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Transcriptional regulation of the mouse deoxycytidine kinase: identification and functional analysis of nuclear protein binding sites at the proximal promoter

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Abstract

Deoxycytidine kinase (EC 2.7.1.74, dCK) catalyzes the phosphorylation of deoxynucleosides and several nucleoside analogues that are important in antiviral and cancer chemotherapy. The enzyme is predominantly expressed in lymphoid tissue by as yet poorly defined mechanisms. In this work, we have studied the mouse dCK regulatory region to understand the molecular details of the tissue specific expression of the enzyme. DNase I footprinting and electrophoretic mobility shift assays using nuclear extracts from mouse lymphocytes (EL-4, T cells; J558, B cells) and non-lymphoid cells (L929, fibroblasts) demonstrated the existence of at least six *cis*-acting elements (FP-1–FP-6) within the proximal promoter region. Functional analysis revealed that all the elements necessary to promote high level transcription of the mdCK gene are located downstream the transcription start site. 5′-Deletion and site-directed mutagenesis assays demonstrated the importance of four GC-rich regions, which bind Sp-1 and Sp-3 transcription factors. In addition, we identified a site (FP-3) located at the −282 to −310 nucleotide region of the promoter, which binds NF-1, only in B cells. Analysis of point mutations introduced at the different regions revealed functional differences in their role in mdCK transcription in the cell lines used.

Keywords: Deoxycytidine kinase; Promoter analysis; Sp-1/Sp-3; NF-1 and E2F

1. Introduction

Deoxycytidine kinase (dCK, EC 2.7.1.74) is a salvage pathway enzyme that catalyzes the phosphorylation of deoxycytidine into dCMP, using ATP or UTP as phosphate donors [1,2]. The enzyme has broad substrate specificity, including deoxyadenosine, deoxyguanosine and plays a role in the maintenance of normal deoxyribonucleotide pools in the cell. In addition, dCK has a pharmacological importance where it phosphorylates and thus activates several important anti-cancer and anti-viral drugs, such as 2′-2′-difluoro-deoxycytidine, 2-chloro-2′-deoxyadenosine, and 1-β-D-arabinofuranosyl-cytidine [3–5]. Deficiency of dCK activity is associated with a disturbance of the deoxynucleotide pools and resistance to therapeutic drugs [6–8]. The kinetic regulatory mechanisms of this enzyme have been established [9–11], and recently, the crystal structure has been determined [12]. A relatively constant amount of the dCK protein in proliferating and resting lymphocytes suggests that its expression is not strictly cell cycle regulated [10,13]. On the other hand, dCK was found to be expressed predominately in lymphoid tissues, which indicates a cell type-specific regulation of the gene [1,10]. In addition, up-regulation of dCK expression was observed in certain solid tumors [6]. However, the molecular mechanisms that lead to tissue specific and

Abbreviations: 5′-UTR, 5′-untranslated region; dCK, deoxycytidine kinase; EMSA, electromobility shift assay; nt, nucleotide; FP, footprint; TSS, translation start site; RT-PCR, reverse transcriptase-polymerase chain reaction

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proliferation dependent transcription of dCK are not well understood.

The molecular bases for the regulation of dCK activity and the expression of human dCK (hdCK) gene have been studied [14–16]. However, no ultimate conclusions were reached concerning the tissue specific expression and/or the up-regulation of hdCK in neoplastic cells. Sequence analysis indicated that the hdCK promoter does not possess a typical TATA-box or CAAT-box. Nevertheless, it is highly GC-rich (71%), similar to several TATA-less promoters [14]. Four major elements with the capacity to mediate transcriptional activation were mapped at the proximal hdCK regulatory region. The human dCK promoter contains two GC-rich motifs, which are canonical binding sites for the Sp-1 transcription factor family [15]. In vitro DNase I footprint assays demonstrated the existence of an imperfect E2F site, an element that could be responsible for transcription activation at the G1/S boundary [15]. The fourth promoter element is an E-box, which is a binding site for basic helix–loop–helix (bHLH) proteins e.g., USF family members. It was proposed that the functional interaction between Sp-1 and USF factors plays important role in the hdCK promoter [16].

Biochemical and molecular genetic studies have shown differences in the substrate specificity between hdCK and mdCK [10,11]. Differences in substrate specificity between enzymes from different species are of importance when using mouse models to evaluate effects and toxicity in the development of therapeutic nucleoside analogues. In addition, inter-species variations were detected in dCK expression in different tissues [10], pointing to a potential difference in the mechanisms involved in dCK transcription regulation. In this work, we cloned and characterized the mouse dCK 5′-flanking region and determined transcriptional factors that confer tissue specific expression of the gene. We describe important differences between the factors involved in human and mouse dCK promoter regulation, as well as the involvement of NF-1 in B lymphocyte-specific regulation of the mouse dCK promoter.

2. Materials and methods

2.1. Cell lines and materials

Mouse cell line L929 (ATCC-CCL-1) was obtained from American type culture collection (ATCC), mouse lymphoid cells (EL-4 and J558) were gifts from Dr. Lars Hellman (Uppsala University, Sweden). Cell culture media and fetal calf serum were obtained from Invitrogen. [α-^{32}P]-dCTP, [α-^{32}P]-dATP and [methyl-^{3}H]-dCyd were obtained from Amersham International (USA). Chemicals were of analytical grade and commercially available. Antibodies against c-Jun, c-Fos and Sp-3 were purchased from Santa Cruz Biotechnology; and anti-Sp-1 antibodies were purchased from Geneka Biotechnology Inc.

2.2. Isolation of a mouse dCK genomic clone and plasmid constructions

Approximately 1×10^6 plaques of the mouse OLA-129 liver genomic library were screened with a cDNA probe encompassing the entire coding region (780 bp) of mdCK as described in [17]. The genomic clones obtained were characterized by restriction enzyme mapping and Southern blot analysis. A fragment from one clone containing the upstream 4.23-kb region of the mdCK was subcloned into the pGL3 basic vector (Promega). The resulting plasmid contains the region from kb −4.23 to nt −1 (numbered according to the translation start site (TSS) as nt = 0) of the mouse dCK gene in front of the firefly Luciferace reporter cassette. Restriction enzyme and progressive unidirectional digestions with exonuclease III (Erase-a-Base system, Promega) were used to generate 3′- and 5′-deletion mutants,

Table 1
Oligonucleotides used in this study

Description	Purpose	Primer sequence
Exon-1 primer	Primer ext.	5′-GCCCTTCTCCCTCGACCAGCTCC
Primer −359 to −333	Primer ext.	5-CCCGCCCTCCCACTGCTCATCCCGCAG
FP-1 (E2F)	EMSA/Mut	5′-CTCCGCGCGCCAAAGTCAAAGTGCG
FP-2 (Sp-1/Sp-3)	EMSA/Mut	5′-TCTCCTCACAAGCCCCGCCCCCTACCC
FP-3 (NF-1)	EMSA/Mut	5′-GCTCGTCGCGCACGGTCAGTCGCCCGGCT
FP-4 (Sp-1/Sp-3)	EMSA/Mut	5′-ATCCCGCAGGCCCCGCCCCCTC
FP-5 (Sp-1/Sp-3)	EMSA/Mut	5′-GCGCAAGGCCCGCCCCCCGCCCT
FP-6 (Sp-1/Sp-3)	EMSA/Mut	5′-CTTCGCTCAAGCTCCGCCCCTGCC
FP-7 (NFAT)	EMSA/Mut	5′-TACTTTTCTTCCCGGAAAAGTTCAAACCACGA
Sp-1 consensus	EMSA	5′-GCTCGCCCCGCCCCGATCGAAT
NF-1 consensus	EMSA	5′-TCAACCGACGCACGGTTTGTCGTGTCGAC
AP-1 consensus	EMSA	5′-CGCTTGATGACTCAGCCGGAA
USF-1 consensus	EMSA	5′-CGGTCACGTGGCCTACACCTG
dCK 5′-primer	RT-PCR	5′-GGACTCTGAAAACCAGCTTTGATT
dCK 3′-primer	RT-PCR	5′-CCAGGCTTTCGTGTTTGTCTTTA

The oligonucleotides listed represent the sense strand. Underlined sequences indicate the mutations introduced to the oligonucleotides for the mutagenesis (Mut) study.

respectively. Site-directed mutagenesis of the foot-printed regions was performed as described previously [17–19] using mutagenic primers listed in (Table 1).

2.3. Primer extension assays

Oligonucleotides (10 pmole) corresponding to nt 26 to 48 of exon 1 and nt −359 to −333 of the mdCK 5′-untranslated region (5′-UTR) region, was end-labeled using T4 polynucleotide kinase (Clontech) and 250 μCi [γ-^{32}P] ATP (Amersham) following the protocol described in [18]. The labeled oligonucleotides were hybridized with 5 μg poly(A) RNA prepared from mouse spleen and thymus as described in [20] with some modifications. Hybridization buffer contained 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 400 mM NaCl and 80% formamide. Annealed fragments were precipitated and resuspended in a buffer containing 50 mM Tris, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM deoxynucleotide triphosphate mix, 10 U RNasin and 10 U SuperScript reverse transcriptase (Invitrogen). The reaction was allowed to proceed for 60 min at 37 °C, followed by 30 min digestion with 5 μg pancreatic DNase free RNase. The complementary cDNA was extracted with equal volume phenol–chloroform, precipitated with ethanol at −80 °C, and resuspended in Tris–EDTA-formamide loading buffer. 10^5 cpm from the radiolabeled cDNA was loaded on a 6% sequencing gel [18].

2.4. In vitro DNase I footprinting and electrophoretic mobility shift assays

Nuclear extracts from mouse fibroblasts, spleenocytes, thymocytes and EL-4, J559 and L929 cell lines were prepared as described previously [18,19]. DNase I footprint analysis was performed as described [20], using 5′-end labeled probes encompassing nt −421 to −194 and −194 to −1. Electrophoretic Mobility shift assays with end-labeled double stranded oligonucleotide probes were performed as described previously [20,21]. The oligonucleotide sequences are shown in Table 1. For supershift (ss) assays, antibodies against c-Jun, c-Fos and Sp-3 were used and obtained from Santa Cruz Biotechnology (USA); anti-Sp-1 antibodies were from Geneka Biotechnology Inc. (Canada).

2.5. Cell culture and transfection methods

The mouse cell lines (J558 and L929) were grown in Dulbecco's modified Eagle's medium (DMEM) and the EL-4 cells were grown RPMI 1640 medium (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. Both media were supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. L929 cells were transfected by the calcium phosphate-DNA co-precipitation method [18]. The J558 and EL-4 cells were transfected by electroporation [22]. The electroporation conditions were 280 and 300 mV at 975 and 950 μF for J558 and EL-4, respectively. The pCMV-β-galactosidase (β-gal) plasmid was co-transfected with the luciferace reporter constructs in all experiments to monitor transfection efficiency. β-Gal assays were performed as described previously [18]. Luciferace activity was measured by the Enhanced Luciferace Kit, in accordance with the protocol described by the manufacturer (BD Biosciences).

2.6. Total protein extracts and the dCK activity assay

Cells grown in the appropriate culture media were harvested and washed once with PBS. Proteins were extracted as described previously [23]. The extraction buffer contained 50 mM Tris–HCl, pH 7.6, 2 mM DTT, 20% glycerol, 0.5% NP-40, and protease inhibitors mix (Qiagen Inc.). For the dCK activity assay [24], total protein extracts (10 μg) were added to a reaction mixture containing 50 mM Tris–HCl, pH 7.6, 2 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 5 mM ATP, 0.5 mg/ml BSA, 10 μM deoxycytidine (dCyd), 1 μM [methyl-^{3}H]-dCyd, and 1.8 mM thymidine. The latter was added to block the interference of the mitochondrial thymidine kinase (TK2) activity [23]. The reaction mixture was incubated at 37 °C, and 10 μl were drawn from the reaction mixture and applied to DEAE-filter membranes (Millipore) at time intervals of 0, 20, 40, and 60 min. Dried filters were washed with 5 mM ammonium formate and the radiolabeled monophosphate nucleosides were detected by scintillation counting.

2.7. RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen Inc.) following the protocol described by the manufacturer. First-strand cDNA was then synthesized from RNA by reverse transcription using the Superscript Reverse Transcription kit (Clontech). The PCR reaction was performed as described [25]. The primers used for amplification of mdCK are shown in Table 1. Glycerol-3-P dehydrogenase (G3PDH) was used as an internal control and primers used for the amplification of G3PDH were obtained from Promega. The reaction conditions used were 2 min at 95 °C, and then 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, for 20 cycles. PCR amplified products were separated on 8% non-denaturing polyacrylamide gel.

3. Results

3.1. The in vivo levels of mdCK mRNA and enzyme activity in the cell models used

The mdCK activity and mRNA levels were determined in the cell lines utilized for the promoter studies. The lymphoid cell lines, EL-4 and J558, contained two-fold

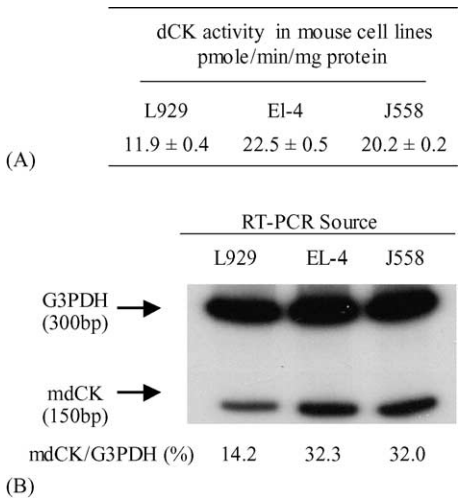

Fig. 1. The mdCK activity and mRNA levels in mouse cell lines. (A) dCK enzymatic activity was determined in total protein extracts from mouse cell lines as described in Section 2. The activity is expressed as pmole/min/mg protein. (B) Semi-quantitative RT-PCR assays were used to determine the levels of dCK mRNA in the mouse cell lines as described in Section 2, using the primers outlined in Table 1, amplified G3PDH mRNA was used as internal control.

higher dCK enzyme activity levels than the fibroblast cell line (Fig. 1A). To rule out possible post-translational regulation, the mRNA levels of dCK were determined by semi-quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assays. As shown in Fig. 1B, the levels of dCK mRNA were about two-fold higher in El-4 and in J558 cells compared to that in L929 cells. This result is in line with the earlier studies demonstrating a close correlation between the activity and the mRNA expression of dCK [26,27]. The cell models used were derived from cancer cells and have relatively high dCK levels. Therefore, we could not perform dCK promoter analysis using cell lines with low endogenous dCK expression.

3.2. Cloning and mapping the upstream regulatory region of the mouse dCK gene

A mouse liver genomic OLA 129-λGEM library was screened with a 780 bp fragment containing the coding sequence of the mdCK cDNA. Restriction mapping and Southern blot analysis of the positive clones identified one clone, which contained the first exon and an approximately 4.23 kb upstream region of the mdCK gene. We determined the nucleotide sequences of this fragment and compared the results with the mouse chromosome 4 working sequence (Gen-Bank accession no. AF260315). All the sequences determined were identical to the deposited sequence.

Primer extension analysis was preformed to identify the transcription initiation site. Using poly(A) RNA extracts from mouse spleen and thymus, and an end-labeled primer generated form exon 1, one major band of 430 bp was detected. The exact position of the transcription initiation site was verified by performing the same assay with a second primer that is located at the 5′-untranslated region (nt −359 to −333 with respect to translation start site, nt = 0). The same intense band corresponding to the translation start site was mapped in both samples at nucleotide position 421 upstream of the ATG codon (Fig. 2A).

3.3. Functional analysis of the 5′-flanking region

To identify the minimal regulatory region of dCK, we fused the 4.2 kb upstream sequence to a luciferace reporter vector. 5′- and 3′-deletion derivatives of this clone were constructed and used in transient transfection assays in mouse El-4, T-cell lymphoblastoma, J558, B-cell lymphoblastoma and the non-lymphoid L929 mouse fibroblast cell line. As shown in Fig. 2B, the results of this assay indicated a complex pattern of transcription regulation by the dCK 5′-flanking region. The promoter activity of the full-length construct (containing 4.2 kb upstream TSS) was two-fold higher in EL-4 and J558 cells compared to the non-lymphoid L929 cell line. In all cells tested, luciferace activity was almost completely abolished when 200 bp were deleted from 5′-flanking region of the full-length clone, pointing to the presence of an enhancer element within this region (Fig. 2B). Interestingly the reporter activity reappeared selectively in the lymphatic cell lines (EL-4 and J558) but not in L929 upon further 5′-deletions to −3200 bp upstream TSS. Progressive deletions from the 5′-end of the distal promoter led to increases in the transcriptional activity of the reporter constructs in the L929 cell line, and to a varying extent in the EL-4 and J558 cell lines as the deleted region approached the transcription start site. This suggests the existence of cell type-specific transcription activator and repressor elements in the tested promoter regions.

In all cell lines tested, maximal luciferace activity was detected in constructs extending downstream of the transcription start site. Further deletions abolished the promoter activity. Interestingly, the 3′-deletion construct containing a 3.8 kb upstream transcription region showed a severe drop in the luciferace activity in all cell lines, suggesting that the 5′-untranslated region contains regulatory elements sufficient to drive the transcription of the dCK gene.

Taken together, these results suggest that the region located downstream of the transcription start site (nt −421 to −1) of the mdCK gene contains all the necessary elements for the transcriptional activation of the gene and the sequences located upstream of the TSS may serve as modulators of the proximal promoter.

3.4. Transcription factors binding to the proximal promoter region

To identify the transcription factor binding sites within the proximal dCK regulatory region DNase I footprinting

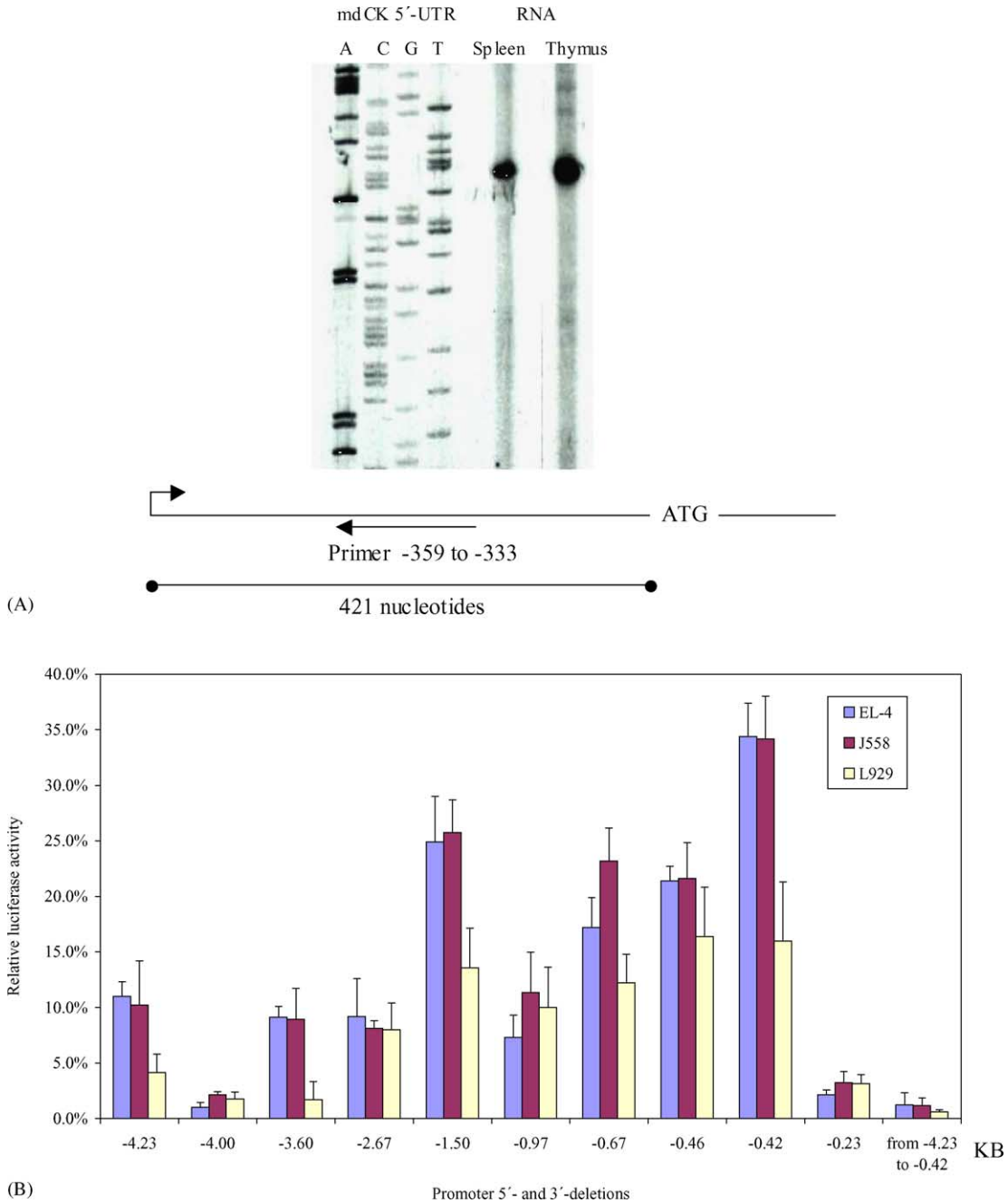

Fig. 2. Cell specific regulation of the mdCK promoter in different mouse cell lines. (A) Identification of the transcription start site of mdCK RNA by primer extension analysis. The end-labeled primer generated from the 5′-UTR (nt −359 to −333) was annealed to poly(A) RNA prepared from mouse spleen and thymus, and extended by reverse transcriptase and analyzed in a sequencing gel. (B) Transient transfection assays of the promoter constructs into lymphoid (El-4 and J558) and non-lymphoid (L929) cell lines. Promoter deletion constructs were generated as described in Section 2. Values indicate normalized luciferace activities expressed as a percentage compared to these found with the SV-40 promoter. The results represent the mean ± S.D. of at least three independent experiments, each performed in duplicate.

analysis was performed with nuclear extracts prepared from the mouse fibroblasts as well as from mouse spleen and thymus lymphocytes.

The first DNase I protected region was observed with a probe spanning nt −194 to −1 upstream the TSS. This protected region designated footprint 1 (FP-1) extends from nt −114 to −94. Sequence analysis indicated that it corresponds to a putative E2F like motif (Fig. 3A). A weak protection was detected in nuclear extracts from mouse spleen and thymus but not in extracts prepared from fibroblasts.

Four major DNase I-protected regions (FP-2, FP-4, FP-5, and FP-6) were detected in all nuclear extracts tested using a probe extending from nt −421 to −194 upstream of the TSS (Fig. 3B). In addition, an extra-protected site (FP-3) was detected only with nuclear extracts from spleen

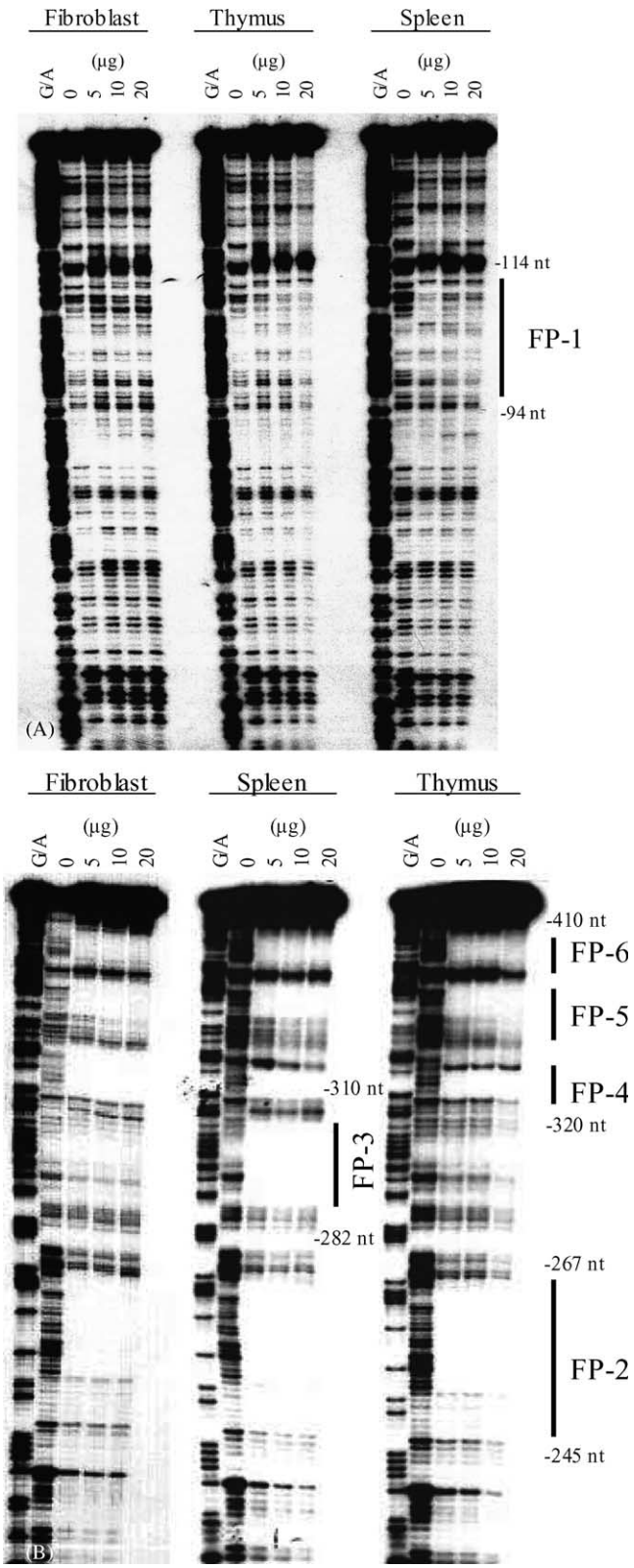

Fig. 3. Identification of DNase I protection regions within the proximal mdCK gene promoter. The footprint analyses were performed with the 5′-end labeled probes described in Table 1. DNA probes were end-labeled and incubated with the indicated amount of nuclear extracts from mouse spleen, thymus and fibroblast cells before DNase I digestion. Cleavage products were separated on 6% denaturing acrylamide gel along with a Maxam and Gilbert (G/A) ladder. (A) The mdCK regulatory region probe extended from nt −194 to −1. (B) The probe extended from nt −421 to −194.

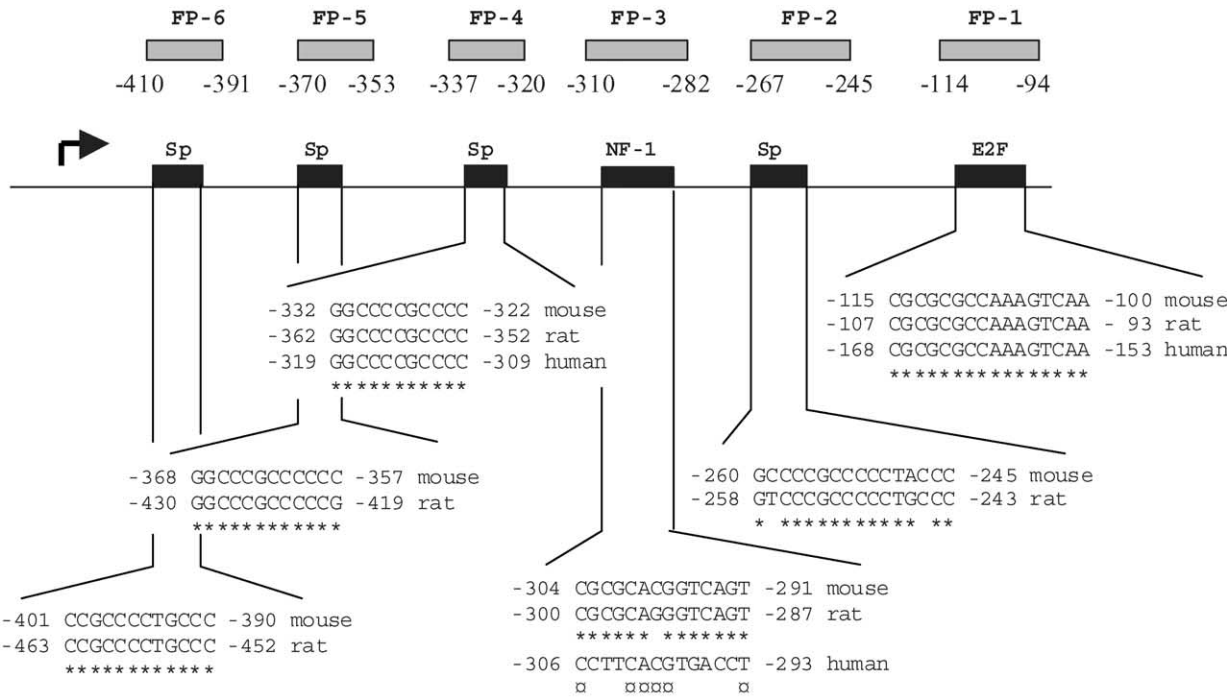

Fig. 4. Sequence alignment of the dCK regulatory region from mouse, rat and human. Similarities were detected in the core sequences of the potential regulatory elements identified in mouse dCK promoter using the Clustal W (1.82) multiple sequence alignment program. In the top panel, the FPs were numbered with respect to nucleotide position of the TSS = 0. To facilitate sequence comparison between the different species, position +1 in the lower panel corresponds instead to the ATG translation initiation codon.

cells. Footprint 2 extends from nt −267 to −245, footprint 4 from nt −337 to −320, footprint 5 from nt −370 to −353, and footprint 6 from nt −410 to −391. Footprint 3, which was detected with nuclear extracts from spleenocytes, extends from nt −310 to −282. Careful analysis of the nucleotide sequences of the protected regions revealed that footprints 2, 4, 5, and 6 are GC-rich motifs, which contain canonical binding sites for the Sp-1 transcription factor family. The sequence selectively protected by spleen nuclear extracts showed a high degree of similarity to the consensus-binding site for the leucine zipper factor family AP-1 (ACTG) adjacent to an NF-1 binding site (Fig. 3B).

Sequence alignment of the mouse, rat and human proximal-promoter region (Gen-Bank accession no. AF-260315, NW_04742 and NP_000779, respectively) by the Clustal W (1.82) multiple sequence alignment program revealed an over all identity of 75.4% between the mouse and rat, and 50.7% between the mouse and human regulatory regions. Interestingly, the nucleotide sequences of the core binding motifs of FP-1 and FP-4 were identical in the species, although, they had different locations within the promoter regions (Fig. 4). Similarly FP-2, FP-5, and FP-6 were found in both the mouse and rat promoters but were absent in the human proximal promoter. The sequences within the footprinted regions FP-3 were 87.5% identical in both mouse and rat promoters, whereas the human promoter showed only 31% homology to the mouse FP-3 region (Fig. 4).

To confirm the identity of the transcription factors that binds to the above footprinted regions, we performed antibody-mediated supershift assays. Antibodies against Sp-1 and Sp-3 were able to partially supershift or eliminate the complexes formed on FP-2- (Fig. 5A), FP-4-, FP-5-, and FP-6-derived double stranded oligonucleotide (data not shown).

Antibodies against the AP-1 proteins, c-Jun and c-Fos, failed to supershift or eliminate the complex formed on the FP-3 probe (data not shown). We also performed competition assays using double stranded oligonucleotides containing consensus sites for some transcription factors of the b-HLH family members (CREB-1, Delta-1, XBP-1, USF-1, and E-element), as well as AP-2 and AP-4. This study indicated that none of the oligonucleotides for the above mentioned transcription factors were able to disrupt the complex formed with FP-3 (Fig. 5B). However, when double stranded oligonucleotides designated for nuclear factor 1 (NF-1) were used, a significant reduction of the intensity of the radiolabeled band corresponding to the protein-DNA complex formed on FP-3 was observed, indicating that the sequence motif of FP-3 is recognized by members of the NF-1 family transcription factors (Fig. 5B).

3.5. Functional analysis of the proximal promoter region

To ascertain the potential functional role of the protein binding sites identified by the DNase I protection assays,

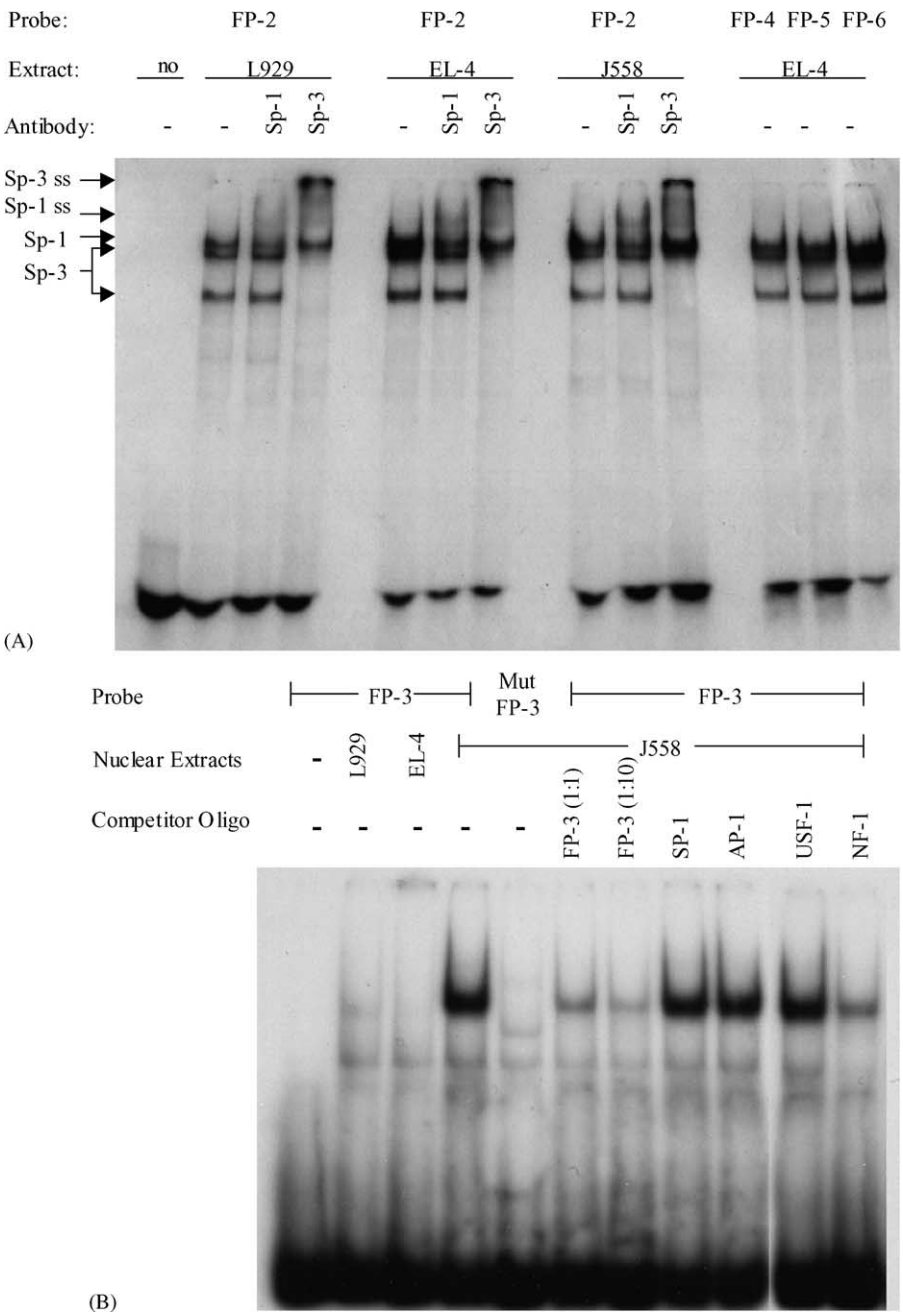

Fig. 5. Electromobility shift assays of Sp-1/Sp-3 and NF-1 binding in the presence of antibodies or competitor oligonucleotides. (A) Double stranded probes with the sequences of the respective FPs (Table 1) were incubated with nuclear extracts from different cell lines in the absence or presence of anti-Sp-1 and Sp-3 antibodies as described in Section 2. The arrows indicate the DNA/protein interactions and supershift in the presence of the antibodies. (B) FP-3 double stranded probes were incubated with the nuclear extracts in the absence or presence of non-radioactive double stranded consensus oligonucleotides for Sp-1 (non-specific), AP-1, USF-1, and NF-1, respectively.

we introduced mutations into the individual footprinted regions that abolish the binding of the corresponding transcription factors. Transient transfections assays with EL-4, J558 and L929 cells revealed that mutations in the E2F binding site (FP-1) reduced the promoter activity to 60% of the wild type value in both lymphoid cell lines, but not in L929 cells (Fig. 6). Base substitutions within the Sp-1/Sp-3 binding motifs (FP-2 and FP-4–FP-6) showed varying levels of luciferace expression in the different cell types. Mutations in the most proximal and the distal GC-rich motifs (FP-2 and FP-6) reduced promoter activity three- to four-fold in all cell lines. Mutations in the Sp-1/Sp-3 consensus site (FP-4) severely reduced the promoter strength in T cells, but not in B cells, whereas the promoter activity was increased two-fold in L929 cells. Mutations in the GC-rich region (FP-5) resulted in four-fold reduction in luciferace activity in L929 but not the EL-4 and J558 cells (Fig. 6). Mutations within the NF-1 binding site (FP-3) reduced the promoter activity to 50%, selectively in B-lymphoblasts, without

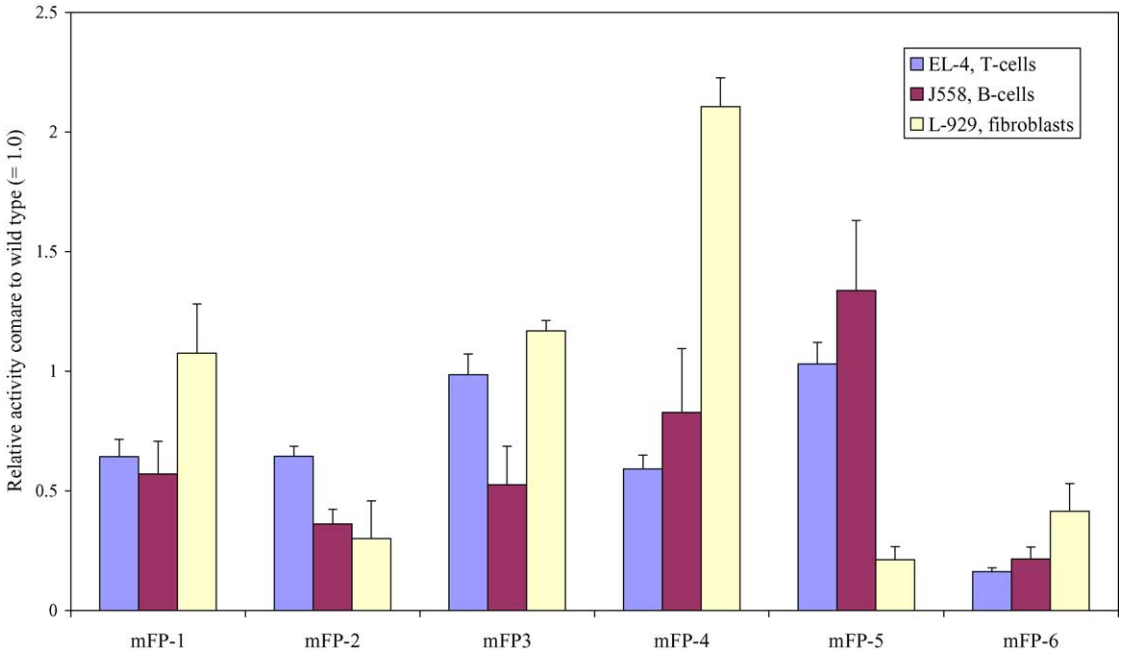

Fig. 6. The effect of mutagenesis of transcription factor binding sites on the mdCK promoter activity. Transcription factor binding sites were subjected to site-directed mutagenesis using the oligonucleotides outlined in Table 1. The values are normalized luciferace activities expressed as a percentage compared to the wild type promoter fragment nt −1500 to −1. The results represent the mean ± S.D. of at least three independent experiments, each performed in duplicate.

any change in the promoter strength in both T cells and L929 cells.

These results suggest that Sp-1 and Sp-3 transcription factors may differentially regulate the proximal mdCK promoter via binding to different GC boxes. Binding of NF-1 contributes to promoter activation in B-lymphoid cells, but not in T cells or fibroblasts.

4. Discussion

In spite of the extensive studies concerning the biochemical properties and the genomic organization of dCK, relatively little information is known about its regulation at the transcriptional level. In this report, we describe the first detailed functional analysis of the mouse dCK promoter. Comparison of the mouse and human dCK regulatory sequence revealed similarities in that both lack TATA and CAAT-boxes, contain several GC-rich motifs and an element resembling an E2F-binding site. Previous studies have indicated that the transcription of hdCK is initiated at nucleotide 146 upstream of the ATG codon [14]. In this study, the mdCK transcription initiation site was mapped to nucleotide 421 upstream of the translation start site. This is in accordance with northern blot analysis, which showed that the mdCK mRNA is about 3.4 kb, whereas, the open reading frame and 3′-UTR is 2.8 kb [10]. Taking into consideration an about 200 bp poly(A) tail, transcription initiation site should occur approximately 400 bp upstream of the ATG codon, in good agreement with the experimental results.

The results presented in this study show that sequences located down stream of the transcription start site are essential for mdCK promoter activity. Transient transfection assays using promoter construct lacking this region resulted in a complete loss of promoter activity. Furthermore, DNase I protection and electromobility shift assays demonstrated the existence of several important motifs in this region. Other regulatory regions further upstream of the transcription start site may have modulatory roles in the regulation of the tissue specific expression of the mdCK gene. This assumption is supported by promoter activity variations observed in transient transfection assays of the 3.8 kb promoter fragment and its 5′-deletion derivatives. The results indicate the existence of positive enhancer elements in the region between −4.23 and −4.0 kb and negative elements between −3.6 and −1.5 kb regions.

The mdCK core promoter, which is situated at the 5′-UTR of the gene, consists of six DNase I protected regulatory elements. The first protected region (FP-1) has an imperfect E2F binding site. Mutations within this site decreased the promoter activity to 60%, particularly in EL-4 and J558 cells. The E2F DNA-binding site has been identified in promoters of many S phase-regulated genes [28,29]. Recent microarray studies revealed that E2F binds to the promoters of a number of other genes, such as those regulating DNA repair proteins, including dCK [30,31]. However, the role of E2F in the transcriptional regulation of constitutively expressed genes, which are not regulated during the cell cycle, is not yet clear.

The other DNase I protected regions of the mdCK core promoter contained four GC boxes, which bind Sp-1 and

Sp-3 transcription factors and an NF-1 binding site. Of the four GC boxes only one (FP-4) is conserved with the human dCK promoter. Sp-1 and Sp-3 factors are known to activate transcription [32], but in case of promoters containing multiple adjacent binding sites, Sp-3 can repress transcription activation mediated by Sp-1 or other transcription factors [33], as seen for the mdCK promoter. Interestingly, mutations within the FP-4 increased the promoter activity only in non-lymphoid cells suggesting that Sp-3 proteins preferably bind to this site in these cells. Mutations within two of the other Sp-binding sites (FP-2 and FP-6) decreased promoter activity in all cell lines tested, indicating that Sp-1 is the key element binding to these sites. Finally a mutation of the GC box situated at FP-5 decreased promoter activity only in L929 cells, indicating that this site is mainly recognized by Sp-1 in fibroblasts, whereas interplay between Sp-1 and Sp-3 factors is taking place in lymphoid cells at this site. In general and because these elements can equally associate with the positive regulator Sp-1 and the negative regulator Sp-3 [32], it is tempting to speculate that the observed effects are exerted by the competition of these two factors for binding to specific regions in a cell type-specific manner.

Of particular interest is the functional role of the FP-3 region. In the human promoter this region contains an E-box, which is a binding site for the USF transcription factor family [34]. It has been demonstrated that USF synergies with Sp-1 proteins via physical interactions to drive high levels of expression of the human dCK promoter [16]. Sequence alignment of the hdCK and the mdCK promoters indicated that the observed E-box consensus site; CACGTG found in the hdCK gene is modified to CACGGT, in the mouse dCK gene, which represents part of a core element for the binding of NF-1 family members. We found no evidence for the involvement of USF proteins in the regulation of the mouse dCK promoter. On the other hand, competition DNA-binding assays revealed that FP-3 is a bona fide NF-1 binding site. Of particular importance is the finding that FP-3 was protected only in B-lymphoid cells and its mutation caused decreased promoter activity only in B cells.

These findings establish a model for the regulation of the mouse dCK promoter, substantially different from that suggested for the regulation of human promoter. The human gene is regulated by the synergistic action of USF with Sp-1 proteins in all cell types, while the mouse dCK gene is differentially regulated in non-lymphoid and B- or T-lymphocytes (Fig. 7). In non-lymphoid cells the promoter is mainly controlled by the interplay of Sp-1 and Sp-3 transcription factors binding to FP-2, FP-4, FP-5, and FP-6. In T cells, transcription is regulated by E2F and Sp-1 binding to FP-1 and FP-2 or FP-6, respectively, while in B cells regulation is mainly achieved by NF-1, which binds to FP-3, together with E2F and Sp-1, which bind to FP-1 and FP-2 or FP-6, respectively.

The differential role of the recruitment of Sp-1 onto different regions and the cell type-specific involvement of E2F and NF-1 transcription factors on mdCK regulation, point to that different pre-initiation complexes are formed on the mdCK promoter in different cell types. Further analysis of the upstream regulatory elements needed to moderate the overall transcription of the mdCK is under investigation.

A better understanding of the molecular details of the tissue specific regulation of dCK may lead to novel targets for therapeutic interventions that can modulate dCK expression at the transcriptional level. Furthermore, understanding interspecies differences in dCK expression are of

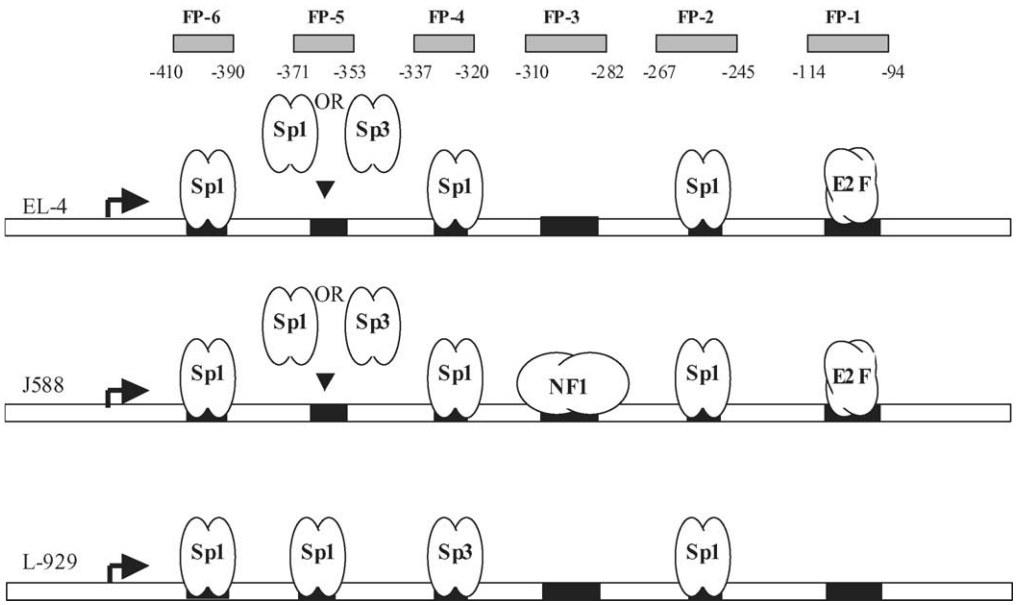

Fig. 7. An overview of the transcription factors involved in the regulation of the mouse deoxycytidine kinase in different mouse cell lines, El-4, T-lymphoblasts; J558, B-lymphoblasts and L929, fibroblasts.

importance when using mouse models to evaluate the potential effect and toxicity of therapeutic nucleoside analogues that are aimed for tissue and cell type-specific targets.

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