCCACGCCCTGCTTGGC-CACGCCCTGCTTGGCCACGCCCTGCTT-3' for pSp1 \times 4. These were designed in such a manner so that, following annealing, they had overhangs which allowed their direct cloning into the *Xma*I and *Xho*I sites in the pGL2-promoter vector (28).

Cell culture and transient transfection assays

Monolayers of human hepatoma cell line Hep3B were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated (30 min, 56°C) fetal calf serum (HI-FCS), 100 μ g/ml streptomycin and 100 U/ml penicillin, at 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air. The *Xenopus* A6 cell line was grown in medium containing 75% (v/v) NCTC-135 media and 15% (v/v) sterile water supplemented with HI-FCS, streptomycin and penicillin, as above, at 26°C in an incubator containing 20 mM HEPES. DNA transfections were carried out by the calcium phosphate precipitation method (30) and utilised 2–6 μ g of reporter plasmid, 0–4 μ g of expression construct and 0.5 μ g of CMV- β -galactosidase (28) to provide an internal control for transfection efficiency. After 16 h, the cells were washed with phosphate buffered saline and left in fresh culture medium for 36 h. The luciferase and the β -galactosidase activity in cell extracts were then determined using commercially available kits (Promega). The luciferase activity was normalised to the β -galactosidase value, and each transfection was repeated at least three times.

Electrophoretic mobility shift assays (EMSA)

The -82/+41, -218/-82 and -321/-218 xC/EBP α promoter fragments were used for EMSA. For the -82/+41 fragment, the -634/+41 promoter construct in the pGL2-Basic vector was digested with the enzymes *Bgl*III and *Sty*I (*Bgl*III site occurs in the polylinker of the vector), size-fractionated by electrophoresis on a 2% (w/v) agarose gel and the resulting fragment of ~125 bp was purified. Isolation of the -218/-82 and -321/-218 fragments required two distinct restriction endonuclease digestions and gel purification: (i) digestion of recombinant -634/+41 plasmid with *Sac*I and *Sty*I, purification of the insert of ~550 bp, its digestion with *Hph*I and isolation of the -218/-82 fragment; (ii) digestion of recombinant -321/+41 DNA construct, containing the promoter fragment in the opposite orientation to that used for transfection experiments, with *Nhe*I and *Hind*III, purification of the fragment of ~360 bp, further digestion with *Hph*I and isolation of the -321/-218 insert. The sequences of the oligonucleotides used for EMSA were: 5'-TGCAGATTGCGCAAT-3' and 5'-TGC-ATTGCGCAATCT-3' (D-C/EBP) (16,25), 5'-CATAGTGG-CGCAAACCTCCCTTACTG-3' and 5'-CAGTAAGGGAGTT-TGCGCCACTATG-3' [C-reactive protein (CRP)] (24,25), 5'-GCAGAATTTCTTGGGAAAGAAA-3' and 5'-GCTATTT-TCTTTCCCAAGAAATT-3' [β -casein (β -cas)] (25), 5'-GATCC-TTCGTGACTCAGCGGGATCCTTCGTGAG-3' and 5'-CCGC-TGAGTCACGAAGGATCCCGCTGAGTCACG-3' (AP-1) (25), 5'-TCGACGGTGTAGGCCACGTGACCGGGTGT-3' and 5'-CGCACACCCGGTCACGTGGCCTACACCGT-3' (USF) (23), 5'-TAGATTTCGATCGGGGCGGGGCGAG-3' and 5'-GCCC-

TCGCCCCGCCCCGATCGAAT-3' (Sp1) (23), 5'-TAGCTT-GGCATTAGGACCCAGTCGAAGGGCAA-3' and 5'-GGC-TTGCCCTTCGACTGGGTCCTAATGCCAAG-3' (NS), 5'-CAGTGTTCAGAC-3' and 5'-TTGGTCTGGAAACA-3' (wtC/EBP), 5'-AAGCAGGGGCGTGGC-3' and 5'-GAG-GCCACGCCCT-3'(wtSP1), and 5'-GCCTTGGCATT-3' and 5'-GAACCGTAATCG-3' (NF-1). These were radiolabelled by 'fill-in' reactions using [α -³²P]dCTP and Klenow DNA polymerase.

Nuclear and whole cell extracts were prepared essentially as described by Ramji *et al.* (24) and Timchenko *et al.* (23), respectively. The protease inhibitors (0.5 mM PMSF, 1 μ g/ml pepstatin A, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml I-S soybean trypsin inhibitor) and DTT (0.5 mM) were added to all the buffers before use. The protein concentration of the nuclear extract was determined using the BCA protein assay kit as described by the manufacturer (Pierce).

For EMSA analysis, 4 μ g of protein was incubated in a 20 μ l total reaction volume containing 34 mM potassium chloride, 5 mM magnesium chloride, 0.1 mM DTT and 3 μ g poly(dI-dC). After 10 min on ice, ³²P-labelled probes (40 000 c.p.m.) were added and the incubation continued for 30 min at room temperature. Following the addition of 5 μ l of a 20% (w/v) Ficoll solution to each sample, the free probe and the DNA-protein complexes were resolved on 6% (w/v) polyacrylamide gels in 0.25 \times TBE (22.5 mM Tris base, 22.5 mM boric acid, 0.5 mM EDTA). The gels were dried under vacuum and exposed to X-ray film. For antibody supershift assays, samples of the nuclear extracts and appropriate antisera were incubated for 30 min on ice prior to the addition of the radiolabelled probe (24,25).

RESULTS

Cloning and sequence analysis of the xC/EBP α promoter

The xC/EBP α genomic clone was isolated as described in Materials and Methods and its identity confirmed by sequence analysis of fragments that contained the entire coding region (data not shown). An overall identity of 93% was found to exist between the predicted amino acid sequence of the coding region present in the cDNA and genomic clones, with almost total identity in the various domains/regions that have been implicated as being important for the function of C/EBP α , including the basic region, the leucine zipper, the three putative *trans*-activation domains, the two in-frame methionine residues in the coding region and a sequence in the putative 5'UTR with a potential to code for a short upstream open reading frame that has been shown to act *in cis* to inhibit its translation in several cell lines (1,2,4,31). It is likely that allelic variations, which are relatively common in *X.laevis* because of a genomic tetraploidization event that occurred over 30 million years ago (32,33), are responsible for some variation seen between the xC/EBP α cDNA and genomic sequences.

In order to investigate the regulation of the xC/EBP α promoter, the nucleotide sequence was determined of both strands of a 1.5 kb *Pst*I genomic fragment that contained ~1.1 kb of the putative promoter region. The sequence has been deposited in the EMBL, GenBank and DDBJ databases under the accession number AJ250304. Before carrying out detailed analysis of the sequence elements that are present in this region, the transcription start site was determined by primer extension analysis. At least

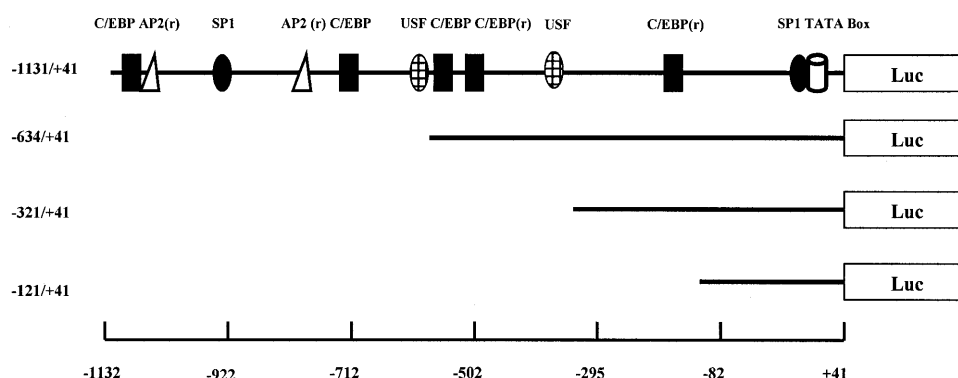


Figure 1. Schematic representation of the xC/EBP α promoter–luciferase DNA constructs. The relative position of the putative TATA box and binding sites for the transcription factors C/EBP, Sp1, Ap2 and USF are shown with r indicating that the consensus sequence occurs in the reverse orientation. See text for details.

four extension products that differed by a single nucleotide were obtained in three independent experiments (data not shown), and indicated that the transcription of the xC/EBP α gene begins 125–128 bp from the first translation initiation codon in the coding region. This compares well with the corresponding distance of 126 bp identified for the human, mouse and rat C/EBP α genes (17,18,22,23). The A residue at position 125 was therefore arbitrarily assigned as +1.

Despite a high level of conservation between the predicted amino acid sequence of xC/EBP α and homologous genes from other species (e.g. 81% identity with the *Rana catesbeiana* homologue, 69–75% with human, rodent and avian members) (2,7,15,34,35), the degree of sequence identity in the putative promoter regions was relatively low (35–42%) (data not shown). Such a low level of sequence identity has also been seen between the promoter regions of the mouse and the human C/EBP α genes (53%) (23). Despite this low overall sequence similarity, a computer search (36) of the xC/EBP α promoter region for the presence of consensus recognition sequences of known transcription factors in the TRANSFAC database showed the existence of putative sites for several factors that have been demonstrated to be important for the expression of C/EBP α genes (7,17–23,34). Figure 1 shows a schematic representation of the putative sites of four such transcription factors (i.e. C/EBP, USF, Sp1, AP2) along with the xC/EBP α promoter–luciferase constructs used in this study.

Analysis of the xC/EBP α promoter activity in transfected cells

In order to identify the regions in the xC/EBP α promoter that were important for the transcription of this gene, three different promoter–luciferase DNA constructs were prepared initially. Thus, the –1131/+41, –634/+41 and –321/+41 promoter regions were amplified by PCR and subcloned upstream of the luciferase gene in the pGL2-Basic-luciferase vector as described in Materials and Methods. The human hepatoma Hep3B and the *Xenopus* kidney epithelial A6 cell lines were used for all the transfection experiments. The Hep3B cells were chosen because they are used widely as a model for the analysis of promoter elements that are involved in both the constitutive and inducible expression of genes in hepatocytes, including the C/EBP family members, from different species

(see 37,38 and references therein) and, additionally, possess a relatively low level of endogenous C/EBP α expression, thereby allowing auto-activation to be monitored relatively easily. The A6 cells represent the only extensively characterised *Xenopus* cell line that has been used for promoter-dissection experiments (e.g. 39), and provides an ideal host model system for the analysis of the xC/EBP α promoter.

Thus, the three xC/EBP α promoter–luciferase DNA constructs were transfected into the two cell lines along with the CMV- β -galactosidase expression plasmid in order to provide an internal control for transfection efficiency. The RSV luciferase and the pGL2-Basic-luciferase vectors were used as positive and negative controls, respectively. The cells were harvested 36 h after transfection, and the luciferase activity was determined and normalised to the β -galactosidase value. The values obtained using the pGL2-Basic-luciferase vector were subtracted from those obtained using the various xC/EBP α promoter–luciferase DNA constructs in the same vector (typically 3–5%). As shown in Figure 2, a concentration-dependent increase in the relative luciferase activity was obtained in both cell lines. Additionally, in contrast to the activity of the pGL2-Basic-luciferase and the CMV- β -galactosidase vectors, the xC/EBP α promoter activity was substantially higher in the homologous A6 cell line (Fig. 2). The deletions also had no significant effect on the relative luciferase activity, thereby indicating that the –321/+41 region was sufficient to drive the constitutive C/EBP α promoter activity.

Both C/EBP α and C/EBP β can activate the xC/EBP α promoter

The mouse and the rat C/EBP α promoters have been shown to be activated by both C/EBP α and C/EBP β (17,18,22). In contrast, the human promoter can only be activated by the C/EBP α expression vector (23). In order to examine the auto-activation properties of the xC/EBP α promoter, the transfection experiments with the three promoter–luciferase DNA constructs, detailed above, were repeated in the presence of co-transfected expression plasmids coding for xC/EBP α or the LAP form of xC/EBP β , which has been demonstrated to act as a potent transcriptional activator (16). The parent pCS2+ plasmid (16), without any cloned insert, was used as a control. Figure 3 shows that all three xC/EBP α promoter–luciferase DNA constructs could be

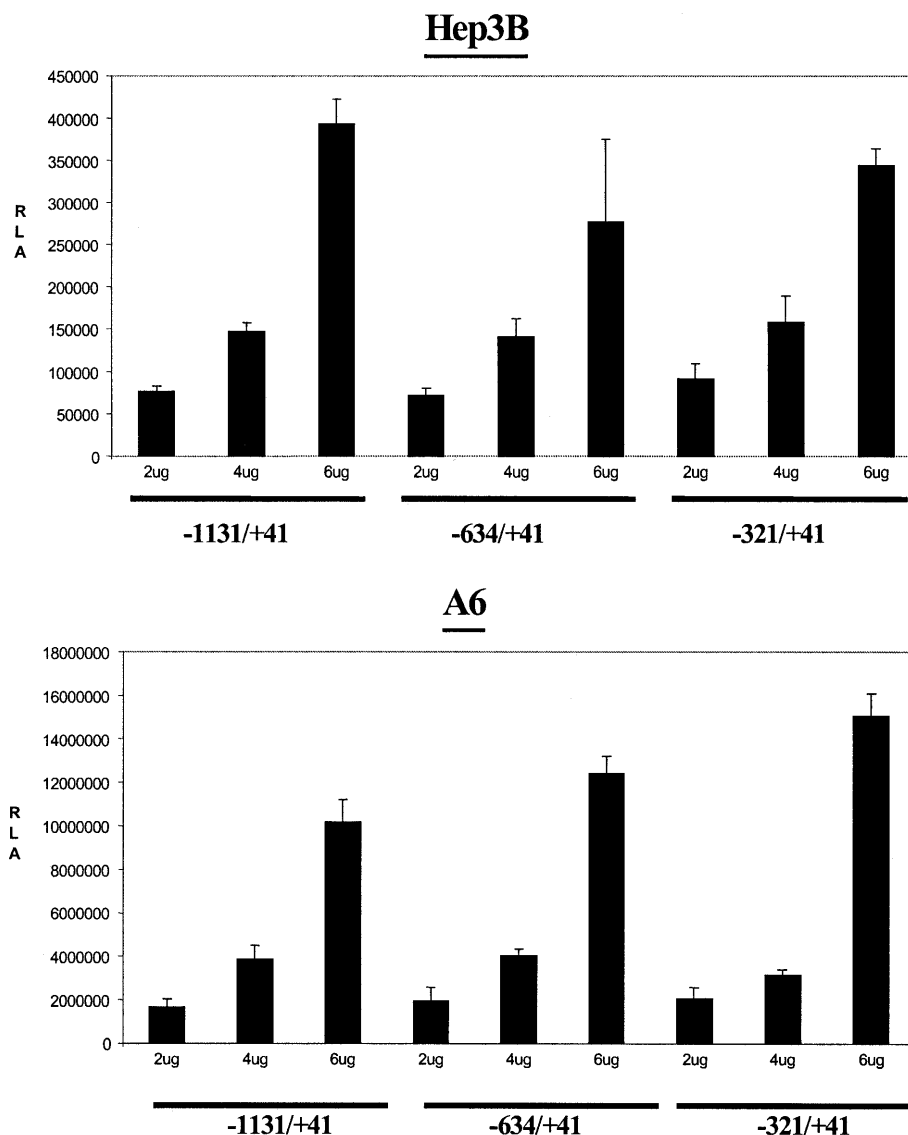


Figure 2. The activity of xC/EBP α promoter deletion constructs in transfected cells. Three different concentrations of the xC/EBP α promoter constructs, -1131/+41, -634/+41 and -321/+41, were transfected into Hep3B or A6 cells as indicated. The luciferase activity at each point was normalised to the β -galactosidase activity, and the values are expressed as relative luciferase activity (RLA). Each value represents the mean \pm SD from three independent experiments.

auto-activated by the xC/EBP α expression plasmid, with an ~3.5- to 5-fold induction of luciferase activity in Hep3B cells and ~4- to 5.5-fold increase in the A6 cell line. This level of induction was therefore higher than the ~3-fold increase seen with the mouse and the human promoters (17,18,23). A slightly different profile was obtained when an expression plasmid coding for the LAP form of xC/EBP β was used in the transfection experiments. In Hep3B cells, although only a marginal increase in the luciferase activity was seen with the -1131/+41 region, an increase between 3- and 7.5-fold was obtained with the two deletion constructs (Fig. 3). In contrast, all three xC/EBP α promoter-luciferase DNA constructs were activated in A6 cells by the xC/EBP β expression plasmid (approximate induction of 3.5- to 6-fold). As expected, co-transfection of the parent pCS2+ expression plasmid did not produce any significant

changes in the luciferase activity with all the three xC/EBP α promoter constructs (Fig. 3). Overall, therefore, these results indicate that the xC/EBP α promoter can be activated by both the xC/EBP α and the xC/EBP β expression plasmids, and that the -321/+41 region contains sufficient information for this activation. Additionally, deletions of the putative USF binding sites (i.e. absent in the -321/+41 DNA construct) (see Fig. 1) have little effect on the auto-activation of the xC/EBP α promoter.

Analysis of DNA-protein interactions with the minimal xC/EBP α promoter region

In order to examine the interaction of DNA binding proteins with the -321/+41 xC/EBP α promoter sequence, EMSA were carried out using three fragments from this region (-82/+41, -218/-82

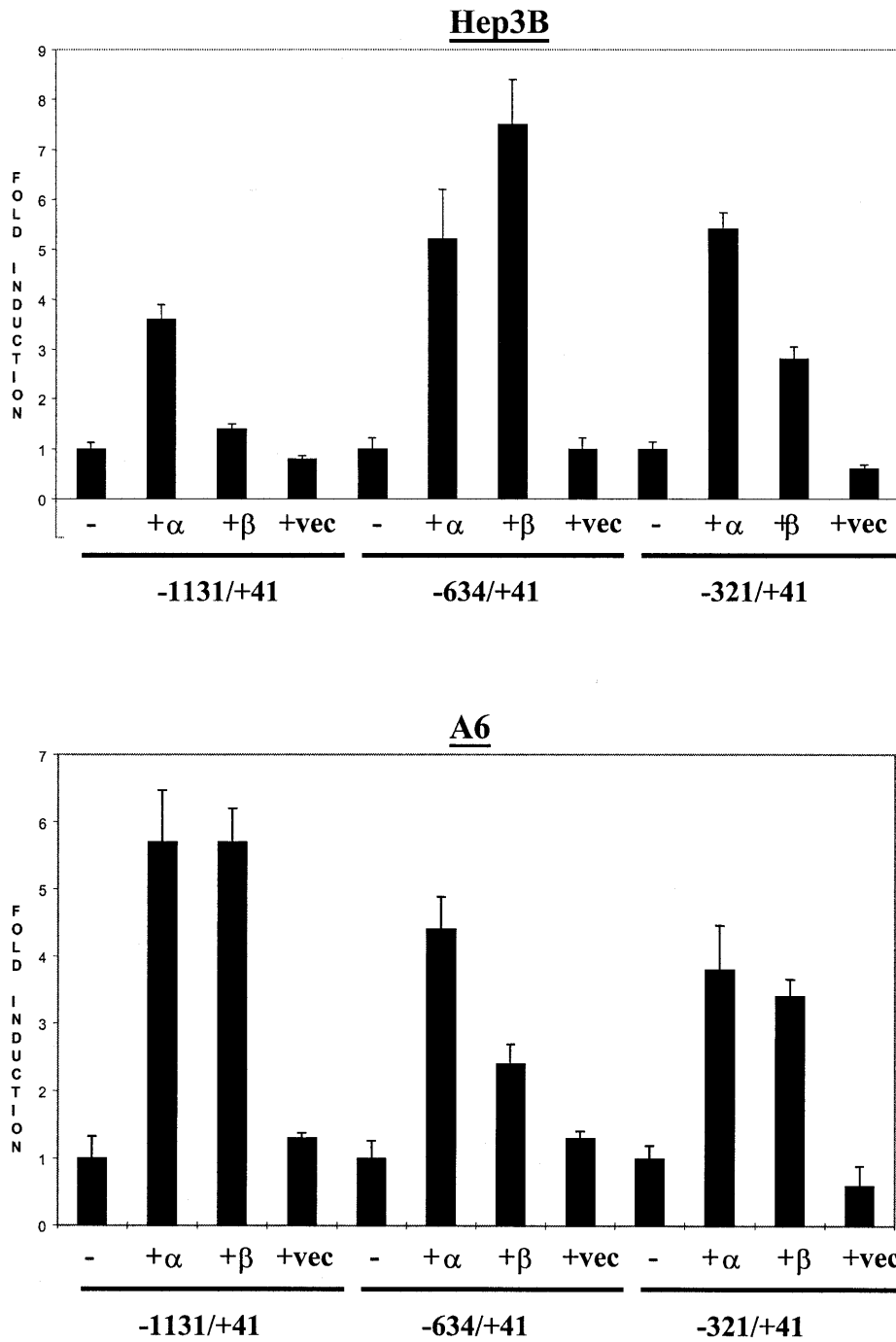


Figure 3. Autoregulation of the xC/EBP α promoter. The three xC/EBP α promoter constructs, -1131/+41, -634/+41 and -321/+41, were co-transfected into Hep3B or A6 cell lines along with recombinant plasmid DNA driving the expression of either xC/EBP α (+ α), the LAP form of xC/EBP β (+ β) or the pCS2+ vector (+vec). The relative luciferase activity obtained using the promoter-less pGL2-Basic vector, in the absence or the presence of the various pCS2+ plasmids, was subtracted from the corresponding values obtained using the xC/EBP α promoter constructs. The normalised activity for each of these promoter constructs alone (-) has arbitrarily been assigned as 1, with those obtained in the presence of the co-transfected expression plasmids being shown as fold induction with respect to this value. Each value represents the mean \pm SD from three to four independent experiments.

and -321/-218) that were produced by restriction endonuclease digestions as described in Materials and Methods. Sequence analysis indicated the absence of any transcription factor binding sites between the junctions of these fragments.

Extracts with high levels of xC/EBP α binding activity were used for the experiments and were obtained by transfection of Hep3B or A6 cells with the pCS2+x α plasmid that directs the expression of the xC/EBP α protein. Initial analysis showed a

similar pattern with both nuclear and whole cell extracts and the former were therefore used for all subsequent experiments. The strategy was based on similar studies that have been carried out on the human *C/EBP α* promoter (23). In order to examine whether the various DNA–protein complexes represented specific interactions of the proteins with the radiolabelled probe, competition experiments were performed using an excess of the appropriate unlabelled fragment. Additionally, to identify whether any of the DNA–protein complexes represented binding by the *C/EBP* family members, the competition analysis was extended to include an excess of double stranded oligonucleotides for a high affinity *C/EBP* binding site (D-*C/EBP*; see Materials and Methods) (16,25), with an AP-1 binding site oligonucleotide acting as a control.

For all three fragments, at least two DNA–protein complexes were obtained using extracts from Hep3B or A6 cells, each of which could be competed by an excess of the corresponding unlabelled fragment (data not shown). From these, only a single DNA–protein complex, obtained using the $-218/-82$ promoter fragment, could be competed specifically by an excess of the *C/EBP* binding site oligonucleotide (data not shown).

Both *C/EBP α* and *C/EBP β* interact with the -218 to -82 promoter region

Because the *xC/EBP α* promoter could be auto-activated by expression plasmids specifying for both *C/EBP α* and *C/EBP β* and EMSA on the minimal promoter region showed the interaction of the *C/EBPs* with the $-218/-82$ sequence (Fig. 3; data not shown), we next decided to investigate further the role of the *C/EBPs* in autoregulation. Oligonucleotide containing the putative recognition sequence present in this region, designated as wt-*C/EBP*, was synthesised and used for EMSA with nuclear extracts from Hep3B or A6 cells that had been transfected with either the pCS2+ α expression plasmid or the parent pCS2+ vector. As shown in Figure 4A, the *C/EBP* DNA binding activity was substantially higher in extracts from Hep3B or A6 cells that had been transfected with the *xC/EBP α* expression plasmid compared to the parent control vector. Such a dramatic increase in DNA binding activity was specific to the *C/EBPs* and was not seen when a control NF-1 binding site oligonucleotide was included in the analysis (Fig. 4A). The DNA–protein complex could be competed by an excess of the corresponding unlabelled wt-*C/EBP* oligonucleotide or those containing the *C/EBP* binding site from the promoter of the CRP or the β -cas genes, but not by an Sp1 or NF-1 binding site oligonucleotide (Fig. 4B). In order to investigate further the interaction of the *C/EBPs* with the wt-*C/EBP* oligonucleotide, antibody interference EMSA experiments were carried out with antisera against the two *C/EBP* isoforms (α and β) using D-*C/EBP* oligonucleotide and pre-immune rabbit IgG as controls. The extracts and antisera were incubated together prior to the addition of the wt-*C/EBP* oligonucleotide. Despite the relatively low signal intensity obtained from the DNA–protein complexes with wt-*C/EBP* oligonucleotide in this experiment, the production of the antibody–protein–DNA ‘super-shift’ complex was seen with both antisera but not the pre-immune serum (Fig. 4C), thereby indicating that both *C/EBP α* and *C/EBP β* interacted with this site.

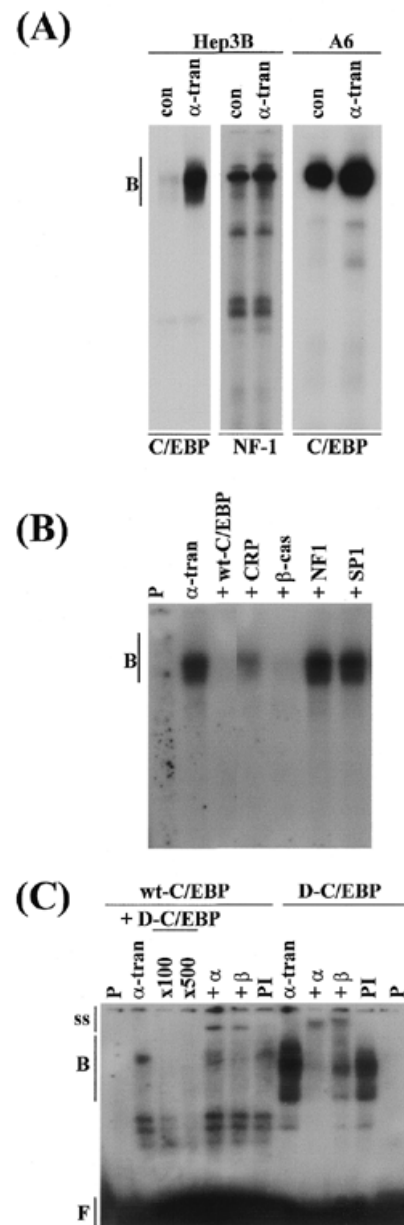


Figure 4. Interaction of the *C/EBPs* with the $-218/-82$ sequence. (A) Nuclear extracts from Hep3B or A6 cells, which had been transfected with the control pCS2+ vector (con) or the *xC/EBP α* expression plasmid (α -tran) were used for EMSA with radiolabelled wt-*C/EBP* or NF-1 binding site oligonucleotides. (B) Competition experiments were carried out using extracts from Hep3B cells transfected with the *xC/EBP α* expression plasmid and a 400-fold molar excess of oligonucleotides for: the corresponding unlabelled wt-*C/EBP* sequence (+wt-*C/EBP*), a *C/EBP* binding site from the C-reactive protein gene promoter (+CRP), a *C/EBP* binding site from the β -cas gene promoter (+ β -cas), a NF-1 recognition sequence (+NF1) and a Sp1 binding site (+SP1). (C) Antibody interference EMSA was carried out using extracts from Hep3B cells transfected with the *xC/EBP α* expression plasmid and radiolabelled wt-*C/EBP* or D-*C/EBP* oligonucleotides, in the absence (α -tran) or the presence of antisera against *C/EBP α* (+ α) or *C/EBP β* (+ β), or pre-immune serum (PI). For EMSA with wt-*C/EBP* oligonucleotide in (C), competition experiments were also carried out using a 100- and 500-fold molar excess of unlabelled D-*C/EBP* oligonucleotide (+D-*C/EBP*). Vertical arrows labelled B, ss and F indicate the position of the major DNA–protein complexes, antibody–DNA–protein supershifted complexes and free probe, respectively. P represents the profile obtained using the radiolabelled oligonucleotide alone; the free probe in (A) and (B) has migrated from the gel.

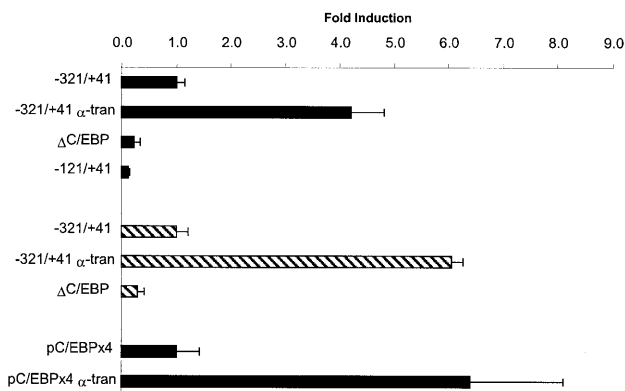


Figure 5. Analysis of the importance of the C/EBP binding site in the minimal xC/EBP α promoter region. The -321/+41, -121/+41, Δ C/EBP and pC/EBP \times 4 promoter-reporter DNA constructs were transfected into Hep3B or A6 cell lines (filled and hatched histograms, respectively) in the presence of the pCS2+x α plasmid that drives the expression of xC/EBP α (α -tran) or the control parent pCS2+ vector. The control activity obtained with the -321/+41 or pC/EBP \times 4 DNA constructs has arbitrarily been assigned as 1, with the others being shown with respect to this value. Each value represents the mean \pm SD from three independent experiments.

The C/EBP recognition sequence is required for both constitutive expression and auto-activation

In order to investigate further the importance of the C/EBP recognition sequence in the minimal xC/EBP α promoter, an internal deletion mutant that lacked this site was prepared by PCR using the del-C/EBP oligonucleotide (see Materials and Methods). EMSA experiments confirmed that this deletion abolished the interaction of the C/EBPs, present in nuclear extracts from Hep3B or A6 cells, with the wt-C/EBP sequence (data not shown). The DNA construct, Δ C/EBP, was then used in transient transfection assays in both Hep3B and A6 cell lines along with the -321/+41 promoter construct. As shown in Figure 5, deletion of the C/EBP site resulted in a drastic reduction in the constitutive xC/EBP α promoter activity. Such a dramatic reduction in reporter gene activity was also seen when another 5' deletion construct that lacked this C/EBP site (i.e. -121/+41) was included in the analysis (Fig. 5). In order to examine whether this C/EBP site is essential for auto-activation, four copies of the sequence were linked to the heterologous SV40 promoter in the pGL2-promoter vector (28) and used for transient transfection assays in Hep3B cells. As shown in Figure 5, a 6-fold increase in luciferase gene activity was obtained in the presence of co-transfected pCS2+x α plasmid.

Sp1 binds to the -82 to +41 region of the xC/EBP α promoter

Analysis of the sequence of the -82 to +41 region showed the presence of a consensus Sp1 recognition sequence, GGGGCGTGG, at position -42 to -34. Although consensus Sp1 binding sites have been identified in the promoter of the human, mouse, rat and chicken C/EBP α genes (7,17-23,34), their precise function has only been determined in detail in the murine promoter. Here, Sp1 was shown to bind to a GC box at the 5' end of the C/EBP recognition sequence and, thereby, repress C/EBP-mediated promoter activation (21). In the light of this finding, we decided to investigate the role of the Sp1 site in the xC/EBP α promoter further and in more detail. In order to identify whether Sp1 was involved in the various

DNA-protein interactions seen with the -82/+41 promoter region, competition EMSA were performed using extracts from Hep3B or A6 cells that had been transfected with the pCS2+x α expression plasmid, and a 500-fold molar excess of a consensus Sp1 binding site oligonucleotide. As a control, a similar molar excess of oligonucleotides corresponding to a high affinity C/EBP binding site (D-C/EBP), sequences corresponding to the -53 to -81 region of the xC/EBP α promoter (i.e. outside the consensus Sp1 site; NS) and a consensus USF site were included. As shown in Figure 6A, all the DNA-protein complexes obtained with the -82/+41 xC/EBP α promoter region could be competed out using an excess of Sp1 binding site oligonucleotide but not with the other sequences. In order to further confirm the interaction of Sp1 with this region, competition experiments were carried out using oligonucleotide designed against the Sp1 site present in the -34 to -42 region of xC/EBP α promoter (designated as wt-Sp1) and a slightly larger oligonucleotide that contained deletion of this sequence (referred to as del-Sp1). As shown in Figure 6B, the wt-Sp1, but not del-Sp1, competed for the binding of nuclear proteins to this region. Additionally, when both the wt-Sp1 and del-Sp1 oligonucleotides were used for similar EMSA with extracts from Hep3B or A6 cells, which had previously been transfected with the pCS2+x α expression plasmid, specific DNA binding was only seen with wt-Sp1 oligonucleotide (data not shown). In order to confirm the interaction of Sp1 with the wt-Sp1 oligonucleotide, antibody supershift assays were performed using antisera against Sp1. As shown in Figure 6C, antibody-DNA-protein supershifted complex was obtained using extracts from both cell lines.

The Sp1 binding site is necessary for constitutive expression but not auto-activation

In order to investigate the importance of the Sp1 binding site in the xC/EBP α promoter, co-transfection experiments were initially carried out using the -321/+41 promoter-luciferase DNA construct and increasing concentrations of Sp1 expression plasmid. As shown in Figure 7A, the Sp1 expression plasmid was found to increase the xC/EBP α promoter activity. To analyse the importance of the Sp1 site further, an internal deletion mutant that lacked the putative recognition sequence was prepared by PCR using the del-Sp1 oligonucleotide that curtails the binding of this factor (data not shown). Transient transfection assays into both Hep3B and A6 cell lines showed that the deletion of this site produces a drastic reduction of the constitutive xC/EBP α promoter activity (Fig. 7B). To examine whether this Sp1 site is also involved in the auto-activation of the xC/EBP α gene, four copies of the recognition sequence were linked to the heterologous SV40 promoter in the pGL2-promoter vector. As shown in Figure 7B, the luciferase activity was similar both in the absence or the presence of co-transfected xC/EBP α expression plasmid, thereby indicating that the site was involved in constitutive promoter activity but not auto-activation.

DISCUSSION

We report in this paper the organisation of the xC/EBP α promoter and an investigation of the nuclear factors that regulate the expression of this gene. Transient transfection assays showed that the -321/+41 region contained sufficient information for

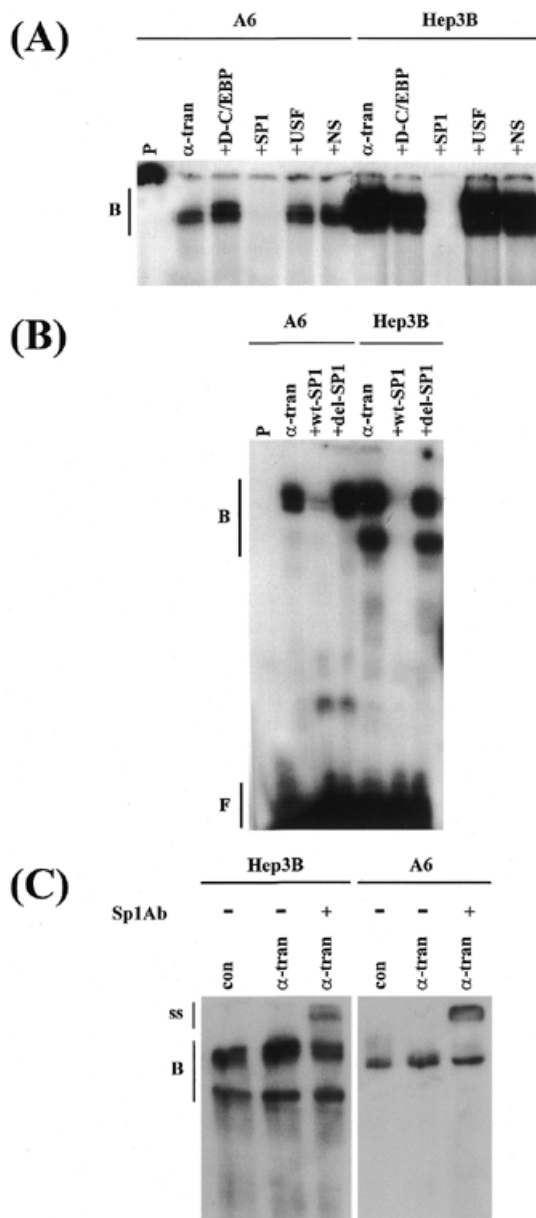


Figure 6. Binding of Sp1 to the $-82/+41$ region. EMSA were carried out using nuclear extracts from Hep3B or A6 cells that had been transfected with the pCS2+ control vector (con) or the pCS2+x α expression plasmid (α -tran) and either $-82/+41$ promoter fragment (A and B) or an oligonucleotide containing the putative Sp1 recognition sequence (C). Competition experiments used a 500-fold excess of oligonucleotides for: a high affinity C/EBP binding site (+D-C/EBP), consensus Sp1 recognition sequence (+SP1), consensus USF binding site (+USF), sequences corresponding to the -53 to -81 region of the xC/EBP α promoter (+NS), the Sp1 site from the xC/EBP α promoter (+wt-SP1) or a larger sequence containing deletion of this site (+del-SP1). In (C), + indicates the profile obtained with extracts from pCS2+x α -transfected cells in the presence of antiserum against Sp1. Vertical lines labelled B, ss and F show the position of the DNA-protein complex, the antibody-DNA-protein-supershifted complex and the free probe, respectively.

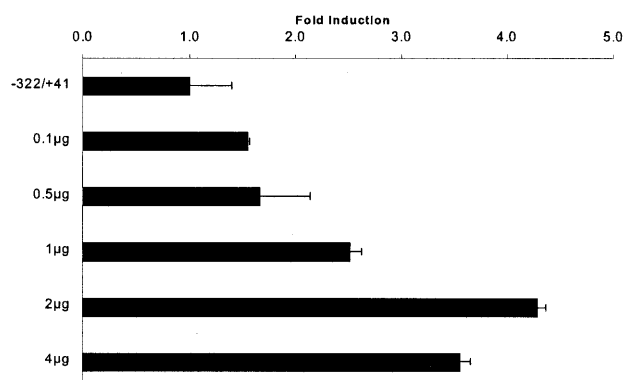
both the constitutive promoter activity and activation by xC/EBP expression plasmids. Both Sp1 and the C/EBPs were identified as important contributors to the regulation of xC/EBP α expression with both sites being involved in constitutive

expression and the C/EBP recognition sequence being absolutely essential for autoregulation. In addition, the studies demonstrate that the regulation of the C/EBP α gene involves species-specific mechanisms for both auto-activation and the action of Sp1.

Autoregulation is relatively common in the control of genes encoding transcription factors from several species, including *X.laevis*, and includes basal transcription factors, cell cycle regulated factors, stimulus-responsive factors and cell-type-specific factors (40,41). The advantages for such type of control are enormous (40). First, it offers the cells a relatively simple regulation mechanism without the involvement of a battery of other factors, which in turn, would need to be subject to another hierarchy of regulation. Thus, given that eukaryotes may contain thousands of independent transcriptional activators, autoregulation may be necessary to limit the complexity required for completely independent regulation. Secondly, it may provide the cell with a direct means to sense and control the cellular concentration of a given factor. Thirdly, autoregulation of cell-type-specific factors could represent a form of memory that may contribute, or even define, a determined state. Fourthly, autoregulation of stimulus responsive factors may serve to amplify cellular signals transiently and, additionally, allow attenuation of the response irrespective of the presence or absence of a given inducer.

Analysis of the effects of both mutations and multimerisation of C/EBP recognition sequence in the xC/EBP α promoter (Fig. 5) show that, in contrast to the human gene but in common with the mouse and rat genes, the *Xenopus* homologue is subject to direct autoregulation. In addition, we have also identified a potential species-specific mode of action of Sp1 in the regulation of C/EBP α gene expression. For example, the mouse C/EBP α promoter contains a Sp1 consensus sequence between the -185 to -194 region that overlaps with a consensus C/EBP recognition sequence (21). A similar configuration also occurs in the rat promoter (22). Tang *et al.* (21) have shown recently that Sp1 competes for the binding of the C/EBPs to this site and, thereby, represses C/EBP α promoter activity. Because the expression of Sp1 was found to be higher in preadipocytes and to decrease during the early phases of differentiation, it was suggested that Sp1 may contribute, at least in part, to the repression of C/EBP α gene transcription prior to adipocyte differentiation (21). The decrease in Sp1 levels seen early in the differentiation programme may facilitate the access of other C/EBP family members to the C/EBP regulatory element and, thereby, cause derepression of the C/EBP α gene (21). This mechanism has been suggested to act in conjunction with the repressor AP2- α , the binding site of which is present in the human, rat and the xC/EBP α promoters, and its expression levels are also high in pre-adipocytes and decrease dramatically during differentiation (7,18-20). Our studies, on the other hand, demonstrate Sp1 as an activator of xC/EBP α gene transcription with deletion of its recognition sequence producing a drastic reduction in activity (Fig. 7). Such a mode of Sp1 action may also be the case for the human and the chicken promoters; the former contains a consensus Sp1 site but lacks C/EBP recognition sequence and the putative C/EBP and Sp1 sites present in the chicken gene are non-overlapping (23,34). Therefore, at least for the human, *Xenopus* and chicken C/EBP α genes, Sp1 may act as an activator and its regulation during adipogenesis may rely solely on the action of AP-2 α .

(A)



(B)

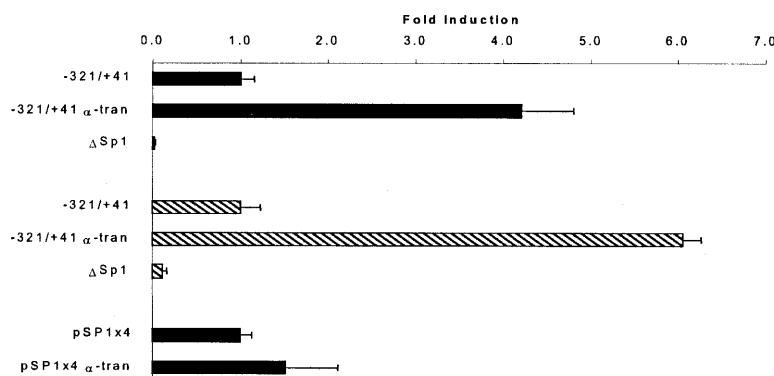


Figure 7. Analysis of the importance of the Sp1 binding site in the minimal xC/EBP α promoter region. (A), the -321/+41 construct was transfected in the absence or presence of different concentrations of the Sp1 expression plasmid as indicated and (B), the -321/+41, Δ Sp1 and pSP1 \times 4 promoter-reporter DNA constructs were transfected into Hep3B or A6 cell lines (filled and hatched histograms, respectively) in the presence of the pCS2+x α plasmid (α -tran) or the parent pCS2+ vector. The control activity obtained with the -321/+41 or pSP1 \times 4 DNA constructs has arbitrarily been assigned as 1, with those obtained in the presence of the co-transfected pCS2+x α or Sp1 expression plasmid being shown with respect to this value. Each value represents the mean \pm SD from two independent experiments.

In conclusion, we have demonstrated that the xC/EBP α gene is subject to direct autoregulation through the binding of the C/EBPs to a recognition sequence present in the proximal promoter region. In addition, we show that Sp1 activates its expression. Thus, the species-specific modulation of C/EBP α gene expression involves both autoregulation and the mode of action of Sp1.

ACKNOWLEDGEMENTS

We thank Prof. Guntram Suske for the Sp1 expression plasmid, Bob Jones for the preparation of the figures and Scott Irvine for help with computer analysis. F.T.K. was supported

by a PhD studentship from the University of Balikesir. D.P.R. acknowledges financial support from the Biotechnology and Biological Sciences Research Council (BBSRC).

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