PHYSICAL AND FUNCTIONAL INTERACTIONS BETWEEN USF AND SP1 PROTEINS REGULATE HUMAN DEOXYCYTIDINE KINASE PROMOTER ACTIVITY*

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SUMMARY

Deoxycytidine kinase (EC 2.7.1.74; dCK) is central to drug activity of anticancer and antiviral agents such as cytosine arabinoside (ara-C) and gemcitabine. HepG2 hepatocellular carcinoma cells were used to study the transcriptional regulation of dCK. 5’-Deletion and site-directed mutagenesis of the dCK upstream region (positions –464 to –27) confirmed the importance of two GC-boxes (positions –317 to –309 and –213 to –206) and two E-boxes (positions –302 to –297 and –278 to –273). In vitro electromobility shift assays with HepG2 nuclear extracts and in vivo chromatin immunoprecipitation assays with HepG2 chromatin extracts confirmed the presence of bound Sp1/Sp3 and USF1/2. Cotransfections in HepG2 cells showed that USF1 and USF2a stimulated and Sp1 repressed promoter activity from a dCK-luciferase reporter gene construct. In Sp- and USF-null Drosophila Mel-2 cells, both Sp1 and USF1 stimulated dCK promoter activity in a dose-dependent manner, however, both Sp3 and USF2a were effectively inert. Combined Sp1 and USF1 showed additive transactivation at lower concentrations of Sp1. Sp1 was inhibitory at higher levels. Stimulation by combined USF1/USF2a with Sp1 was similar to that for USF1 alone with Sp1, whereas transactivation by Sp1 and USF2a without USF1 was synergistic. Physical interactions between USF and Sp proteins were confirmed by immunoprecipitations with Sp- and USF-specific antibodies. Domain mapping of USF1 and USF2a localized the functional interactions between USF and Sp proteins to the DNA binding domain of USF. Identifying the physical and functional interactions between Sp and USF proteins may lead to a better understanding of the basis for differential expression of the dCK gene in tumor cells, and may foster strategies for up-regulating dCK gene expression and improving chemotherapy with ara-C and gemcitabine.
INTRODUCTION

Deoxycytidine kinase (NTP: deoxycytidine 5′-phosphotransferase, EC 2.7.1.74; dCK) is responsible for the phosphorylation of several deoxyribonucleosides such as 2′-deoxycytidine and purine nucleosides including 2′-deoxyadenosine and 2′-deoxyguanosine. dCK also catalyzes the rate-limiting step in the phosphorylation of important anticancer and antiviral agents such as cytosine arabinoside (ara-C), difluorodeoxycytidine (gemcitabine), 2-fluoro-ara-AMP (fludarabine), 2′,3′-dideoxycytidine, and 2-chlorodeoxyadenosine (Cladribine; CdA) (1,2). dCK levels are critical determinants of ara-C and gemcitabine antitumor activities, and resistance to nucleoside analogs is frequently associated with decreased dCK (3,4). Further, transfection of the dCK cDNA into dCK-deficient tumor cell lines restores in vitro sensitivities to ara-C (5,6).

The product of dCK gene is a 30.5 kDa polypeptide that is present at low levels in most tissues (7-10). The human dCK gene spans over 34 kb on chromosome 4 (4q13.3-q21.1) and includes a coding region consisting of 7 exons, ranging in size from 90 to 1544 bp (11). Promoter activity was localized to a 697 bp upstream fragment, including 386 bp of 5′ upstream region, 250 bp of exon 1, and 61 bp of intronic sequence (11). The dCK promoter is highly GC-rich and lacks a TATA-box but contains a transcription initiator region located adjacent to the major transcription start site at position –146 relative to the start of translation. The initiation region also contains an imperfect E2F binding site (12). In vitro DNase I footprint and electromobility shift assays (EMSAs) demonstrated binding of Sp1 to two GC-boxes and upstream stimulatory factor (USF) to a critical E-box. Although site-directed mutagenesis of these cis-elements implied their transcriptional importance, the detailed mechanisms by which these binding associations regulate dCK gene expression have not been established.

The Sp and USF proteins are ubiquitously expressed, yet both are implicated in the regulation of genes characterized by tissue-specific or developmental patterns of expression (13,14). Sp1 and the related Sp3 proteins can exert activating or inhibiting effects on transcription, depending on the cell or promoter context, through binding to GC- or GT-box elements (13). The USF proteins belong to the
class of b-HLH-ZIP (basic helix-loop-helix leucine zipper) transcription factors including the nuclear proteins Myc, Max, Mad, Mxi1, AP4, TFEB, TFE3, MiTF, and ADD1 (15). USF was first identified by its capacity to stimulate transcription from the adenovirus late promoter and was purified from HeLa cells as two polypeptides, designated USF1 (43 kD) and USF2a (44 kD) (16,17). Subsequently, a 38 kDa form, designated USF2b, was described (15). USF1 and USF2 proteins are encoded by two separate genes, and USF2a and 2b represent alternate splice forms of USF2 (15). USF1 and USF2 proteins have similar DNA binding domains (67% homology) but differ in their N-terminal transactivation domains, yet all forms bind to the E-box motif (CACGTG) as homo- and heterodimers (18,19).

An attractive biological feature of USF and Sp proteins is their abilities to mediate a wide range of transcriptional activities via protein-protein interactions with other families of transcription factors. Moreover, evidence for cooperative interactions between USF and Sp proteins has been described, via binding to juxtaposed E-box and GC- elements (20,21). Given the proximity of the essential GC- and E-box elements in the dCK promoter, co-operative interactions between USF and Sp proteins can, likewise, be envisaged to regulate expression of this gene. In the present paper, we significantly extend earlier reports of dCK promoter structure and function in lymphoid cells (12). We characterize the major cis-elements and transcription factors that regulate dCK in HepG2 human hepatoma cells, and document the existence of physical interactions between the USF and Sp proteins that can potentially regulate dCK promoter activity over a wide range.
MATERIALS AND METHODS

Chemicals and Reagents. [γ-32P] ATP (3000 Ci/mmols) was purchased from Dupont-New England Nuclear (Boston, MA). Synthetic oligonucleotides were purchased from Genosys Biotechnologies (The Woodlands, TX). Restriction and modifying enzymes, reporter gene vectors (pGL3-Basic, pRLSV40), and other molecular biologicals were purchased from Promega (Madison, WI). The pPacSp1, pPacO, and pCMV-Sp1 plasmid constructs were provided by Dr. Robert Tjian (University of California, Berkeley, CA), and the pPacSp3 and pPacUSp3 constructs were provided by Dr. Guntram Suske (Philipps-Universitat, Marburg, Germany). The pPacUSF2b plasmid construct and the mammalian (CMV) expression plasmid construct of USF2a were provided by Dr. Shiqing Yan (Wayne State University, Detroit, MI).

Cell Culture. The human HepG2 hepatocellular carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD) and was cultured as previously reported (21). Drosophila Mel-2 (D. Mel-2) cells were purchased from Invitrogen (Carlsbad, CA) and maintained in Schneider’s insect medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine plus 100U/ml penicillin and 100µg/ml streptomycin at 28°C.

Construction of dCK Promoter-Luciferase Reporter Plasmids and Site-Directed Mutagenesis. The dCK promoter was PCR amplified from human genomic DNA isolated from HL-60 promyelocytic cells using forward (5’-gcccactgcaggtgacgccctct-3’, dCK/F2) and reverse (5’-tggagattttcttgatgcgggtcccctca-3’, dCK/R2) primers. The PCR products were first cloned into pGEM-T-Easy vector. An antisense pGEM-T-Easy clone of the dCK promoter was identified and digested by NcoI and SaI and the dCK promoter fragment was then subcloned into pGL3Basic at the XhoI and NcoI restriction sites to generate pdCK-464/+75. To generate the 5’-deletion constructs, pdCK-359/+75, pdCK-298/+75, pdCK-179/+75, and pdCK-27/+75, the pdCK-464/+75 was digested by Smal,
SmaI and Eco72I, SmaI and Bpu10I, and SmaI and SacI, respectively, and religated (the Bpu10I and SacI sites were blunt-ended with T4 DNA polymerase before ligation).

Promoter constructs harboring nucleotide substitutions in putative transcription factor binding elements were prepared using an overlap extension PCR protocol (22). Separate amplifications were performed for sense and antisense mutagenesis primers (Table 1) with dCK/R2 or dCK/F2, respectively, using pdCK-464/+75 as template. The two products were mixed, and a second PCR was performed using the dCK/F2 and dCK/R2. The mutant amplicons were blunt-ended with T4 DNA polymerase and subcloned into the SmaI site of pGL3-Basic. All the deletion and site-directed mutagenesis constructs were sequenced to confirm the intended deletions or mutations.

**Construction of USF1 and USF2 Drosophila and Mammalian Expression Plasmids.** The USF1 coding cDNA was generated by RT-PCR from HepG2 total RNA with forward (5’-ttagatatcataagggcagcagaaaaacagct-3’, USF1atg) and reverse (5’-ttacctgagtagttctgtctattctgtagacgacctc-3’, USF1taa) primers. BamHI and XhoI restriction sites, respectively (underlined) were incorporated into the primers for subcloning. To generate pPacUSF1, the PCR products were digested with BamHI/XhoI and inserted into the *Drosophila* pPac expression vector after excision of the Sp1 coding sequence from pPacSp1 (23). To construct the N-terminal deletion plasmids of USF1, PCR reactions using the full-length pPacUSF1 as a template were carried out with a GC-Rich Kit (Roche). The following oligonucleotides were designed as upstream PCR primers: Δ1-15, 5’-ttagatatccagattcagaggtgctgctgct-3’; Δ1-39, 5’-ttagatatccagattcagaggtgctgctgct-3’; Δ1-80, 5’-ttagatatccagattcagaggtgctgctgct-3’; Δ1-100, 5’-ttagatatccagattcagaggtgctgctgct-3’; Δ1-130, 5’-ttagatatccagattcagaggtgctgctgct-3’; Δ1-156, 5’-ttagatatccagattcagaggtgctgctgct-3’; Δ1-175, 5’-ttagatatccagattcagaggtgctgctgct-3’; Δ1-196, 5’-ttagatatccagattcagaggtgctgctgct-3’. The USF1taa oligonucleotide was used as the reverse primer.
for each amplification. The PCR products were digested with BamHI/XhoI and cloned into pPac, as described above.

To generate the mammalian expression pcDNA3-USF1 construct, the USF1 cDNA from HepG2 cells was amplified with forward (5'-ggatccacagagagatgaaggggcagcagaaaacagct-3’) and reverse (USF1taa) primers and the amplicon inserted into pcDNA3 vector (Invitrogen) at the BamHI and XhoI restriction sites.

Full length pPacUSF2a and its N-terminal deletion constructs were generated by PCR using the mammalian (CMV) expression construct as template and USF2tga as downstream primer (5' - ttaaggctttcactgcggggtgcctgccc-3’, the HindIII site is underlined) and the following forward primers (the XhoI site in each oligonucleotide is underlined): USF2a, 5'-ttactcgagatggacatgctggacccg-3’ (designated USF2atg); Δ1-36, 5’-ttactcgaggaagccgggagccggccagga-3’; Δ1-76, 5’-ttactcgaggtacataccgcgtagtcagttggtcc-3’; Δ1-143, ttactcgaggtgcagttctatggatatccctca-3’; Δ1-193, 5’-ttactcgagggccactttcactgcggggtgcctgccc-3'; Δ1-223, 5’-ttactcgagccgttctacgtcatgatga-3’; and Δ1-223, 5’-ttactcgagaaatgtggaccaacaggaacccc-3’. The pPacUSF2Δ1-36&76-143 construct was generated by PCR with the Δ1-36 forward primer and USF2tga reverse primer, and pPacUSF2b (Δ76-143) as a template. The amplicons were digested with XhoI and HindIII and inserted into pPac at the XhoI/HindIII site.

Overlap-extension PCR was used to create the USF2a internal deletions. Separate amplifications were performed with the internal forward and reverse deletion primers (Δ143-193, forward 5’-cccttgccaggtctagcagc-3’ and reverse 5’-gctggcagggcgcctgggaccgc-3’; Δ193-223, forward 5’-tttcatgtggagttagcaggaagcgctg-3’ and reverse 5’-ttttctacgtcagcatgaagaggtgcg-3’; Δ223-242, forward 5’-cccttctgtggaggggttgaggcgggagg-3’ and reverse 5’-cccttgccaggtctagcagc-3’) and USF2tga and USF2atg, respectively. The products were mixed, and a second PCR was performed using the USF2atg and USF2tga primers. The amplicons were cloned into the XhoI and HindIII sites in pPac. All constructs were all confirmed by DNA sequencing.
**Transient Transfections and Luciferase Assay.** Transient transfections of dCK-luciferase reporter gene constructs (in pGL3-Basic) into HepG2 cells and reporter gene assays were performed as previously reported (21). Firefly luciferase activities were normalized to Renilla luciferase activities. D. Mel-2 cells were cotransfected with 1 µg of the dCK-luciferase reporter gene constructs and Sp1 (10 to 500 ng pPacSp1) or Sp3 (25 ng pPacUSp3 and pPacSp3, for the long and short isoforms, respectively), and/or USF1 (100-500 ng pPacUSF1) or USF2a (100-500 ng pPacUSF2a), using Fugene™ 6 reagent (Roche Diagnostics Corporation, Indianapolis, IN). Cells were harvested after 24h for luciferase assays using the Single Luciferase Assay System (Promega). Luciferase activities were normalized to total cell protein, measured by the Bio-Rad protein assay system. The expression levels of Sp1, USF1 and USF2a (including mutant forms) in D. Mel-2 cells were monitored by Western blotting (see below).

**Preparation of Nuclear Extracts and Electromobility Shift Assays.** Nuclear extracts from HepG2 cells or D. Mel-2 transfectants were prepared by standard methods (24). Electromobility shift assays with HepG2 and D. Mel-2 nuclear extracts were performed as previously described (21). For supershift experiments, reaction mixtures were pretreated for 30 minutes with 2 µg of rabbit polyclonal antibodies [Sp1 (Geneka Biotechnology, Inc.); USF-1 and USF2 (Santa Cruz Biotechnology)].

**Chromatin Immunoprecipitation (ChIP) Assay.** ChIP assays were performed in HepG2 cells as previously described (39), with Sp1 (rabbit polyclonal antibody, Active Motif), USF-1, or USF2 antibodies (rabbit polyclonal antibody, Santa Cruz). Standard PCR for the dCK promoter region was performed with forward (dCK/F2) and reverse (dCK/R2) primers spanning positions −464 to +75. An unrelated human gene (reduced folate carrier coding exon 1) was also amplified with forward (5′-cagttgcacctgctccctccg-3′, KS43/P8) and reverse (5′-ggtgatgaagctctcccctgg-3′, RFCPoly1) primers to validate the specificity of the ChIP assays.

**Immunoprecipitation and Western Blot Analysis.** Nuclear extracts (300 µg) from D. Mel-2 cells, cotransfected with pPacSp1 and pPacUSF1, or pPacSp1 and pPacUSF2a, were diluted to 1 µg/µl with
immunoprecipitation buffer [10 mM Tris/HCl, pH7.5, 150 mM NaCl, and 1% Nonidet P-40, containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin) and phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride)]. Samples were pre-cleared with protein A-agarose beads for 1 h at 4 °C, then treated overnight at 4 °C with 10 µg of rabbit USF1 or USF2 antibody (Santa Cruz Biotechnology), or normal rabbit IgG, followed by incubation with 100 µl of protein A-agarose beads for 90 min at 4 °C. The beads were collected by centrifugation (8,000 rpm) and washed (4 times) with immunoprecipitation buffer, and the immunoprecipitates were eluted by boiling for 5 min in 30 µl of 2× Laemmli sample buffer. The eluates were electrophoresed through 12% SDS-polyacrylamide gels, and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were developed with Sp1 (Geneka Biotechnology, Inc.), USF1 or USF2 antibodies (in TTBS containing 0.5% fat-free dried milk powder) and Lumi-Light Western Blotting Substrate (Roche), and exposed to x-ray film.
RESULTS

*Localization of the major cis-elements in the dCK promoter by 5'-deletion and mutation analysis.* A previous report demonstrated that two GC-boxes (positions –317 to –309 and –213 to –206; designated GCa and GCb, respectively) and one E-box [positions –302 to –297; designated E-box (a)] (Figure 1) are important for dCK promoter activity in human lymphoblast cell lines (12). To further investigate the interrelationships between these elements and their bound transcription factors, a 539 bp upstream fragment of the dCK gene was amplified from HL-60 genomic DNA (including 464 bp of 5'flanking sequence and 75 bp of the first coding exon) and subcloned into pGL3-basic to generate pdCK-464/+75 (Figure 1). Alignment of our dCK promoter sequence to the previously published sequence (ref. 12) revealed variations at three positions (not shown). However, no difference was observed between our dCK promoter sequence and the human genome sequence (GenBank accession #: AC093851). In addition to GCa and GCb-boxes and the E-box (a) elements, database analysis revealed a previously unrecognized E-box [positions –278 to –273, designated E-box (b)] (Figure 1).

Transient transfections of pdCK-464/+75 into HepG2 cells resulted in high levels of promoter activity (Figure 2). Deletion of 105 bp from the 5'-end of dCK-464/+75 (i.e., dCK-359/+75) slightly decreased activity (~25%) (Figure 2). Further deletion of 61 bp (i.e., pdCK-298/+75), including the GCa and the E-box (a) elements, resulted in a significant loss of promoter activity, to ~15% of the level for the full-length construct. This decreased to 8% upon removal of an additional 113 bp of 5' sequence (pdCK-179/+75), including GCb and E-box (b) (Figure 2). Deletion of the E2F half site (pdCK-27/+75) did not result in any further change in promoter activity.

Similar to results with lymphoid cells, site-directed mutagenesis of either the GCa and E-box (a) elements in pdCK-464/+75 was notably inhibitory in reporter gene assays (decreases of 33% and 45%, respectively, relative to the wild type construct). However, the effect of mutating both the GCa and E-box (a) elements was essentially the same as those for the individual mutants. Mutation of E-box (b) resulted in ~20% decrease of dCK promoter activity. Mutation of GCb resulted in increased luciferase
activity (~17%) over the wild type pdCK-464/+75 construct, albeit less than reported for lymphoblast cells (12). The double GCa/GCb and E-box (a)/GCb mutants were also slightly activating. Interestingly, double mutation of the two E-boxes resulted in additive loss of dCK promoter activity (to 35% of the wild type). The double GCa/E-box (b) and E-box (b)/GCb mutants were slightly repressive (30 and 15% decrease, respectively).

Collectively, these results implicate a 61 bp stretch beginning 298 bp upstream of the translation start and including the GCa and E-box (a) elements as important to dCK promoter transactivation. Further, they imply that the downstream GCb-box is repressive and that mutating this element can override the inhibitory effects resulting from mutation of either the GCa or the E-box elements. The failure of the double GCa/E-box (a) mutant to repress activity to an extent similar to the pdCK-298/+75 deletion construct may reflect (i) the presence of E-box (b) between the GCb and E-box (a) and/or (ii) the repressive effects of GCb on pdCK–298/+75 activity.

In vitro and in vivo binding of transcription factors bound to the GC-box and E-box elements in the dCK promoter. Gel shifts were used to identify the major transcription factors that bound to the dCK promoter. When a labeled oligonucleotide (dCK-328/-289, positions: -328 to -289) containing both the GCa and the E-box (a) elements was incubated with nuclear extracts from HepG2 cells, 5 distinct DNA/protein complexes (labeled 1 to 5) were detected (Figure 3A, lane 2), which were completely abolished by treatment with excess unlabeled dCK-328/-289 probe (Figure 3A, lane 3). Competition and supershift results suggested a complex interplay between USF binding to E-box (a), and Sp proteins binding to the GCa element. Thus, unlabeled Sp1 consensus oligonucleotide completely abolished complexes 1, 2, 3, and 5 (lane 4), whereas a USF1 competitive oligonucleotide completely eliminated complexes 1, 2, and 4 (lane 5). Complexes 1, 2, 3, and 5 were variably supershifted by Sp1 and/or Sp3 antibodies (lanes 6 and 7), whereas USF1 or USF2 antibodies supershifted complexes 1, 2, and 4 and significantly decreased complexes 3 and 5 (lanes 8 and 9). Finally, competition with dCK-328/-289 containing mutated GCa (GCa mt, lane 10) abolished the formation of complexes 1, 2, and 4, and
addition of dCK-328/-289 including a mutated E-box (a) [E-box (a) mt, lane 11] eliminated complexes 1, 2 and 5 and significantly decreased the signal of complex 3.

When a labeled oligonucleotide spanning the E-box (b) (dCK-291/-260, positions: -291 to -260) was incubated with HepG2 nuclear extracts, one major DNA/protein complex was detected, which was completely abolished by excess unlabeled dCK-291/-260 or a USF1 consensus oligonucleotide (Figure 3B, lanes 2, 3, and 4). However, competition was ineffective with the dCK-291/-260 oligonucleotide including a mutated E-box (b) [E-box (b) mt, lane 7]. Addition of USF1 or USF2 antibodies supershifted the DNA/protein complex (lanes 5 and 6), confirming that this contains both USF1 and USF2 proteins.

Gel shifts were also performed using HepG2 nuclear extracts and a labeled oligonucleotide spanning the GCb box (dCK-223/-181, positions: -223 to -181). Two DNA/protein complexes (complexes 1 and 2) were readily detected (Figure 3C, lane 2) and were effectively competed by either excess unlabeled dCK-223/-181 or a Sp1 consensus oligonucleotide (lanes 3 and 4). However, competition was ineffective if a dCK-223/-181 oligonucleotide was used in which the GCb element was mutated (GCb mt) (lane 7). Addition of Sp1 or Sp3 antibody supershifted both complexes (lanes 5 and 6), thus confirming the competition results and establishing that both Sp1 and Sp3 were bound to the GCb element.

In vivo binding of the transcription factors to the dCK promoter in HepG2 chromatin extracts was confirmed by the ChIP assays (Figure 3D). These results demonstrate binding of the Sp and USF families of proteins to the critical GC and E-box elements in the dCK promoter.

Co-transfections of Sp1 and USF1/USF2a in HepG2 cells. To determine the functional significance of Sp1/3 and USF1/2 binding to the dCK promoter, transient co-transfections were performed in HepG2 cells using the pdCK-464/+75 reporter construct with Sp1, USF1, or USF2a expression constructs. Sp1 alone effected a 36% decrease of dCK promoter activity (Figure 4). Overexpression of USF1 or USF2a, in the absence of Sp1, resulted in a potent stimulation of luciferase activity (12- and 9-fold,
respectively). When USF1 or USF2a was co-expressed with Sp1, the maximal transactivation response compared with USF1 or USF2a, alone, was decreased by ~25%.

These results document an apparent repressor role for Sp1 binding, consistent with the findings of our mutagenesis experiments with the GCb element (Figure 2). Moreover, they support a functional interaction between Sp and USF proteins, consistent with the results of our gel shift experiments.

**Functional interactions of Sp1/Sp3 and USF1/USF2a in D. Mel-2 cells.** To further explore the functional relationships between Sp and USF proteins in regulating the dCK promoter, additional experiments were performed in *Drosophila* Mel-2 cells, which provide a null background for these transcription factors (23,25,26). The pdCK-464/+75 reporter gene construct was co-transfected with expression constructs for Sp1 (pPacSp1) or Sp3 (pPacSp3 and pPacUSp3), and USF1 (pPacUSF1) or USF2a (pPacUSF2a). Luciferase activities were compared with those obtained with the empty pPacO expression vector.

The potent transactivation by Sp1 in *D. Mel-2* cells showed a strong dose dependence from 10 to 50 ng. At 50 ng Sp1, a net 105-fold stimulation of promoter activity was observed over empty pPacO vector (Figure 5A). Likewise, USF1 alone (100-500 ng) showed a dose-dependent transactivation with a maximum stimulation of ~50-fold. Transactivation by combinations of Sp1 and USF1 was completely additive at levels of Sp1 to 25 ng, however, at 50 ng Sp1, promoter activity progressively declined with increasing USF1 (Figure 5A). This effect became even more pronounced at higher levels of Sp1 (200-500 ng), since under these conditions inhibition of promoter activity occurred at all doses of USF1 (not shown). In contrast to USF1, USF2a up to 500 ng was incapable of transactivating the pdCK-464/+75 reporter gene construct (Figure 5B). However, when Sp1 was cotransfected with USF2a, a strong synergistic transactivation of promoter activity was observed at all levels of Sp1 and USF2a (Figure 5B).

Additional experiments were performed with combined USF1 and USF2a, thus permitting comparisons between transactivating potentials of the USF1 and USF2a homodimers, and USF1/USF2a...
heterodimers. In this series of experiments, combined USF1/USF2a (250 ng each), without Sp1, was essentially equivalent to 500 ng of USF1. Together with Sp1, combined USF1/USF2a was ~25% less transactivating than homodimeric USF2a (500 ng) and approximately equivalent to the effects of homodimeric USF1 (500 ng) (data not shown).

Although Sp3 binding to the GCa and GCb elements was detected on gel shifts with HepG2 nuclear extracts (Figure 3, panels A and C), neither the long (USp3) nor the short forms of Sp3 (25 ng) transactivated dCK promoter activity when transfected individually into D. Mel-2 cells. Whereas a small (1.2-fold) stimulation of promoter activity was observed when Sp3 or USp3 was combined with USF1, there was no effect of combined Sp3/USp3 with USF2a on promoter activity (data not shown).

To verify the binding function of these ectopically expressed transcription factors in these experiments, nuclear extracts were prepared from D. Mel-2 cells transfected with USF and Sp1, and used for gel shifts with the labeled dCK-328/-289 probe. Predictably, no specific DNA/protein complexes were detected with extracts from mock-transfected cells (Figure 6, lane 2). For the dCK/USF1/Sp1 transfectants, specific protein binding was detected (lanes 3, 5, 7, 9, and 12). The identities of the bound proteins were confirmed as Sp1, USF1, and USF2a by competitions with Sp1 and USF consensus oligonucleotides (lanes 4, 6, 8, 10, 11, 13, and 14). Notably, in cotransfections with Sp1 and USF proteins, competition and supershift assays for USF1 and USF2a binding not only perturbed the complexes identified as USF1/USF2a, but also decreased the binding of Sp1 (lanes 11 and 14).

These results directly support the notion that functional antagonistic or synergistic relationships exist between Sp and USF proteins with the dCK promoter (Figure 5) and that these effects are reflected in physical interactions between these proteins detected on gel shifts. The net transcriptional response observed appears to be specific to Sp1, since Sp3 was functionally inert, and thus varies in both magnitude and direction with the different Sp proteins and with the hetero- versus homodimeric composition of the USF complex.
Co-immunoprecipitation assays of Sp1 and USF proteins. To provide evidence for the existence of direct physical associations between Sp1 and USF1 and/or USF2a, co-immunoprecipitations were performed from nuclear extracts prepared from D. Mel-2 cells co-transfected with expression constructs for these transcription factors. As shown in Figure 7 (lower panels for both A and B), USF1 and USF2a were immunoprecipitated with antibodies to these proteins. Moreover, both USF1 (panel A, lower) and USF2 (panel B, lower) antibodies also immunoprecipitated Sp1, demonstrating that Sp1 physically interacts with the USF proteins.

Identification of the USF1 and USF2a domains responsible for E-box-dependent dCK transactivation or repression with Sp1. Our functional studies with HepG2 and D. Mel-2 cells suggested a potential for wide ranging regulation of the dCK promoter by the Sp and USF families of proteins. For instance, whereas USF1 and Sp1 were both potently transactivating, USF2a and Sp3 were effectively inert. For USF1, promoter activity was repressed at elevated levels of Sp1, and Sp1 combined with USF2a was highly synergistic. Direct binding associations between Sp1 and USF1/USF2a proteins were strongly implied by gel shifts and were confirmed by co-immunoprecipitations with USF-specific antibodies.

To identify the USF domains responsible for dCK transactivation and the cooperative or antagonistic functional interactions between Sp1 and USF proteins, a series of USF deletion clones were prepared (8 for USF1, 11 for USF2) and analyzed by transient co-transfections of D. Mel-2 cells with pdCK-464/+75, with or without Sp1. Both USF1 and USF2a bind to DNA as homo- and heterodimers, involving the leucine zipper and helix-loop-helix regions (18,19). Thus, except for the Δ223-242 mutant of USF2a, all of our USF mutant constructs were designed to contain the basic region and the helix-loop-helix domains, two cysteines (229 and 248 for USF1; 265 and 284 for USF2a) required for oligomerization, and the C-terminal leucine zipper.
Among the transfections with USF1 constructs, levels of USF1 and Sp1 proteins were essentially equal on western blots (Figures 8B and C). Deletion of the first 15 amino acids from the N-terminus of USF1 (Δ1-15) significantly increased USF1 transactivation, with and without Sp1, suggesting a repressor function for this region (Figure 8A). For both conditions, promoter activity was unchanged accompanying loss of an additional 24 amino acids (Δ1-39). Further deletions to position 80 (Δ1-80) resulted in a ~60% decrease in the extent of USF1 transactivation (but only in the absence of Sp1). Loss of 20 more amino acids (Δ1-100) was accompanied by a nearly total loss of response to USF1, alone, and completely abolished the additive effect of USF1 with Sp1. Thus, it appears that amino acids 80-100 are involved in a functional interaction between USF1 and Sp1. The USF1 Δ1-130, Δ1-156, Δ1-175 and Δ1-196 mutants (including the basic, helix-loop-helix, and leucine zipper domains) were transcriptionally inert. Moreover, they all exerted dominant negative effects on Sp1 transactivation, suggesting that the interaction between Sp1 and USF1 also involves the DNA binding domains.

For the transactivations with assorted USF2a deletion constructs, USF2a and Sp1 levels on westerns were constant (Figures 9B and C). With the exception of Δ76-143 mutant (USF2b), none of the USF2 constructs by themselves significantly transactivated the dCK promoter (Figure 9A). The effects of full length USF2a with Sp1 on dCK promoter activity were again synergistic (Figure 9A, right panel) and this was only slightly affected by deletion of exons 1-4 (Δ1-143). However, with further deletions (Δ1-193 and Δ1-223), activity somewhat decreased and plateaued at ~70% of the level for full length USF2a.

Interestingly, internal deletion of exon 4 (Δ76-143; USF2b) not only completely eliminated the synergistic activation with Sp1 but also decreased promoter activity below that with Sp1, alone (although higher than for empty pPacO and the other USF2 deletion mutants). Internal deletion of exon 5 (Δ143-193) resulted in 30% decreased activity compared to the full length construct and closely
approximated the results with the Δ1-193 construct. Deletion of exons 1, 2 and 4 (Δ1-36 & 76-143) resulted in a similar activity to the Δ1-143 mutant. Deletion of exon 6 (i.e., USR; Δ193-223) did not affect activity, whereas deletion of the basic region (Δ223-242) completely abolished the synergistic transactivation with Sp1, indicating that DNA binding of USF2a is essential for its functional interaction with Sp1.

Thus, as with USF1, for USF2a, the DNA binding domains are sufficient to functionally interact with Sp1. However, in the case of USF2a, the effects of the DNA binding domains involve a synergistic transactivation rather the repression. The N-terminal exon 5 is clearly an activator, whereas exon 4 functions as a repressor in the presence of exon 5. Both exon 4 and the basic region are critical for the synergistic response with Sp1.
DISCUSSION

Phosphorylation of a variety of nucleoside analogs (e.g., ara-C and gemcitabine) that are important to cancer chemotherapy by dCK is a rate-limiting step in drug activation (1,2). Moreover, dCK levels in leukemia cells correlate with clinical responses to ara-C (28). Interestingly, our previous study of determinants of ara-C response in DS myeloblasts compared to non-DS myeloblasts, described a significant median 2.6-fold increased dCK expression in DS myeloblasts accompanying dramatically enhanced ara-C sensitivities (29).

dCK promoter activity was previously localized to a 697 bp upstream fragment, including 386 bp of 5’ sequence flanking 250 bp of exon 1, and 61 bp of intronic sequence (11,12). The dCK promoter is highly GC-rich and lacks a TATA-box. Binding of Sp1 to two GC-boxes and USF to a single E-box appeared to be critical for promoter activity in lymphoblast cultures (12). Based in part on the detection of high levels of dCK transcripts in HepG2 cells by RT-PCR (not shown), we used this cell model to further explore the transcriptional regulation of the dCK gene. Deletion and mutation analysis confirmed that the two GC-boxes (designated GCa and GCb) and the E-box element [E-box (a)] previously reported (12) comprised important regulatory regions, and that GCa and E-box (a) were activators and that GCb was repressive. An additional functional E-box [E-box (b)] was also identified, which appeared to play an important transactivating role.

An attractive biological feature of USF and Sp proteins involves their abilities to mediate a wide range of transcriptional activities via protein-protein interactions with other families of transcription factors including Ets-1 (30,31), MTF-1 (32), hepatic nuclear factor-4 (33), and the basal transcription factors TFIID (34,35) and TFII-I (36,37). In the dCK promoter, the GC-box elements and the E-box elements are juxtaposed. Thus, co-operative functional interactions between Sp1/Sp3 proteins bound to the GC-boxes, and USF1/USF2 bound to the E-box elements can easily be envisaged to regulate gene expression over a wide range.
To test this hypothesis, we performed gel shifts with the dCK \(-328/-289\) oligonucleotide including both the GCa and E-box (a) elements and nuclear extracts from HepG2 cells. In addition to the three major complexes previously reported and identified as containing Sp and USF proteins (12), two slower migrating DNA/protein complexes (1 and 2) were identified that by competition and supershift assays were found to contain both the Sp (Sp1 and Sp3) and USF (USF1 and USF2a) families of proteins, suggesting physical interactions between these two families of proteins. USF1/USF2 binding to the E-box (b) and Sp1/Sp3 binding to the GCb box were demonstrated on gel shifts with the dCK-291/-260 and dCK-223/-181 probes, respectively. *In vivo* binding of Sp1, USF1, and USF2 to the dCK promoter between positions \(-464\) and \(+75\) was confirmed by ChIP assays. Our transient cotransfections of HepG2 cells with a dCK promoter reporter gene construct and Sp1, USF1, and USF2a expression vectors suggested that functional interactions occurred between Sp1 and USF1/USF2.

To further investigate the physical and functional interactions between Sp proteins and USF1/USF2a, experiments were extended to *D. Mel-2* cells. Both Sp1 and USF1 could each transactivate the dCK promoter, however, Sp3 and USF2a were largely inert. Combinations of Sp1 and USF1 were additive at low levels of Sp1, but promoter activity decreased at higher Sp1 levels. In contrast, the effects of Sp1 and USF2a on dCK promoter activity were highly synergistic. The addition of USF1 (thus forming USF1/USF2a heterodimers) decreased the synergism between Sp1 and USF2a to a level essentially indistinguishable from that for USF1 and Sp1. Thus, USF and Sp proteins could potentially regulate dCK promoter activity over a wide range, reflecting relative levels and tissue distributions of Sp1 and Sp3, as well as of USF1 and USF2a.

Sp and USF expression constructs were co-transfected into *D. Mel-2* cells and nuclear extracts were prepared for gel shift and co-immunoprecipitation studies. Physical interactions between Sp1 and USF1 or USF2a were suggested by the decreased levels of Sp1 binding to GCa on gel shifts in the presence of competitive oligonucleotides and by supershifts with USF1 and USF2a antibodies. Direct
evidence for an association between Sp1 and USF1/USF2a was provided by co-immunoprecipitations of Sp1 with USF1 and USF2a. Taken together, these results strongly suggest that both Sp and USF proteins are essential for high level transactivation of the dCK promoter and that direct physical interactions between these proteins contribute to this response.

Cooperative interactions between Sp1/Sp3 and USF proteins were previously suggested to be required for the full activation of human transcobalamin II promoter (20). Likewise, our previous studies of the human cystathionine- -synthase (CBS) –1b promoter suggested that cooperative interactions between USF1 binding to the E-box and Sp1/Sp3 binding to the GC-c box element were involved in the transcriptional regulation of this important gene (21). Our recent studies on the human reduced folate carrier B promoter have also implicated Sp1-USF interactions as critical to high level transactivation (J.R. Whetstine, S.G. Payton, R.M. Flatley, M.J. Liu, Y. Ge, J.W. Taub, L.H. Matherly, manuscript submitted). Thus, cooperative interactions between Sp1/Sp3 and USF proteins can easily be envisaged to be a global mechanism for regulating tissue-specific gene expression, presumably by protein-protein interactions. Notably, our finding that Sp1 and USF proteins can be co-immunoprecipitated from D. Mel-2 cotransfectants provides the first direct evidence that a physical interaction between these proteins may contribute to a functional response.

The domains of USF1 and USF2a responsible for dCK transactivation and functional interactions with Sp1 were mapped by deletional mutagenesis and transient co-transfections with pdCK-464/+75 in Sp1- and USF-null D. Mel-2 cells. Our results with USF1 identified both repressor (amino acids 1 to 15) and activator (residues 39 to 100) domains, and suggested that amino acids 80 to 100 also were critical to functional interactions between USF1 and Sp1. Interestingly, the USF1 DNA binding domains (including the basic region, helix-loop-helix, and leucine zipper domains), alone, significantly repressed the activating effects of Sp1 on the dCK promoter, strongly implying a functional interaction between this region and Sp1.
For USF2a, two repressor (exons 1, 2, and 4) and two activator (exons 3 and 5) domains were identified, yet the DNA binding domains, by themselves, were sufficient to mediate a synergistic transactivation response in combination with Sp1. This difference from USF1 was surprising in light of the close homology between the USF2a and USF1 DNA binding domains (67%), yet further documents significant functional differences between these closely related transcription factors. Interestingly, USF2b, a naturally occurring USF2 splice form, resulting from internal deletion of exon 4 (Δ76-143), not only completely eliminated the synergistic activation with Sp1 but also decreased promoter activity below that with Sp1, alone. This suggests another regulatory component that may come into play for tissues in which USF2b is present at high levels, involving a significant repressive effect by USF2b on the extent of dCK transactivation by Sp1, and USF1 and USF2a. Indeed, USF2b has been suggested to modulate E-box-mediated transcriptional activity of the major histocompatibility complex class I gene in this fashion (38).

In summary, our results significantly extend earlier studies of transcriptional regulation of dCK in lymphoid cells. They document transcriptionally important roles for Sp and USF1/USF2a proteins, via binding to essential GC- and E-box elements, and provide the first direct evidence for a physical association between these proteins that results in promoter transactivation/repression. Co-operative interactions between Sp and USF proteins, including Sp1, Sp3, USF1, USF2a, and USF2b, can be envisaged to effectively regulate dCK expression over a wide range and contribute to tissue-specific patterns of expression of this critical gene. Better understanding of the major determinants of dCK gene expression may lead to opportunities for therapeutic interventions by modulating dCK activity at the transcriptional level in combination with cytotoxic antiviral and antitumor nucleosides. Given our interest in the basis for the disparate therapeutic responses of Down syndrome acute myeloid leukemia patients to chemotherapy, including ara-C (29), it seems reasonable that alterations at the level of dCK
transcription may, in part, provide a molecular explanation for this unique clinical finding (29), as well.

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## Table 1. Summary of Oligonucleotides for Gel Shifts and Mutagenesis.

| Oligonucleotide    | Sequence                                      |
|--------------------|-----------------------------------------------|
| dCK-328/-289       | CCACACGCGCGGCCGCCCCGCCCCGCTTCACGTGACCTGCCT   |
| dCK-223/-181       | GGCTTGAGGAGGGCCGCCGGCCGCCCCGCCCCGAGGCCAGTGTCC |
| dCK-291/-260       | CGTGCGGAGCGCGCACCGGGAAACCCGCGCT              |
| dCK Gca mt         | CCACACGCGCGGGGCGCGCGCCCCGCCAGGCCAGTGTCC      |
| dCK E-box (a) mt   | CCACACGCGCGGCCCCCGCCCCCGGCTTTGACCTGCCT      |
| dCK E-box (b) mt   | CGTGCGGAGCGCGTTTTGGAAACCCGCGCT              |
| dCK GCb mt         | GGCTTGAGGAGGTTTTGGCCGCCCCCGCCAGTGTCC        |

Sense DNA sequences for wild-type double-stranded oligonucleotides were used as probes and/or competitive oligonucleotides in gel shift assays. Sense and antisense single stranded mutant oligonucleotides were used in the preparation of mutant reporter gene constructs for transient transfection experiments. The consensus sequences of the GC-box and E-box elements are underlined. Lowercase letters indicate the bases mutated.
FIGURE LEGENDS

Figure 1. DNA sequence of the dCK promoter. The critical cis-regulatory elements [GCa, GCb, E-box (a) and E-box (b) and the E2F binding site] on the plus (+) and minus (-) DNA strands are underlined and shown in bold. The major transcription start site reported previously (12) is shown by an arrow. Numbering is relative to the dCK translation start site.

Figure 2. Functional analysis of the cis-regulatory elements in HepG2 cells. A series of 5’-deletion and site-directed mutational constructs were prepared, as described in the Materials and Methods section, and transiently transfected into HepG2 cells. Numbering is based on the dCK promoter sequence in Figure 1. Luciferase activities for the deletion and mutation constructs were expressed relative to the activity for the full-length pdCK-464/+75. The data are presented as the means ± S.E. for three experiments.

Figure 3. Gel shift assays with the dCK-328/-289, dCK-291/-260, and dCK-223/-181 probes. Gel shift assays were performed with HepG2 nuclear extracts and the 32P-labelled dCK-328/-289 (A), dCK-291/-260 (B), and dCK-223/-181 (C) probes in the absence and presence of molar excess of commercial consensus oligonucleotides or mutants of dCK-328/-289, dCK-291/-260, and dCK-223/-181 oligonucleotides. DNA sequences for the wild-type and mutant dCK-328/-289, dCK-291/-260, and dCK-223/-181 were shown in Table 1. NS indicates non-specific complex. Free indicates free probe. (D), in vivo binding of Sp1 and USF proteins to the dCK promoter was confirmed by ChIP assays, as described in the “Materials and Methods”.

Figure 4. Functional interactions between Sp1 and USF1/USF2a in HepG2 cells. The full-length dCK promoter construct pdCK-464/+75 (1µg) was co-transfected into HepG2 cells along with 10 ng of
pRLSV40 and expression constructs for Sp1 (100 ng), USF1 or USF2a (500 ng), or Sp1 (100 ng) with
USF1 or USF2a (500 ng). Luciferase activity is expressed relative to a mock transfection with the
empty vector. For all transfections, constant plasmid was maintained at 600 ng with empty pcDNA3
plasmid. Data are presented as the means ± S.E. from three independent experiments.

**Figure 5. Functional interactions between Sp1 and USF1 (Panel A) or USF2a (Panel B) in *D. Mel-2* cells.** *D. Mel-2* cells were co-transfected with 1 µg of the dCK full-length promoter construct (pdCK-464/+75) with pPacSp1 (10-50 ng), pPacUSF1 or pPacUSF2a (100-500 ng), or pPacSp1 with
pPacUSF1 or pPacUSF2a. For all transfections, constant plasmid was maintained (at 550 ng of the
pPac series) with pPacO vector. At 24 h posttransfection, *D. Mel-2* cells were harvested and lysed for
luciferase assays. Data from three experiments are presented as means ± S.E. fold induction in
luciferase activity relative to the mock transfection with 550 ng of pPacO.

**Figure 6. Gel shift assays of DNA binding of ectopically expressed Sp1, USF1, USF2a, Sp1 and
USF1, and Sp1 and USF2a in *D. Mel-2* cells.** *D. Mel-2* cells (3 × 10^7 cells/30 ml of media) were
transiently transfected with pPacSp1 (1.5 µg), pPacUSF1 or pPacUSF2a (30 µg each), or cotransfected
with both pPacSp1 and pPacUSF1 or pPacUSF2a. Nuclear extracts were prepared and incubated with
32P-labelled dCK-328/-289 probe in the absence or presence of Sp1 or USF1 consensus
oligonucleotides or commercial antibodies for Sp1, USF1, or USF2. Specific DNA/protein complexes
are noted. NS indicates non-specific complex. Free indicates free probe.

**Figure 7. Co-immunoprecipitation of Sp1 and USF1 or USF2a in *D. Mel-2* cells.** Nuclear extracts
(Sp1+USF1) and (Sp1+USF2a) used in Figure 6 were co-immunoprecipitated by anti-USF1 and anti-
USF2 antibodies, as described in the Materials and Methods. The immunoprecipitates were analyzed
by Western blotting. The co-immunoprecipitated Sp1 along with USF1 (A) or USF2a (B) is shown.

NR: non-related; WB: Western blot.

**Figure 8. Identification of USF1 functional domains that interact with Sp1 in D. Mel-2 cells.** (A)
A series of N-terminal deletion constructs of pPacUSF1 were generated as depicted in the left panel. The full-length dCK promoter construct (pdCK-464/+75) was co-transfected into D. Mel-2 cells along with 500 ng of pPacUSF1 or mutant pPacUSF1 constructs in the absence or presence of 25 ng of pPacSp1. At 24 h post transfection, cells were lysed for luciferase assays. Luciferase activities from three experiments for USF1 alone (open bars) and for both Sp1 and USF1 (closed bars) are shown as the means ± S.E. fold induction relative to that of the mock transfection with 525 ng of pPacO. (B and C) The cell lysates from (A) were analyzed by Western blotting to monitor the expression levels of USF1 and its mutants (B) and levels of Sp1 (C).

**Figure 9. Identification of USF2 functional domains that interact with Sp1 in D. Mel-2 cells.** (A)
A series of N-terminal and internal deletion constructs of pPacUSF2a were generated as depicted in the left panel. The full-length dCK promoter construct (pdCK-464/+75) was co-transfected into D. Mel-2 cells along with 500 ng of pPacUSF2a or mutant pPacUSF2a constructs in the absence or presence of 25 ng of pPacSp1. At 24 h post transfection, cells were lysed for luciferase assays. Luciferase activities from three experiments for USF2a alone (open bars) and for both Sp1 and USF2a (closed bars) are shown as the means ± S.E. fold induction relative to that of the mock transfection with 525 ng of pPacO. (B and C) The cell lysates from (A) were analyzed by Western blotting to monitor the expression levels of USF2a and its mutants (B) and levels of Sp1 (C).
Figure 1

-458 ctgcaggtga cgccctctgc cctccacgcgc cctcaggcct ctgggttccag ccccttcctcc
-398 ccacccgact ccgggaaccttc ttcccgccgc ctgccccgggc gcctggctgct ttgggggtaga
-338 ggcctttcgcg cacacgcgacg gcccccccc cc cgccttcacg tgcctgcgt gccccgggagcgc
GCa(−) E-box(a)(+) E-box(b)(+)
-278 cacgcgggaa cccgcgctgg aggccgggga gggccggaggg gcagctaggg gggcgggttt
-218 gagggagggcgg cccgcgttccccgcagcgcgcgc gccgttcctc agctgcctcc gcgcgcacaaa
GCb(+) E2F(+) E2F(+)
-158 gtcaaaaccgc gacaccggcc gcggggcgcc tggacctact agctgaccccg gcaggtcagg
-98 atctgggtta gcggcgccgc gcagctcagg tggcgcaccc gttggccgctt cccagccccctc
-38 ttgtcgggac gagctctgggg cggccacaag actaagggaAT Ggcacccccg ccccaagagaa
+23 gctgccccgtc tttctcagcc agctctgagg ggacccgcat caagaaaaatc tcc
Figure 2

Relative luciferase activity

- pdCK-464/+75
- pdCK-359/+75
- pdCK-298/+75
- pdCK-179/+75
- pdCK-27/+75
- GCa mt
- E-box (a) mt
- E-box (b) mt
- GCb mt
- GCa + E-box (a) mt
- GCa + E-box (b) mt
- Double GC mt
- Double E-box mt
- E-box (a) + GCb mt
- E-box (b) + GCb mt
- pGL3-Basic
Figure 3A

Probe: dCK-328/-289

| Ab      | Sp1-ab | Sp3-ab | USF1-ab | USF2-ab |
|---------|--------|--------|---------|---------|
|         | -      | -      | -       | -       |
|         | -      | -      | +       | -       |
| dCK-328/-289 | -      | +      | -       | -       |
| Sp1     | -      | -      | +       | -       |
| USF1    | -      | -      | -       | -       |
| GCa mt  | -      | -      | -       | +       |
| E-box (a) mt | -  | -  | -  | +  |
| HepG2   | -      | +      | +       | +       |

Free
Figure 3B

Probe: dCK-291/-260

| NE CompetitorsAb | USF1-ab | USF2-ab | dCK-291/-260 | USF1 | E-box (b) mt | HepG2 |
|------------------|---------|---------|--------------|------|-------------|-------|
|                  | -       | -       | -            | -    | -           | +     |
|                  | -       | -       | -            | -    | -           | +     |
|                  | -       | -       | -            | -    | -           | +     |

1 → NS → Free

1 2 3 4 5 6 7
Figure 3C

Probe: dCK-223/-181

| NE CompetitorsAb | Sp1-ab | Sp3-ab | dCK-223/-181 | Sp1    | GCb mt | HepG2    |
|------------------|--------|--------|--------------|--------|--------|----------|
| Free             | -      | -      | -            | -      | -      | -        |

1 2 3 4 5 6 7
Figure 3D

| IP antibodies | Input | No.48 | IgG | Sp1 | USF1 | USF2 |
|---------------|-------|-------|-----|-----|------|------|
| dCK           |       |       |     |     |      |      |
| RFC           |       |       |     |     |      |      |
Figure 4

|        | Vector | Sp1 | USF1 | USF2a |
|--------|--------|-----|------|-------|
|        | 1100   | -   | -    | -     |
|        | 1000   | -   | -    | -     |
|        | 600    | -   | 500  | -     |
|        | 600    | -   | 500  | -     |
|        | 500    | -   | 500  | -     |
|        | 500    | -   | 500  | -     |

Relative luciferase activity
Figure 5

A

B

| Protein | No addition | With 100 ng of USF1 | With 250 ng of USF1 | With 500 ng of USF1 |
|---------|-------------|---------------------|---------------------|---------------------|
| PPacO   |             |                     |                     |                     |
| USF1    |             |                     |                     |                     |
| Sp1-10  |             |                     |                     |                     |
| Sp1-25  |             |                     |                     |                     |
| Sp1-50  |             |                     |                     |                     |

Fold induction

"Downloaded from highwire press via IP by guest on March 24, 2020"
Figure 6

Probe: dCK-328/-289

|       | Ab       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|-------|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|       | Sp1      | USF1  | USF2  | Sp1   | USF1  |       |       |       |       |       |       |       |       |       |
|       | -        | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     |
|       | CP       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       | Sp1      | USF1  |       |       |       |       |       |       |       |       |       |       |       |       |
|       | -        | -     | +     | -     | +     |       | +     | +     | +     | +     | +     | +     | +     | +     |
|       | NE       |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       | Sp1+USF2a| USF1  |       |       |       |       |       |       |       |       |       |       |       |       |
|       | -        | -     | -     | -     | -     | +     | +     | +     | +     | +     | +     | +     | +     | +     |
|       | Sp1+USF1 | USF2a |       |       |       |       |       |       |       |       |       |       |       |       |
|       | -        | -     | -     | -     | +     | +     | +     | -     | -     | -     | -     | -     | -     | -     |
|       | USF1     |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       | -        | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     | +     |
|       | Sp1      |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       | -        | +     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     |
|       | PacO     |       |       |       |       |       |       |       |       |       |       |       |       |       |
|       | -        | +     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     | -     |

Probe: dCK-328/-289
Figure 7

A

Sp1 → [Blot Image] → IgG

WB: Sp1

USF1 → [Blot Image]

WB: USF1

B

Sp1 → [Blot Image] → IgG

WB: Sp1

USF2a → [Blot Image]

WB: USF2a
Figure 8A
Figure 8B&C

B

USF1

C

Sp1
Figure 9A

A
Figure 9B&C
Physical and functional interactions between USF and SP1 proteins regulate human deoxycytidine kinase promoter activity
Yubin Ge, Tanya L. Jensen, Larry H. Matherly and Jeffrey W. Taub

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