Mechanism for basal expression of rat mitochondrial branched-chain-2-oxo-acid dehydrogenase

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The rat branched-chain-2-oxo-acid dehydrogenase (BCOD) kinase mRNA is transcribed from a TATA-less promoter that has GC-rich sequences and two putative Sp1 binding sites near the transcription start site. We demonstrated previously that the 5' region of the kinase gene, base pairs –128 to +264, contained promoter activity when assayed using luciferase as a reporter (Huang and Chuang (1996) *Biochem. J.* **313**, 603–609). To define DNA elements required for efficient expression of the kinase gene, nested deletion constructs of the above promoter region fused with a luciferase reporter gene were transfected into cultured H4IIE (hepatoma) and NRK-52E (kidney) cells. The results showed that the region between nucleotides –58 and +21 was indispensable for the kinase basal promoter activity. Methylation-interference and mutagenesis-promoter assays identified nucleotides –50 to –40 (ACAACCTCCCA) as *cis*-acting DNA sequences that are required for nuclear protein binding

and efficient promoter activity. Gel-supershift analysis with anti-Sp1 antibody suggested that the nuclear protein capable of binding to the –58 oligonucleotide (bp –58 to –34) was immunologically related to the Sp1 protein. The –58 oligonucleotide formed a DNA–protein complex with recombinant Sp1 protein with an affinity approximately ten-fold lower than that of the consensus Sp1 oligonucleotide. Co-transfection of the Sp1 expression plasmid and the –58 promoter construct into *Drosophila* Schneider cells revealed that Sp1 contributed to the kinase basal promoter activity by binding to the non-consensus site in the –58 region. Deletion of two consensus Sp1 binding sites (bases –150 to –140 and bases +29 to +38) in the kinase gene did not affect the basal promoter activity. Therefore binding of Sp1 or Sp1-like proteins to the above single non-consensus Sp1 sequence in the –58 region plays a major role of trans-activating basal expression of the BCOD kinase.

INTRODUCTION

The mammalian mitochondrial branched-chain-2-oxo-acid dehydrogenase (BCOD) complex catalyses the oxidative decarboxylation of the branched-chain 2-oxo acids derived from leucine, isoleucine and valine. The mammalian BCOD complex is a macromolecule (molecular mass 4×10^6 Da) comprising multiple copies of five component enzymes. The three catalytic components are: a thiamine pyrophosphate (TPP)-dependent decarboxylase (E1, branched-chain 2-oxo acid decarboxylase) with two E1 α (molecular mass 45 500 Da) and two E1 β (molecular mass 37 500 Da) subunits; a dihydrolipoyl transacylase (E2), which contains 24 lipoate-bearing polypeptides (monomer, molecular mass 46 500 Da) and a dihydrolipoyl dehydrogenase (E3), which is a flavoprotein of homodimeric structure (monomer, molecular mass 55 000 Da) [1,2]. In addition, the BCOD contains two regulatory enzymes, a specific kinase [3] and a specific phosphatase [4], which control enzyme activity through a reversible phosphorylation (inactivation)–dephosphorylation (activation) of E1 [5]. The BCOD complex is organized around the 24-mer dihydrolipoyl transacylase cubic core (molecular mass 1.1×10^6 Da), to which E1, dihydrolipoyl dehydrogenase, the kinase, and the phosphatase are attached through ionic interactions [1,6].

The BCOD complex-specific kinase and phosphatase activities are influenced by metabolic nutritional states and hormones [5,7–10]. Administration of glucagon or adrenaline was shown to result in a four-fold increase in the percentage of active or dephosphorylated enzyme complexes (i.e. the activity state) in the liver of rats fed a low protein diet [11]. Similar effects on the activity state occurred with insulin and cAMP, but to a lesser extent [11]. The kinase activity in the liver of rats fed diets devoid of protein increased 3- to 4-fold compared with rats on a 50%

protein diet [10]. The increase in kinase activity was accompanied by a corresponding elevation in the levels of kinase protein and mRNA. This provides an effective mechanism to down-regulate the flux of branched-chain amino acids through the enzyme complex. Moreover, the phosphorylated state (inactive state) of the complex varies significantly in rat tissues and correlates with the amounts of kinase protein and mRNA. The same study [10] also showed that the kinase protein and mRNA levels were low in the liver and high in the kidney and heart of rats fed a normal diet.

To better understand the transcriptional regulation of the BCOD kinase gene, we have cloned and partially characterized the promoter region [12]. The wide tissue distribution of the kinase mRNA and TATA-less, GC rich sequences in the vicinity of the transcription start site indicate that the kinase is a housekeeping gene. In the present study, DNA elements required for efficient expression of the rat BCOD kinase gene were further defined by promoter assays. The region required for high promoter activity was also essential for the binding of nuclear proteins, as demonstrated by gel-shift and methylation-interference assays. Moreover, it was shown that a single, low affinity Sp1 binding site was sufficient for the efficient expression of the BCOD kinase gene in both kidney and hepatoma cell lines.

MATERIALS AND METHODS

Construction of promoter–luciferase vectors

The promoter constructs –255 and –128 were prepared as described previously [12]. To make internally deleted constructs, –128 Δ –33 to +37, –128 Δ Sp1 (+22 to +37), –255 Δ –128 to –100, and –255 Δ –99 to –59, PCR amplification

Abbreviations used: BCOD, branched-chain-2-oxo-acid dehydrogenase; BrdU, 5-bromo-2'-deoxyuridine; E1, branched-chain-2-oxo acid decarboxylase; DTT, dithiothreitol; EMSA, electrophoresis mobility-shift assays; SL2, Schneider cell line 2.

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was used to generate specific promoter segments. The sense primer Luc P₁: 5'-CCAACTCATCAATGTATC-3' (corresponding sequences in the upstream of polylinker *Bam*HI site of pBS-Luc PA), the antisense primer: 5'-GCCTCCTGGGAGTGTAGTT-3' (bases -34 to -53) and the template promoter construct -128 were used to amplify promoter region (bases -128 to -34) and 30-bp upstream vector sequences. To make the Sp1-deficient construct, the sense primer Luc P₁, the antisense primer 5'-AGCCCGCCTTCGCC-3' (bases +21 to +8) and the template -128 were used to amplify promoter region corresponding to the bases -128 to +21 and 30-bp upstream vector sequences. The two fragments were digested with *Bam*HI and purified by agarose-gel electrophoresis. A DNA fragment, corresponding to +38 to downstream *Pst*I site of pBS-Luc PA, was excised from the promoter construct -128 with *Sal*I, blunt-ended with Klenow and then digested with *Pst*I. The *Sal*I blunt-ended *Pst*I fragment and one of either of the *Bam*HI-digested segments was ligated to a *Bam*HI-*Pst*I cut pBS-Luc PA vector. The resulting constructs were -128 Δ -33 to +37 and -128 Δ Sp1 (+22 to +37). A similar strategy was applied to generate -255 Δ -128 to -100 and -255 Δ -99 to -59. The fragment containing the 30-bp upstream vector sequence and the promoter region of bases -255 to -129 or bases -255 to -100 was amplified by PCR using, as a template, the -255 promoter construct and a pair of primers, Luc P₁ and the antisense primer 5'-CTCTGCCAGCACCCCTACCA-3' (bases -129 to -148), or Luc P₁ and the antisense primer 5'-CCTTCAGATGAGTAGTTGACTCTGCCAG-3' (bases -100 to -128) respectively. The two PCR fragments produced were digested by *Bam*HI and isolated by agarose-gel electrophoresis. The sense primer 5'-GGTAGGGGATACCCCTAAAGA-3' (bases -99 to -80) or 5'-GGTCAAACCTACAACTCCAGGAGGC-3' (bases -58 to -34) and the antisense primer 5'-TGTCCCGCTTCTTTGTTG-3' (bases +264 to +247) were used to PCR amplify the region corresponding to bases -99 to +264 or bases -58 to +264 respectively. The *Bam*HI-digested -255 to -129 fragment and the segment -99 to +264 were ligated into *Bam*HI-*Sma*I digested pBS-Luc PA vector, resulting in plasmid -255 Δ -128 to -100. The same ligation strategy was used to generate construct -255 Δ -99 to -59. The -58 to +264 fragment was ligated to the *Sma*I-digested pBS-Luc PA vector to make plasmid -58. The correct orientation of these constructs was confirmed by DNA sequencing.

Construct -128 to -34 was generated by ligating the *Bam*HI-digested fragment described above into the *Bam*HI and *Sma*I cut pBS-Luc PA vector. Construct -128 to +37 was obtained by ligating a blunt-ended *Sal*I and *Bam*HI fragment (bases -128 to +37) excised from the -128 promoter construct into the *Bam*HI and *Sma*I-digested pBS-Luc PA vector.

Multiple mutations were generated in the bases -58 to -34 promoter region by PCR-based site-directed mutagenesis. To generate -58 mutant constructs, primers containing doubly mutated oligonucleotides in position -53/-52, -51/-50, -49/-48, -47/-46, -45/-44, -43/-42, -41/-40, or -39/-38 and the antisense primer Luc P₂ 5'-TCGGCAAGGTAGGAGATC-3' (corresponding to the pBS-Luc PA sequences located downstream of the *Pst*I polylinker site) were used with the promoter construct -58 as a template for PCR amplification. The amplified product was digested with *Pst*I and ligated into the pBS-Luc PA vector previously cut with *Sma*I and *Pst*I. The mutated sequences were confirmed by DNA sequencing.

Cell culture and transient co-transfection

Rat hepatoma cells H4IIE (generously given by Dr. C. R.

Mendelson, Department of Biochemistry, University of Texas Southwestern Medical Center, TX, U.S.A.) were grown in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) bovine serum and 5% (v/v) fetal-calf serum. Normal rat kidney/epithelial-like cells, NRK-52E, were grown as described previously [12]. Cells were harvested by treatment with trypsin and transfected with the promoter-luciferase vectors by electroporation. About 2×10^6 NRK cells or 2×10^7 H4IIE cells in 1 ml of serum-free Dulbecco's modified Eagle's medium were mixed with approx. 2.5 pmol (about 10 to 15 μ g) of various promoter-luciferase vectors and 5 μ g of cytomegalovirus β -galactosidase plasmid, supplemented with salmon sperm DNA to a total of 100 μ g DNA in each transfection mixture. NRK and H4IIE cells were electroporated at 1180 μ F, 250 V, and 1980 μ F, 250 V respectively. Electroporated cells were cultured in 60-mm dishes with a complete culture medium for 48 h before harvesting.

The *Drosophila* Schneider cell line 2 (SL2) cell line was grown in *Drosophila* media (GIBCO) [13] supplemented with 10% (v/v) heat-inactivated fetal-bovine serum (56 °C, 30 min) and cultured in a tightly closed flask at 25 °C. Cells were plated at a density of 5×10^6 per 60-mm dish on the day before transfection. Expression plasmids containing cDNA encoding the 696 C-terminal amino acids of human Sp1 in-frame with the translation initiation ATG of the *Drosophila* alcohol dehydrogenase (*adh*) gene (pAdhSp1-in) and an out of frame control (pAdhSp1-out) were kindly provided by Dr. Robert Tjian (Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California at Berkeley, CA, U.S.A.). Luciferase reporter plasmids and the Sp1 expression vectors (pP_{adh} Sp1-in or pP_{adh} Sp1-out) and β -galactosidase plasmid, 2 μ g each, were co-transfected into SL2 cells using the calcium phosphate method [14,15]. A positive control pGL 3 containing the SV40 promoter in a luciferase reporter plasmid was obtained from Promega. After incubation with the calcium phosphate-DNA co-precipitate for 30 min at 25 °C, transfected plates were supplemented with 4 ml of a complete medium and incubated for 24 h. The medium was then replaced with fresh complete medium and the plate was incubated for an additional 24 h. Cells were harvested for luciferase and β -galactosidase assays as described previously [16].

Preparation of nuclear extracts

H4IIE cells (1×10^9) were harvested from media by centrifugation for 5 min at 650 g. Pelleted cells were washed with PBS and collected by centrifugation as described above. The nuclear extract of H4IIE cells was prepared as described previously [17], with minor modifications. Cells were suspended in 5 vol. of buffer A [10 mM Hepes, pH 7.9 at 4 °C/1.5 mM MgCl₂/10 mM KCl/0.5 mM dithiothreitol (DTT)] and incubated, on ice, for 10 min. The cells were collected by centrifugation and suspended in two vol. of buffer A and lysed with 10 strokes in a Kontes glass Dounce homogenizer. The homogenate was examined for the degree of cell membrane disruption by microscopy. Broken cells were centrifuged for 10 min at 650 g to sediment nuclei. The pellet was subjected to a second centrifugation for 20 min at 27000 g. Crude nuclei were resuspended in 3 ml of buffer C [20 mM Hepes, pH 7.9/25% (v/v) glycerol/0.42 M NaCl/1.5 mM MgCl₂/0.2 mM EDTA/0.5 mM PMSF/0.5 mM DTT] per 10^9 cells and disrupted again with a Kontes glass Dounce homogenizer. The resulting suspension was stirred gently for 30 min and centrifuged for 30 min at 27000 g. The supernatant was dialysed at 4 °C against buffer D [20 mM Hepes, pH 7.9/10% (v/v) glycerol/50 mM KCl/0.2 mM EDTA/0.5 mM PMSF/0.5 mM DTT]. The dialysate was clarified by centri-

fugation and the supernatant containing the nuclear extract was frozen in liquid N₂ and stored at -70 °C.

Electrophoresis mobility-shift assay (EMSA)

Equimolar amounts of an oligonucleotide and its complementary strand were annealed in water at 90 °C for 5 min and slowly cooled to 25 °C. The double-stranded oligonucleotide at 10 pmol was 5'-labelled by T4 kinase. The binding of nuclear factors to selected oligonucleotide probes was accomplished by adding 5 µg of crude nuclear extract to each gel-shift reaction mixture. The reaction mixture (20 µl) contained 10 fmol (approx. 2.5×10^4 c.p.m.) of the appropriately end-labelled double-stranded oligonucleotide, 10 mM Hepes (pH 7.5), 50 mM KCl, 10 mM EDTA, 0.5 µg poly(dI-dC)·poly(dI-dC), 10% (v/v) glycerol, and 1 mM DTT. After incubation for 20 min on ice, the reaction mixture was loaded on to a 6% polyacrylamide gel in 50 mM Tris base/50 mM boric acid/1 mM EDTA, and electrophoresed at 400 V for 1 h at 25 °C. In competition studies, unlabelled oligonucleotides were added immediately before the probe. To immunologically identify the protein component of protein-DNA complexes, specific antibodies and the nuclear extract were added simultaneously to the reaction mixture and kept on ice for 20 min before electrophoresis.

To carry out EMSA, 8 µl of H4IIE nuclear extract (about 3 to 4 µg/µl) was mixed with 2 µl of 10 mM EDTA and incubated on ice for 30 min. The EDTA-treated nuclear extract was used for EMSA as described above and various divalent cations were added to the reaction mixture. The sequences of the oligonucleotides used for EMSA were as follows:

Kin -58 (5'): GGTCAAACTACAACCTCCAGGAGGC
 Kin -58 (3'): GCCTCCTGGGAGTTGTAGTTTGACC
 Kin -58 mutation 1 (5'): GGTCAAACCAGGGCTCCCA-GGAGGC
 Kin -58 mutation 1 (3'): GCCTCCTGGGAGCCCTGGTTT-GACC
 Kin -58 mutation 2 (5'): GGTCAAACTACAACCTGTGAG-GAGGC
 Kin -58 mutation 2 (3'): GCCTCCTCACAGTTGTAGTTT-GACC
 Sp1 (5'): ATTCGATCGGGGCGGGGCGAGC
 Sp1 (3'): GCTCGCCCCGCCCCGATCGAAT
 Kin -128 (5'): CTGGCAGAGTCAAACCTACTCATCTGA-AGGG
 Kin -128 (3'): CCCTTCAGATGAGTAGTTTGACTCTG-CCAG

Methylation-interference analysis and chemical sequencing

20 pmol of -58 (3') oligonucleotide was 5'-labelled using T4 kinase at 37 °C for 30 min and denatured at 65 °C for 10 min. The radiolabelled oligonucleotide was annealed to the complementary strand oligonucleotide -58 (5'). The asymmetric end-labelled double-stranded oligonucleotide was chemically modified at 37 °C for 30 min with 0.5% (w/v) dimethyl sulphate in 200 µl of buffer [50 mM sodium cacodylate, pH 8.0/10 mM MgCl₂/1 mM EDTA], followed by the addition of 50 µl of a second buffer (1.5 M sodium acetate, pH 7.0/1 M 2-mercaptoethanol) containing 100 µg/ml tRNA to stop methylation. The methylated oligonucleotide was purified by two rounds of ethanol precipitation. The labelled and methylated -58 probe was incubated with the nuclear extract from H4IIE cells, and subjected to EMSA as described above. Bound and free probes were excised from the 6% polyacrylamide gel and eluted with buffer [0.1% (w/v) SDS/0.5 M ammonium acetate/10 mM sodium

acetate] at 37 °C overnight. Eluted oligonucleotides were separated from the gel by centrifugation in a spin column (Millipore), and concentrated by ethanol precipitation. Precipitated radiolabelled DNA samples were cleaved with 10% (w/v) piperidine at 90 °C for 30 min and freeze-dried. The resulting DNA pellet was denatured at 95 °C for 5 min in a loading buffer [98% (w/v) deionized formamide, 10 mM EDTA, pH 8.0, 0.025% (w/v) xylene cyanol FF and Bromophenol Blue] and resolved on 15% DNA sequencing gels consisting of acrylamide/*N,N'*-methylene-bis-acrylamide (30:1) and 8 M urea.

The asymmetric end-labelled double-stranded -58 oligonucleotide (10^5 c.p.m.) in 10 µl water was used for G + A and G chemical sequencing as described before. The piperidine-cleaved product was freeze-dried and analysed on a 15% DNA sequencing gel.

UV cross linking of the oligonucleotide-protein complex

A substituted Kin -58 (3') oligonucleotide was synthesized (Biosynthesis Inc.) for UV cross-linking experiments in which the thymidine residues at positions -40, -46, -47, and -49 were substituted with 5-bromo-2'-deoxyuridine (BrdU). The substituted Kin -58 (3') oligonucleotide was annealed with the Kin -58 (5') oligonucleotide and radiolabelled as described above. A 2-fold scale-up of the EMSA reaction was carried out in 40 µl, the solution was exposed to UV light for 30 min, and products were separated by SDS/PAGE (7.5% gel). Gels were dried and exposed to PhosphorImager cassettes. A 1000-fold excess of unlabelled Kin -58 or Kin -58 mutant 2 oligonucleotide was added to the reaction mixture to determine the specificity of the cross-linked oligonucleotide-protein complex. In parallel, a 10-fold scale-up of the EMSA reaction was carried out in the presence of Sp1 antibody. Bands supershifted by the antibody were extracted from the gel after a 30 min UV-light irradiation, and separated by SDS/PAGE (7.5% gel).

RESULTS

Identification of the rat BCOD kinase promoter region

The promoter activity of the kinase gene was studied by the luciferase reporter assay using H4IIE (rat hepatoma) or NRK-52E (rat kidney/epithelial-like) cells as a host. Promoter constructs were transfected into host cells by electroporation. Luciferase reporter activity was normalized to co-transfected β-galactosidase activity to correct for variations in transfection efficiencies. Except for constructs -128/+37 and -128/-34, promoter constructs used in the present study contained an identical 3' end at base +264, which is 5' contiguous to the initiation Met codon, whereas 5' ends of the constructs varied (Figure 1A).

Figures 1(B) and (C) show that constructs -255, -128 and -58 have high promoter activity in both NRK and H4IIE cells. Constructs -255 Δ -128/-100 and -255 Δ -99/-59 have similar promoter activities in comparison with construct -255. Although sequence analysis of the -128 to -100 region indicated existence of potential activator-protein 1 and cAMP-responsive element binding sites, site-directed mutagenesis of the activator-protein 1 or cAMP-responsive element site did not affect basal promoter activity (results not shown). The loss of 90% promoter activity in construct -128 Δ -33/+37 compared with construct -128/+37 (Figures 1B and 1C) suggests that the region -33 to +37 is needed for constitutive basal promoter activity. Moreover, construct -128 Δ +22/+37 shows full (100%) luciferase activity similar to clones -58 and -128. To examine the necessity of intron 1 for the kinase basal

Figure 1 Promoter activities of 5' and internal deleted mutants measured by the luciferase reporter assay

DNA fragments representing different lengths of the promoter-regulatory region of the rat kinase gene were ligated into the pBS-Luc PA plasmid containing the luciferase reporter cDNA. **(A)** Scheme illustrating where gaps/deletions occur. The constructs are designated according to position of the 5' terminal base of the DNA fragment. The 3' end of all constructs is base +264, except for constructs -128 to +37 and -128 to -34. The open box (not to scale) depicts intron 1 (581 bp) that interrupts the 5'-untranslated region (264 bp). The internal deleted region is expressed as Δ base number to base number. **(B)** Luciferase activity of gap/deletion constructs in NRK cells. Luciferase activity was measured in cell extracts and expressed as a percentage of the activity of clone -128. To correct for variation in transfection efficiency, luciferase activity was normalized to co-transfected β-galactosidase activity. **(C)** Luciferase activity in H4IIE cell extracts. The activity is expressed as a percentage of the activity of clone -255 Δ -128 to -100. Error bars are S.E.M. ($n = 3$).

expression, the promoter construct -128/+37 was analysed and showed similar activity to the -128 construct (Figures 1B and 1C). Therefore intron 1 does not play a role in contributing to basal promoter activity of the kinase. The above data, taken together, suggest that the minimal promoter regulatory region between bases -58 and +21 is both necessary and sufficient for basal transcription of the rat kinase gene in H4IIE and NRK cells.

Since the rat BCO1D kinase promoter lacks a TATA box and has a GC-rich sequence flanking the transcription starting site, we investigated whether the Sp1 transcription factor conferred basal kinase expression. There are two Sp1-binding sites in the proximal promoter region -150 to -140 and +30 to +38. The similar activity between constructs -255 and -128 (Figures 1B and 1C) indicated that the upstream Sp1 in the -150 to -140

region is not essential for basal promoter activity. The absence of the downstream Sp1 site in the construct -128 Δ Sp1 (+22/+37) also did not affect the basal promoter activity in NRK-52E or H4IIE cells (Figures 1B and 1C). Therefore neither of these two consensus Sp1-binding sites participated in the basal expression of the kinase.

Definition of the DNA elements for activation of the kinase basal expression

To test whether the -58 region is required for basal promoter activity of the kinase gene, site-directed mutants were generated. Single or contiguous double-base substitutions in the region between bases -50 and -40 resulted in about 50% to 90% reduction of the promoter activity compared with the wild-type

Figure 2 Sequences required for promoter activity and nuclear protein binding

(A) Promoter activities of substituted mutation constructs in the -58 to -34 promoter region. The double-stranded wild-type sequence in this region is shown at the top. Bases in the box (-49 to -40) are essential for basal promoter activity (see below). All of the constructs contained intron 1 (581 bp), which interrupts the 5'-untranslated region (264 bp), and the 5' and 3' end terminated at -58 and +264 respectively. The mutated nucleotides in each construct are shown in lower case letters and complementary bases are omitted. To correct for variation in transfection efficiency, luciferase activity was normalized to co-transfected β -galactosidase. The relative activity of mutated substitution constructs was determined and expressed as a percentage of the activity of the wild-type -58 construct. The values are the means of two individual transfections. (B) Methylation-interference analysis. The -58 anti-sense oligonucleotide was end labelled and partially methylated as described in the Materials and methods section. The modified probe was incubated with the H4IIE nuclear extract and free and bound complexes were isolated after electrophoresis and cleaved with piperidine. The -58 probe (approx. 10^5 c.p.m.) was modified with formic acid or dimethylsulphate for chemical sequencing of G + A and G residues respectively. Modified G + A (lane 1) or G (lane 2) residues were cleaved with piperidine and resolved in a 15% sequencing gel. Lanes 3 and 4 show the G sequencing of the -58 probe from major (slow migration) and minor (fast migration) shifted bands respectively. Lane 5 shows the chemical sequencing of the free probe. (C) Summary of the methylation-interference data. The promoter sequence of the -58 oligonucleotide is shown with the numbers above the sequence indicating the distance from the mRNA initiation site. G nucleotides protected by proteins from the H4IIE nuclear extract and their relative locations on the sequencing gel are indicated with arrows. *, methylated G residues.

-58 promoter (Figure 2A). Mutations at bases -38/-39 or -52/-53 did not affect promoter activity. Therefore the sequence ACAACTCCCA between -50 to -40 (Figure 2A, boxed) is required for the basal promoter activity of the kinase gene. To define nucleotides between bases -58 and -34 required for binding nuclear proteins, the radiolabelled -58 oligonucleotide probe was used in methylation-interference analysis. Piperidine cleaves sugar-phosphate bonds of methylated residues, whereas non-methylated G residues, protected by the nuclear protein, cannot be cleaved by piperidine. The partially methylated -58 oligonucleotide probe formed a major, slowly migrating and a minor, fast migrating complex with the H4IIE nuclear extract on EMSA. The piperidine-cleaved products from the major and minor retarded bands are shown in Figure 2(B), lanes 3 and 4 respectively; the free probe cleaved by piperidine is depicted in lane 5. The G + A and G chemical sequencings of the -58 oligonucleotide are shown in lanes 1 and 2 respectively. The methylation-interference analysis indicated that the region containing non-methylated G residues at -45, -43, -42 and -41 of the antisense strand is essential for the formation of the two

DNA-protein complexes. Moreover, the G base at position -48 required for the formation of the major DNA-protein complex is also indispensable for the promoter activity, suggesting a strong sequence-dependent correlation between the formation of the major complex and the promoter activity. The results are summarized in Figure 2(C). Thus the nucleotides that confer both the binding of nuclear proteins and promoter activity are located in the region of bases -50 to -40 (Figure 2C).

Correlation between nuclear protein binding and the promoter activity

If binding of the nuclear protein to the -58 region is functionally important, mutations affecting the promoter activity should also affect binding with the nuclear protein. Sequences of the oligonucleotides used for EMSA competition study are shown in Figure 3(A). Although the -128 region shares a 10 bp identical sequence with the -58 region, the -128 oligonucleotide was not able to compete for the binding. This result suggests the 10-bp common sequence GTCAAAC TAC (bases -120 to -111 and

Figure 4 Sp1 competition and supershift analysis

(A) Sequences of the -58 (25 mer) and Sp1 oligonucleotides (22 mer) are illustrated. The 10-bp sequence identified from promoter or methylation-interference assays in the -58 oligonucleotide and the consensus Sp1 binding motif are indicated in the open box. (B) The ^{32}P -labelled -58 (lanes 1-7) or Sp1 (lanes 8-14) oligonucleotide probe was incubated with 5 μg of H4IIE nuclear extract in the absence (lanes 1 and 8) or in the presence of an increasing molar excess (10, 10^2 , 10^3) of the -58 (lanes 2-4 and 9-11) or the Sp1 (lanes 5-7 and 12-14) oligonucleotide. N. E., nuclear extract. (C) The ^{32}P -labelled -58 (lanes 1-7) or Sp1 (lane 8-14) oligonucleotide probe was incubated with either 5 μg of H4IIE nuclear extract or 10 ng of purified Sp1 protein in the absence or in the presence of the Sp1 antibody (Ab). NE, nuclear extract.

Figure 5 Supershift analysis of UV cross-linked -58 oligonucleotide-protein complexes

(A) UV cross linking of EMSA solution. The BrdU-substituted -58 probe was UV cross-linked with the human Sp1 protein (lanes 2–4) or H4IIE nuclear extracts (lanes 5–7) in the absence (lanes 2 and 5) or presence of a 1000-fold excess of unlabelled -58 (lanes 3 and 6) or -58 mut 2 (lanes 4 and 7) oligonucleotide. The UV-irradiated BrdU-substituted -58 probe was loaded in lane 1. Cross-linked oligonucleotide-protein complexes are shown with an arrow. Molecular-mass markers are shown on the left. (B) UV cross linking of the Sp1-antibody supershifted bands. The EMSA supershift experiment was performed using the BrdU-substituted -58 probe complexed with the Sp1 protein (lane 2) or H4IIE nuclear extracts (lane 1) in the presence of Sp1 antibody. The gel was exposed to UV light and the Sp1 antibody supershifted bands were eluted and analysed by SDS/PAGE (7.5% gels). Estimated sizes of the observed oligonucleotide-protein complexes are indicated by arrows. Molecular-mass markers are shown on the left.

bases -57 to -48) itself is not sufficient for specific nuclear protein binding, in agreement with the finding that the essential binding site is located in the -50 to -40 region, as analysed by methylation-interference and promoter assays. EMSA showed that the -58 oligonucleotide formed a DNA-protein complex with the nuclear extract from H4IIE cells (Figure 3B, lane 2). Similar results were obtained with nuclear extracts from FTO-2B cells, rat liver and kidney (results not shown). The association of the -58 oligonucleotide and the unknown nuclear protein is sequence dependent and can be effectively competed for by the -58 wild-type oligonucleotide but not by -58 mutant 1 and 2 oligonucleotides (Figure 3B, lanes 6–11). Moreover, downstream mutations (-58 mutant 2 oligonucleotide) showed more severe defects in the binding ability than upstream mutations (-58

mutant 1 oligonucleotide), when the competitive binding ability of mutant 1 (lanes 6–8) with mutant 2 (lanes 9–11) oligonucleotide is compared. Although the minor DNA-protein complex can be preferentially displaced with the -58 wild-type oligonucleotide at high concentration (1000-fold excess), -58 mutant 2 and -128 oligonucleotides compete for the formation of the minor complex more effectively than that of the major complex.

The CA (sense)- or GT (antisense)-rich sequence in the -50 to -40 region does not match perfectly with any transcription factor binding sites obtained from the National Center of Biotechnology Information database. It was found during an EMSA study that high concentrations of EDTA had deleterious effects on the binding of nuclear protein to the -58 oligonucleotide. To examine the EDTA effect on DNA-protein formation, the nuclear extract was pretreated with EDTA for 30 min and used in EMSA studies. The EDTA-treated nuclear extract failed to bind the -58 oligonucleotide (Figure 3C, lane 3) and the -58 major oligonucleotide-protein complex could be restored by the addition of Zn^{2+} ions (Figure 3C, lanes 10 and 11), but not by the addition of other divalent cations such as Ca^{2+} (lanes 4 and 5), Cd^{2+} (lanes 6 and 7), Mg^{2+} (lanes 8 and 9) or Mn^{2+} (lanes 12 and 13). In contrast, the addition of Zn^{2+} did not enhance the formation of two minor faster-migrating complexes. To confirm that the deleterious effects of EDTA on nuclear protein binding was caused by the removal of Zn^{2+} ions, the Sp1 oligonucleotide was used as a control (Figure 3C, lanes 14–16) to monitor the DNA binding ability of the Sp1 protein in the EDTA-treated nuclear extract. The formation of Sp1 DNA-protein was diminished in the EDTA-treated nuclear extract (Figure 3C, lane 15) but was reversed by the addition of Zn^{2+} ions (lane 16). These data suggest that the binding of nuclear proteins to the -58 oligonucleotide requires Zn^{2+} ions.

Identification of the nuclear protein that binds to the -58 oligonucleotide in EMSA as Sp1 or an Sp1-like protein

The results shown in Figure 3(C) suggest that the protein bound to the -58 oligonucleotide is a zinc-finger transcription factor. Since the kinase promoter lacks a TATA box and contains GC-rich sequences around the transcription starting site, Sp1 may be involved in regulating basal promoter activity of the kinase gene. Although two proximal Sp1 sites in the kinase promoter are not needed for basal promoter activity, Sp1 may activate the kinase promoter by binding to a non-consensus site with low affinity. Sequences of the -58 (25-mer) and Sp1 oligonucleotides (22-mer) are illustrated in Figure 4(A). The 10-bp sequence identified from promoter or methylation-interference assays in the -58 oligonucleotide and the consensus Sp1 binding motif are indicated in the open box. In competition experiments, the -58 oligonucleotide-protein complex could be specifically replaced by an oligonucleotide containing the consensus Sp1 binding site (Figure 4B, lanes 5–7), and vice versa (lanes 9–11). In the presence of a 100-fold higher concentration of Sp1 oligonucleotide or a 1000-fold higher concentration of -58 oligonucleotide, the formation of the -58 DNA-protein complex was abolished (lanes 4 and 6). Similarly, in the presence of a 1000-fold higher concentration of -58 oligonucleotide or a 100-fold higher concentration of Sp1 oligonucleotide, the formation of the Sp1 DNA-protein complex was diminished to the same degree (lanes 11 and 13). The data suggest that the same nuclear protein binds to the -58 and Sp1 oligonucleotide with a 10-fold lower binding affinity for the former.

The results of a supershift experiment, using polyclonal IgG raised against amino acid residues 520–538 of human Sp1, which are conserved in rat Sp1, provided further support that the major

Figure 6 Activation of basal promoter activity of the kinase by Sp1 in *Drosophila* SL2

SL2 were co-transfected with 2 μ g each of a promoter–luciferase construct, the cytomegalovirus β -galactosidase plasmid and either the pAdhSp1-in or pAdhSp1-out plasmid. The SV40–luciferase construct containing triple 21-bp Sp1 sites was used as a positive control. The –4.3 K wrongly-oriented kinase–luciferase construct, with background promoter activity in mammalian cells, served as a negative control. After 48 h, the cells were harvested and luciferase activity was measured. To correct for variation in transfection efficiency, luciferase activity was normalized to co-transfected β -galactosidase and expressed as relative light units/absorbance (O.D.) at 570 nm. Relatively activities in the presence (solid bar or pAdhSp1-in) or in the absence (open bar or pAdhSp1-out) of Sp1 expression are shown. WT, wild-type.

protein–DNA complex formed in the nuclear extract was the result of Sp1 binding. The antibody used for the supershift experiment is specific for Sp1 p95 and p105, and does not cross-react with other transcription factors in the Sp1 family (Sp2, Sp3 and Sp4). As shown in Figure 4(C), the Sp1 antibody itself did not bind to either –58 or the Sp1 oligonucleotide (lanes 2 and 9). In addition, the majority of the –58 oligonucleotide–protein complex and Sp1 oligonucleotide–protein complex were supershifted by Sp1 antibody (lanes 4 and 11). However, the proteins complexed with both oligonucleotides could not be further retarded in the presence of higher concentrations of Sp1 antibody (Figure 4C, lanes 5 and 12) indicating that some proteins in the complexes were not immunologically reactive with the Sp1 antibody. Moreover, the recombinant human Sp1 protein bound to the –58 oligonucleotide with a markedly lower affinity than Sp1 oligonucleotide when signal intensities were compared (Figure 4C, lanes 6 and 14), and the complex was supershifted by Sp1 antibody (lanes 7 and 13).

The molecular masses of the nuclear proteins bound to the –58 sequences were measured by EMSA and UV cross-linking experiments (Figure 5A). Purified full-length human Sp1 was used to compare the gel migration patterns between Sp1 and the nuclear factors in H4IIE cells. Upon UV cross-linking, human Sp1 protein and the BrdU-substituted –58 probe formed a smeared complex (Figure 5A, lane 2), which could be specifically competed for with the –58 oligonucleotides (Figure 5A, lane 3) but not with the –58 mutation 2 oligonucleotide (Figure 5A, lane 4). The nuclear factors and the –58 oligonucleotide also formed a sequence-specific smeared complex on the gel, which had a molecular mass similar to that of the complex formed between the –58 probe and human Sp1 (Figure 5A, lane 5). The nuclear factor–oligonucleotide complex could also be specifically competed for with the –58 oligonucleotide (Figure 5A, lane 6) but not with the –58 mutation 2 oligonucleotide (Figure 5A,

lane 7). UV cross-linking in combination with supershift experiments were carried out to investigate further whether nuclear proteins other than Sp1 bound directly to the –58 oligonucleotide in the Sp1-antibody supershifted complex. This complex was irradiated with UV and subjected to SDS/PAGE. Because of glycosylation, human Sp1 is known to exist with two different apparent molecular masses, 95 and 105 kDa [18]. UV cross linking of purified human Sp1 complexed with the –58 probe yielded two species of approx. 102 kDa and 112 kDa (Figure 5B, lane 2). Similarly, UV cross linking of the –58 probe complexed with the H4IIE nuclear extract produced two complexes of approx. 99 kDa and 108 kDa (Figure 5B, lane 1). The minor differences in molecular masses between the –58 probe and human Sp1 and the –58 probe and the H4IIE nuclear-extract complexes might be due to species differences. Thus the results indicated that the –58 oligonucleotide bound directly to the Sp1 or Sp1-like protein rather than to a ternary or higher-order complex of which Sp1 was a component.

Transactivation of the rat BCOD-kinase promoter in Schneider cells by Sp1

To test whether Sp1 functionally transactivates the –58 region, the Sp1 expression plasmid and the –58 promoter–reporter plasmid were co-transfected into SL2 to determine if Sp1 was needed for the kinase basal promoter activity (Figure 6). SL2 was derived from *Drosophila* embryonic cells and is devoid of endogenous Sp1 protein since no Sp1 homologues have been found in invertebrates. The SV40–luciferase construct, containing triple Sp1 binding sites, served as a positive control. The promoter–luciferase constructs (SV40, –58, –58 mutant, –4.3 K wrongly oriented) with Sp1, the in-frame expression plasmid (pAdhSp1-in) or the out-frame plasmid (pAdhSp1-out), together with the β -galactosidase plasmid were co-transfected into SL2 cells. The SV40 construct with the in-frame Sp1 plasmid showed 7–8 fold higher normalized luciferase activity than SV40 with the out-frame plasmid. Although mutant kinase constructs have an unusually high background activity in SL2 cells, the construct carrying the wild-type –58 sequence showed nearly 1.5 to 4-fold higher activity in the presence of than in the absence of Sp1 expression. Relatively high background activity was observed in the two mutant constructs (–58 mutant and –4.3 K wrongly oriented). However, the promoter activities of the mutant constructs were not altered, whether with or without Sp1 expression. These data support the notion that the sequence of the –58 GC box is required for activation by Sp1 in SL2.

DISCUSSION

We have demonstrated that the protein in H4IIE extracts that binds to the –58 region of the rat kinase promoter is Sp1 or a closely related protein. In gel retardation assays, nuclear proteins in the H4IIE extract formed two specific protein–DNA complexes with an oligonucleotide probe containing the consensus Sp1-recognition sequence. The upper complex migrated similarly to the complex formed between the Sp1 oligonucleotide and purified human Sp1. The formation of both complexes was competitively inhibited by unlabelled oligonucleotides corresponding to the –58 oligonucleotide of the BCOD kinase promoter and was supershifted by polyclonal IgG directed against the Sp1 protein. When used as a probe in EMSA, the –58 oligonucleotide formed the same protein–DNA complex with the Sp1 consensus oligonucleotide and the complex was also recognized by Sp1 antibody. Although *in vitro* binding assays demonstrated that the –58 oligonucleotide binds to Sp1 with markedly lower affinity than the wild-type Sp1 oligonucleotide, replacing the –58 region

with wild-type Sp1 sequence did not enhance the kinase basal promoter activity in H4IIE cells (results not shown). The results suggest that the Sp1 protein in the cell type examined is so abundant that it saturates a weak binding site or that other cellular factors or the chromatin structure may facilitate Sp1 binding [19]. The other two consensus Sp1 sites (−150 to −140 and +30 to +38) in the kinase gene are apparently not needed for basal promoter activity. Constructs containing the −58 region were sufficient to allow transactivation of the BCOD kinase promoter by Sp1 in embryonic SL2. Thus binding and transactivation of the kinase promoter by Sp1 or its related protein may account for the universal expression pattern of the kinase gene.

Sp1 contains three zinc-finger motifs, (Cys)₂ (His)₂, which bind to a consensus GGG GCG GGCG sequence [20]. The nucleotide sequence in the −58 region differs from the Sp1 consensus sequence, with an affinity for the Sp1 protein approx. 10-fold lower than that of the latter. Based on the crystal structure of the protein–DNA complex of the closely related zinc-finger protein Zif 268 [21], and two dimensional NMR structures of the Sp1 zinc fingers 2 and 3 [22], it was proposed that high-affinity binding of Sp1 required zinc finger 3 to contact G residues at positions 2 and 3, and zinc finger 2 to contact G residues at positions 4 and 6 [20]. More conformational flexibility of zinc finger 1 for the DNA interaction would explain the sequence diversity that is tolerated within the 3′ portion of the Sp1 binding site in several natural promoters [23–25]. In the present study, the nuclear protein bound to the −58 oligonucleotide displayed all the binding characteristics of Sp1. The four G residues were conserved in the antisense strand (bases −41, −42, −43 and −46) and were protected by a nuclear protein in the methylation-interference study. Mutations of the three G residues at positions 2, 3 and 4 (bases −41 to −43) in the −58 mutant 2 oligonucleotide, which presumably affect the interaction with zinc fingers 2 and 3 of the Sp1, cannot titrate the −58 DNA–complex binding. The −58 mutant 1 oligonucleotide, carrying mutations at positions 7, 8 and 9 (bases −48, −47 and −46) which are less essential for protein interaction, is able to partially replace the −58 oligonucleotide at a 1000-fold higher concentration. These results are consistent with the view that the 5′ ends of the sequences are more critical than the 3′ end sequences for Sp1 binding. Although nucleotides at positions 7, 8 and 9 are less critical for Sp1 binding in the different sequences that exist in natural promoters [23–25], these nucleotides still play a role in determining overall binding affinity. This is shown by the difference in binding affinity between −58 wild-type and mutant 1 oligonucleotides.

Sp1 is an abundant nuclear protein in cells, but the amount of expression changes during development and in different cell types [26]. A large number of ubiquitous and tissue-specific genes have been suggested to be regulated by Sp1. These were demonstrated by specific binding of Sp1 to *cis* elements that are required for promoter activity [23,27]. Recently, Marin et al. [28] have obtained Sp1^{−/−} knockout embryos. The lethal knockout and abnormal phenotypes in the Sp1^{−/−} embryo support the indispensable role of Sp1 and confirm the notion that no Sp1-knockout mammalian cells can be successfully generated. Reverse transcription-PCR analysis of Sp1-target genes in embryonic stem cells and in mouse embryos at about 8.5 days postcoitum showed that many putative Sp1-target genes are expressed normally in Sp1^{−/−} mice. The data suggest that the number of genes critically dependent on the presence of Sp1 for expression is significantly smaller than expected. It is proposed that other members of the Sp1 family may compensate, in part, for the loss of Sp1 activity in Sp1 knockout cells. Additional members of the

Sp gene family include Sp2, Sp3 and Sp4 [29,30], with one of them, Sp3, probably functioning as an inhibitory member of the family [29,30]. Sp3 and Sp4, but not Sp2, bind to the consensus Sp1 sequence with an affinity comparable with authentic Sp1 [29–32]. Most Sp1-dependent genes contain multiple binding sites required for Sp1 activation [23]. It is of interest that the kinase gene contains only one low-affinity Sp1 binding site, which is apparently sufficient for promoter activation. It remains to be determined, however, whether other Sp1 closely-related proteins bind to and activate the rat BCOD kinase promoter in the absence of Sp1 expression in the Sp1^{−/−} embryo.

Although the kinase is ubiquitously expressed, the amount of kinase mRNA is highly variable and inversely correlates with different activity states of the BCOD complex in rat tissues [5,8,10]. The mechanism for differential expression of kinase mRNA in tissues is unknown. It has been shown, however, that Sp1 binds directly to the TATA-box-protein accessory factor TAFII110 [33]. When bound to distant sites in *cis*, Sp1 can interact with itself and loop out the intervening DNA, suggesting that Sp1 may promote interactions between the basal promoter and distant regulatory elements *in vivo* through such a looping mechanism [34,35]. In fact, recent studies have shown that promoter activation by upstream transcription factors requires the presence of the downstream Sp1 binding sequence, further supporting a co-operation between upstream activators and the Sp1 protein [36,37]. Moreover, the Sp1-responsive element cannot be replaced by a functional TATA box, suggesting that the presence of Sp1 binding sites is important for efficient activation of a promoter by other transcription activators [37]. These findings support the thesis that differential expression of the kinase gene may result from interactions of Sp1 with upstream transcription factors that are specific in different tissues.

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