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Characterization of Sp1, AP-1, CBF and KRC binding sites and minisatellite DNA as functional elements of the metastasis-associated *mts1/S100A4* gene intronic enhancer

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ABSTRACT

The *mts1/S100A4* gene encodes a small acidic calcium-binding protein that is expressed in a cell-specific manner in development, tumorigenesis and certain tissues of adult mice. A composite enhancer that is active in murine mammary adenocarcinoma cells was previously identified in the first intron of the *mts1/S100A4* gene. Here we present a detailed analysis of the structure and function of this enhancer in the Mts1/S100A4-expressing CSML100 and non-expressing CSML0 mouse adenocarcinoma cell lines. In CSML100 cells the enhancer activity is composed of at least six *cis*-elements interacting with Sp1 and AP-1 family members and CBF/AML/PEBP2 and KRC transcription factors. In addition, a minisatellite-like DNA sequence significantly contributes to the enhancer activity via interaction with abundant proteins, which likely have been described previously under the name minisatellite-binding proteins. Extensive mutational analysis of the *mts1/S100A4* enhancer revealed a cooperative function of KRC and the factors binding minisatellite DNA. This is the first example of an enhancer where two nuclear factors earlier implicated in different recombination processes cooperate to activate transcription. In Mts1/S100A4-negative CSML0 cells the strength of the enhancer was 7- to 12.5-fold lower compared to that in CSML100 cells, when referred to the activities of three viral promoters. In CSML0 cells the enhancer could be activated by exogenous AP-1 and CBF transcription factors.

INTRODUCTION

The S100A4 protein belongs to the S100 family of small acidic calcium-binding EF-hand polypeptides. The corresponding gene was cloned under the name *mts1*, as a gene overexpressed in a metastatic mouse carcinoma cell line, CSML100, but down-regulated in its non-metastatic counterpart, CSML0 (1). Using two transgenic models, it was later demonstrated that overexpression of S100A4 stimulates *in vivo* invasiveness and promotes tumor progression (2,3). S100A4, which is mainly detected in the cytoplasm, physically interacts with such molecular targets as the heavy chain of non-muscle myosin (4,5), actin (6) and tropomyosin (7), facilitating cell motility and invasiveness (8–10). The molecular mechanisms underlying the phenotypic effects of S100A4 protein are not clear. However, since S100A4 protein was shown to affect phosphorylation of myosin by protein kinase C (PKC) and casein kinase 2 (CK2), it is thought to participate in PKC- and CK2-mediated signaling pathways by defining the substrate specificity of these enzymes (11).

Thirteen S100 genes have been identified to date in a gene cluster on human chromosome 1q21, called the epidermal differentiation complex (12–14). Although these genes are structurally and evolutionarily related, the intracellular localization and expression pattern of each individual S100 protein is unique in both normal and tumor cells.

Transcriptional regulation of the *S100A4* gene has been studied in mouse and rat mammary adenocarcinoma cell lines (15–18), in NIH 3T3 fibroblasts and renal proximal tubular epithelial cells (MCT) (19) and human lymphoma cell lines (20). In the S100A4-expressing mouse adenocarcinoma cell line CSML100 no sequences significantly contributing to up-regulation of the *S100A4* gene were identified in the 5′-flanking region. However, Okada *et al.* have found a novel *cis*-acting element located at positions –177 to –173, which was active in NIH 3T3 fibroblasts but inert in the S100A4-negative

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epithelial cell line MCT due to lack of the corresponding transcription factor (19).

We have previously reported the presence of a transcriptional enhancer located in a DNase I hypersensitive area in the first intron of the *S100A4* gene (16). The enhancer was active in both S100A4-positive and S100A4-negative mouse adenocarcinoma cells. The enhancer activity was partially formed by a κB-like element, interacting with the nuclear factor κB (NF-κB)/Rel-specific p50–p50 homodimer and p50–p65 heterodimer, as well as with κ recognition component (KRC). Mutagenesis analysis of the κB-like site revealed that KRC, rather than NF-κB/Rel, contributed to enhancer activity in mouse adenocarcinoma cells (16,21). The intronic enhancer was also found to be functional in the non-transformed mouse cell lines NIH 3T3 and MCT (19).

Here we describe in detail the structural and functional organization of the *S100A4* intronic enhancer in the S100A4-expressing highly metastatic adenocarcinoma cell line CSML100 and in its S100A4-negative non-metastatic counterpart, CSML0. In addition to the previously defined κB site, there are six other *cis*-elements in the enhancer interacting with various transcription factors belonging to the Sp1, activating protein-1 (AP-1) and core-binding factor (CBF) families and presumably with minisatellite binding proteins (Msbp). Normalization of the *S100A4* enhancer activity to the activities of three viral enhancers revealed that it drives transcription 7- to 12.5-fold more efficiently in CSML100 than in CSML0 cells. Expression of exogenous CBFA1 and Fra-1 transcription factors, which are deficient in CSML0 cells, partially surpassed the relative invalidity of the *S100A4* enhancer in these cells.

MATERIALS AND METHODS

Cell culture

CSML0 and CSML100 cell lines were grown at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco BRL) containing 10% fetal calf serum (Biological Industries, Israel), 200 U/ml penicillin and 25 μg/ml streptomycin sulfate.

Transient transfection and luciferase assay

CSML0 and CSML100 cells were transfected with 4 μg reporter plasmid and 0.5 μg pCMVβ by electroporation (260 V, 250 μF), using Bio-Rad equipment. In experiments where varying amounts of expression plasmids were used, empty vector was added to maintain an equal amount of expression plasmid in all transfections. Forty-eight hours post-transfection cells were harvested and lysed in Reporter Lysis Buffer (Promega). Luciferase activity was assayed using the Luciferase Assay Reagent (Promega) in a Lumat LB9501 luminometer (Berthold). β-Galactosidase activity was assayed as described (22) and used to correct for variations in transfection efficiency.

Plasmids

The pfLUC reporter construct contains the *Photinius pyralis* luciferase gene controlled by the minimal (–56 to +109) c-*fos* promoter (23). peLUC17E was generated by subcloning the 65–1227 sequence of the *S100A4* gene in the Klenow filled-in *Hin*dIII site of pfLUC upstream from the c-*fos* promoter. peLUC1 and peLUC48 were constructed as described for peLUC17E, but contain only the 782–916 and 732–966 sequence, respectively. Plasmids peLUC2, peLUC3, peLUC8, peLUC10, peLUC11, peLUC14, peLUC19, peLUC22 and peLUC24 were generated by introducing point mutations (*mut2–mut7*, see Fig. 1D) into peLUC1 by *Pfu*-mediated PCR. peLUC20 contains a deletion of nucleotides 803–807 and peLUC21 a deletion of nucleotides 803–812. peLUC32 has an insertion of the nucleotides 5′-CTTAC-3′ between nucleotides 800 and 801, whereas the sequence 5′-CTTAGCTTAC-3′ is inserted in peLUC33. Point mutations, deletions and insertions in all constructs were verified by DNA sequence analysis. pcDNA-CBFA1 was constructed by cloning the 3739 bp *Bgl*II–*Pvu*II fragment of pKSαA1 (24) (kindly provided by Dr Y. Ito) into the *Bam*HI and *Eco*RV sites of pcDNA1.1/Amp (Invitrogen). pCMV-Fra1 has been described earlier (25). pCMVβ was purchased from Clontech. pCMVLuc was constructed by cloning the *Bam*HI–*Xho*I fragment of pfLUC into the *Bam*HI and *Xho*I sites of pcDNA1.1/Amp. pRSVLuc was constructed by excising the β-galactosidase insert of pRSVβ-gal (kindly provided by Dr M. Strauss) using *Hin*dIII and *Bam*HI, filling-in with Klenow and inserting it into *Bam*HI + *Xho*I-digested and blunt-ended pfLUC. pSV40Luc was purchased from Promega Corp. (pGL2-Enhancer Vector).

Nuclear extracts and EMSAs

Nuclear extracts were prepared as described (26). Binding reactions were performed by mixing 10 μg nuclear extract with 2 μg poly(dI·dC)–poly(dI·dC) and 2 μl of 5× binding buffer (150 mM KCl, 50 mM HEPES–KOH pH 7.9, 25 mM Tris–HCl pH 7.9, 5 mM EDTA, 5 mM DTT, 25% glycerol, 1.5 μg/μl BSA) in a total volume of 9 μl. The reaction was incubated for 5 min at 30°C, after which 1 μl of radiolabeled oligonucleotide (5×10^4 c.p.m.) was added and the reaction continued for 15 min. The reactions were analyzed by electrophoresis in a non-denaturing 4% polyacrylamide gel at 4°C in 0.4× TBE buffer (pH 8.0). Sp1/Sp3 binding reactions were performed in the following binding buffer: 0.1 M KCl, 20 mM HEPES pH 8.4, 5% glycerol, 0.1 mM EDTA, 0.25 mM $ZnSO_4$, 0.05% NP-40, 0.3 μg/μl BSA, 1 mM DTT. The sequences of *CBFcons* and *Sp1cons* are 5′-GGGGATATCTGTGGTAAGCACC-3′ and 5′-ATTCGATCGGGGCGGGGCGAGC-3′. Sequences of the remaining oligonucleotides are shown in Figure 1D.

For supershift analysis, antibodies against CBFA1, CBFA2 (Geneka Biotechnology Inc.), p52, Sp1, Sp3 (Santa Cruz Biotechnology) or CBFβ (provided by Dr S. Hiebert) were added when the binding reaction was complete. Incubation was continued for 1 h at 0°C, except for the Sp1 and Sp3 antibodies, where incubation was for 40 min at room temperature.

In vitro DNase I footprint analysis

An aliquot of 20–80 μg nuclear extract was mixed with 6 μg poly(dI·dC)–poly(dI·dC) and 12 μl of 5× footprinting assay buffer (5 mM EDTA, 2.5 mM spermidine, 3.5 mM DTT, 75 mM HEPES–KOH pH 7.9) in a total volume of 60 μl. The reaction was incubated for 5 min at 20°C. Then 1 μl of probe (3×10^4 c.p.m.) was added and incubation continued for 30 min. After adding 25 μl of 24 mM $MgCl_2$ and 1 min incubation, 1 U (for reactions with nuclear extract) or 0.5–2 U (for free probe) DNase I (Boehringer Mannheim) was added. The reaction was continued for 2 min, followed by addition of 145 μl of stop solution (1% SDS, 40 mM EDTA, 0.25 M NaCl, 0.1 μg/μl proteinase K, 0.13 μg/μl tRNA). The samples were

run in a 6% gradient (0.4–0.6 mm) sequencing gel. The fragment analyzed by DNase I footprinting was generated by PCR-mediated amplification of the 732–966 sequence using ^{32}P-labeled 3′-end and unlabeled 5′-end oligonucleotides.

Methylation interference assay

The binding reaction was performed as described above. Methylation interference analysis and A/G cleavage were done as described (27,28). The samples were analyzed in a 12% gradient (0.2–0.6 mm) sequencing gel.

Western blotting

Nuclear extracts (30 µg) were denatured and fractionated on 10% polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Millipore) by the standard procedure and incubated in blocking solution with primary antibody at a dilution of 1:20 000 (for anti-CBFA2; Geneka Biotechnology Inc.), 1:500 (for anti-CBFA1; Geneka Biotechnology Inc.) and 1:500 (for anti-CBFβ, provided by Dr S. Hiebert). Immunoreactive proteins were detected using the enhanced chemiluminescence system (ECL; Amersham).

RESULTS

Enhancer activity is associated with the 782–916 fragment of the *S100A4* gene first intron

We have previously searched for DNase I hypersensitivity sites (DHSs) in the *S100A4* gene as potential indicators of areas involved in transcriptional control. In CSML100 cells DHSs were mapped between +750 and +950. Deletion analysis of the first intron sequences revealed the presence of an enhancer downstream of +781 (16). The 782–916 fragment of the *S100A4* first intron (Fig. 1A) was cloned upstream from the minimal c-*fos* promoter in the pfLUC reporter plasmid (peLUC1). The activity of peLUC1 in CSML100 cells was compared with that of the vector, a plasmid containing the entire *S100A4* first intron sequence except the first 27 bp (peLUC17E) or a construct harboring the 732–966 sequence (peLUC48). In CSML100 cells the 782–916 first intron fragment conferred an ~40-fold increase in reporter activity. peLUC48 had essentially the same activity as peLUC1. On the other hand, peLUC17E was less active than peLUC1 (Fig. 1A). The difference between the activities of peLUC1 or peLUC48 plasmids and peLUC17E can be attributed either to the longer distance between the promoter and enhancer elements in peLUC17E or to the inhibitory activity of sequences in the first intron of *S100A4* outside the enhancer. As peLUC1 and peLUC48 showed nearly equal levels of enhancer activity, we concluded that the *S100A4* intronic enhancer is entirely located within the 782–916 fragment (plasmid peLUC1).

The enhancer is partially cell specific

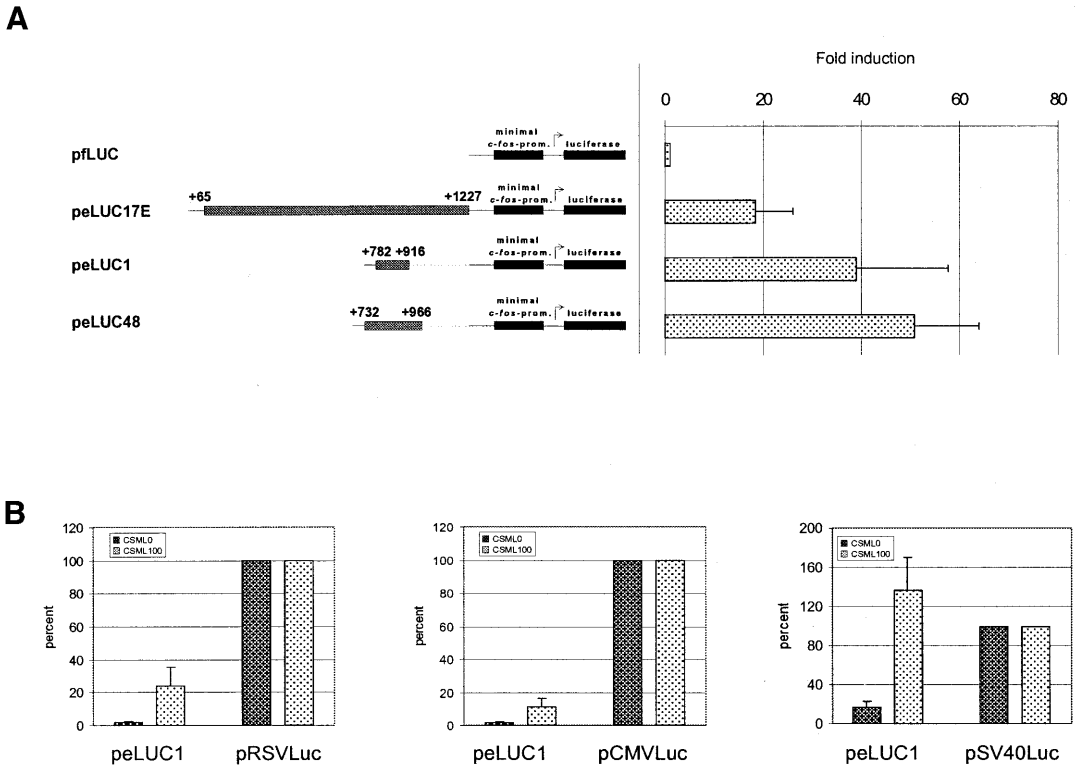
We next studied whether the *S100A4* enhancer acts in a cell-specific manner and thereby contributes to tissue-specific expression of the *S100A4* gene. We compared the activity of peLUC1 in CSML0 and CSML100 cells with the activities of three reporters, pRSVLuc, pCMVLuc and pSV40Luc, where the luciferase gene was controlled by viral enhancers. The activity of each of the viral enhancers was taken as 100% and the strength of the *S100A4* enhancer in CSML0 and CSML100 cells was expressed as a percentage of the viral enhancer strength. Based on these calculations, we could compare the activities of the peLUC1 reporter in S100A4-positive CSML100 and S100A4-negative CSML0 cells. When the measurements were normalized to the pRSVLuc reporter, the activity of the *S100A4* enhancer was found to be 12.5-fold higher in CSML100 than in CSML0 cells (Fig. 1B). When the strengths of the CMV or SV40 enhancers were taken as references, the observed ratios were 7- and 8.4-fold, respectively (Fig. 1B). Since the *S100A4* intronic enhancer, as assayed in transient transfections, is moderately cell specific (7- to 12.5-fold more active in expressing versus non-expressing cells) it can contribute to differential expression of the *S100A4* gene in CSML0 and CSML100 cells.

To characterize the potential interactions of transcription factors with the *S100A4* enhancer, we performed DNase I footprinting experiments with the end-labeled 732–966 fragment as probe. *In vitro* a previously described κB element of the *S100A4* enhancer binds the NF-κB family p50–p50 homodimer and p50–p65 heterodimer, as well as the KRC transcription factor (16,21). The 786–807 sequence was weakly cleaved by DNase I in free DNA. Therefore, we could observe a narrow but clear protection only in the 5′-part of this sequence using both CSML100 and CSML0 nuclear extracts (Fig. 1C). The broadest footprint produced by both CSML100 and CSML0 extracts was observed in the area 810–839, the Sa sequence. This region contained a 10mer element, the Sa core, matching the minisatellite core DNA sequence (29–31), as well as five GGCA/TG motifs resembling the mo-1-related minisatellite core element, GGCAGG (32). Two other protected areas (879–893 and 900–916) were specific for CSML100 nuclear extracts and contained recognition sequences for the CBF and AP-1 transcription factors, respectively (Fig. 1C). We next performed a computer search for transcription factor binding sites in the 782–916 sequence. In addition to the sequences revealed in DNAse I footprinting experiments, the search disclosed a perfect AP-2 binding site (841–848), as well as three GC- or GT/A-rich motifs (835–844, 849–858 and 896–905) whose sequences deviated from the Sp1 consensus, G/TRGGC/AGG/TRRY (33), in only one position (Fig. 1D).

To undertake a detailed study of the *S100A4* enhancer, we measured the contribution of each individual element to enhancer activity by mutating the corresponding sequences in the nucleotide context of peLUC1 (the sequences of the mutated oligonucleotides are given in Fig. 1D) and identified nuclear proteins interacting with these elements.

Characterization of *cis*-elements comprising the *S100A4* intronic enhancer

In our previous study we found that a κB element contributes to *S100A4* enhancer activity via an interaction with KRC protein, a transcription factor whose expression is higher in CSML100 than CSML0 cells (16,21). We designed a point mutation abolishing interaction of the κB element with KRC, *mut2* (16; Fig. 1D). We found that in the context of the whole first intron *mut2* decreased enhancer activity in CSML100 cells to 32% (16). However, in the context of the 782–916 fragment this mutation (peLUC2) reduced enhancer activity to 18% of the wild-type enhancer (see Fig. 5A). In CSML0 cells the effect of this mutation, 43% of peLUC1 (data not shown), roughly coincided with the data obtained in the whole intron



context (16). Therefore, KRC is a more potent activator of the peLUC1 reporter in CSML100 than in CSML0 cells.

We attempted to characterize the proteins producing the strong footprint in the area 810–840 using EMSA and methylation interference assay. An end-labeled 31mer oligonucleotide, the *Sa* oligonucleotide (Fig. 1D), corresponding to the protected sequence, formed abundant and sequence-specific complexes with both CSML0 and CSML100 nuclear extracts (Fig. 2A). Methylation interference analysis of the Ca and Cb complexes revealed 7 nt located within the 10mer, as well as 5 nt in the 5′- and 3′-flanking sequences, directly involved in the formation of both complexes. In addition, the study of complexes specific for CSML100 but not CSML0 cells revealed the presence of two DMS hyper-reactive nucleotides, guanine 838 in the sense strand and guanine 811 in the antisense strand (Fig. 2B and C). Together with the slightly different mobilities of the Ca and Cb complexes (Fig. 2A), these data suggest that the proteins forming Ca and Cb might be different.

The wild-type Sa sequence was replaced by the *mut3* oligonucleotide in the peLUC1 construct (peLUC19). This mutation significantly affected the function of the *S100A4* enhancer in CSML0 and in CSML100 cells, where peLUC19 retained only 7 and 15% of peLUC1 activity, respectively (Fig. 2D). The proteins interacting with the minisatellite DNA are therefore directly involved in maintenance of the functional state of the *S100A4* enhancer.

The search for homologies in the *S100A4* enhancer revealed a GC-rich motif matching the AP-2 binding site, as well as three sites that differed by one substitution from the Sp1 consensus sequence (Fig. 1D). Whereas AP-2 transcription factors are expressed at very low levels in CSML0 and CSML100 cells (data not shown), the Sp1-I, Sp1-II and Sp1-III elements did interact with transcription factors of the Sp1 family. Since the EMSA patterns for all three Sp1-related sequences were similar, only the data for Sp1-II are shown (Fig. 3A). Two slowly migrating complexes could be efficiently competed by a 100-fold molar excess of a Sp1 consensus oligonucleotide, *Sp1cons*, (Fig. 3A, lanes 4 and 11). Mutations nullifying the homology with the Sp1 consensus were designed (*mut4–mut6*, Fig. 1D) and introduced into the Sp1-like sequences. All mutations fully abolished the ability of the resulting oligonucleotides to compete with the wild-type oligonucleotides for complex formation (Fig. 3A, lanes 3 and 10, and data not shown). The upper complexes were completely supershifted with a specific anti-Sp1 antibody (Fig. 3A, lanes 5 and 12). Incubation with an anti-Sp3 antiserum resulted in disappearance of the second complex (Fig. 3A, lanes 6 and 13). The abundance of the third complex from the top formed by the *Sp1-II* oligonucleotide decreased when anti-Sp3 antibody was added (Fig. 3A, lanes 6 and 13). These complexes likely contained unconventional or degraded forms of Sp3. None of the observed complexes were affected by antibodies against the two remaining members of the Sp1 family, Sp2 and Sp4 (data not shown).

In order to examine the contribution of each particular Sp1-binding site to function of the *S100A4* enhancer in both CSML0 and CSML100 cells, we replaced each of the three sites in peLUC1 by *mut4*-, *mut5*- or *mut6*-containing oligonucleotides. Interestingly, the effect of mutation of each of these sites differed and was cell line dependent. Whereas the Sp1-I site played a major role in maintenance of the functional state of the enhancer in CSML100 cells (Fig. 3B, peLUC8), mutating the Sp1-II or Sp1-III sites only moderately affected activity of the reporter

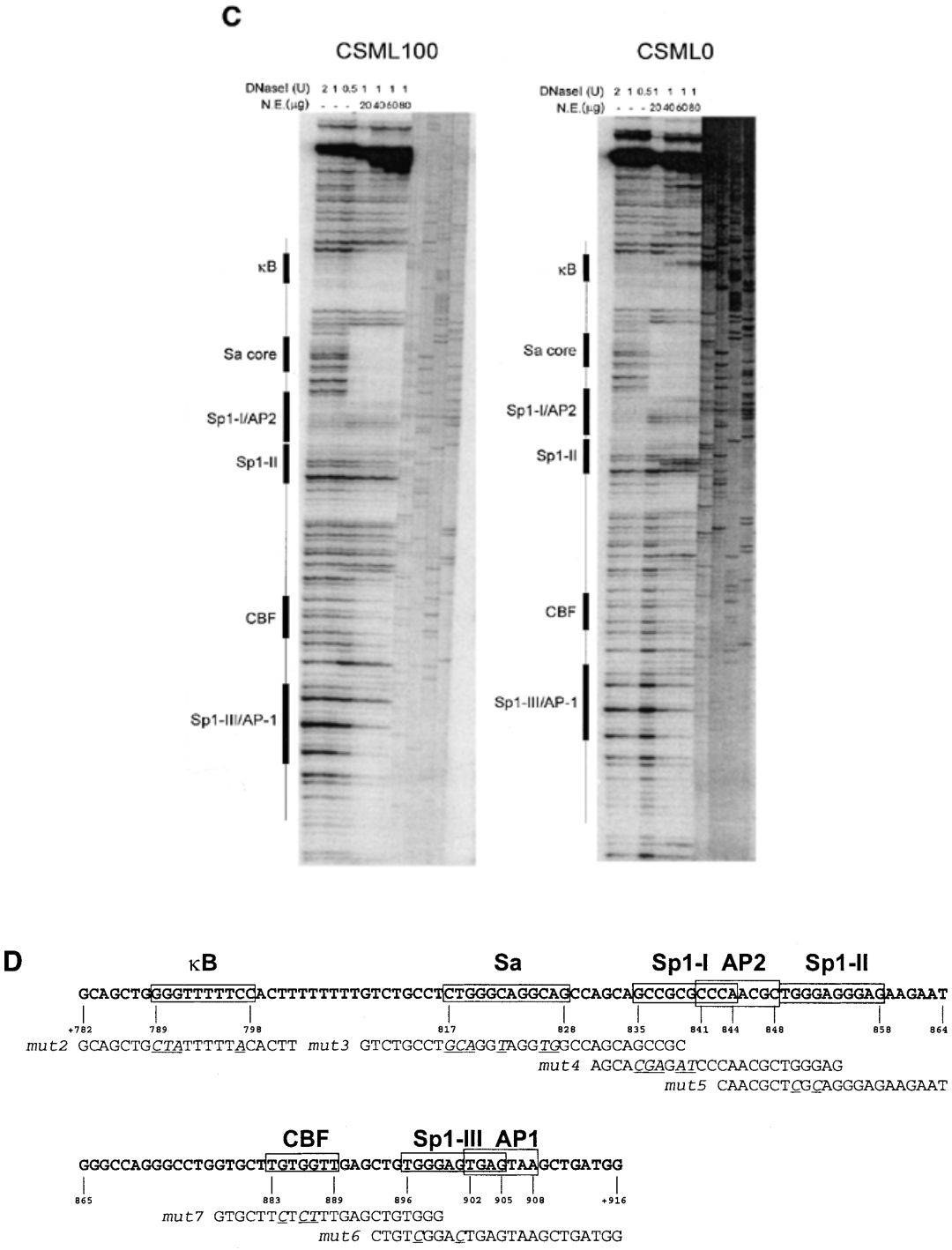


Figure 1. (Opposite and above) The *S100A4* gene intronic enhancer is located within the 782–916 area and exhibits limited cell specificity. (**A**) CSML100 cells were co-transfected with reporter constructs as indicated, along with the β-galactosidase expression vector pCMVβ. Transfections were normalized for β-galactosidase activity and expressed as fold activation relative to the activity of the enhancerless pfLUC plasmid. (**B**) CSML0 or CSML100 cells were transfected with peLUC1 or with constructs where the luciferase gene was placed under control of the RSV (pRSVLuc), CMV (pCMVLuc) or SV40 (pSV40Luc) enhancers. Activities of the viral enhancers were taken as 100% and the activity of peLUC1 was expressed as a percentage of viral enhancer strength. Here and elsewhere the results (means ± SD) of more than three independent transfections are shown. (**C**) *In vitro* DNase I footprint analysis of the *S100A4* 732–966 region using CSML100 or CSML0 nuclear extracts. The non-coding strand of the DNA was labeled. The DNA was incubated with the indicated amounts of nuclear extract, followed by DNase I treatment, as shown. (**D**) The nucleotide sequence of the 782–916 *S100A4* intronic DNA fragment. Potential transcription factor recognition sequences were identified by screening a transcription factor database (54), and are indicated by boxes. Sequences of mutant oligonucleotides used in later experiments are indicated below the sequence. The corresponding wild-type oligonucleotides used for EMSA and other experiments (*Sa*, *Sp1-II* and *CBF* oligonucleotides) have exactly the same length and coordinates as the corresponding mutant ones (*mut3*, *mut5* and *mut7*).

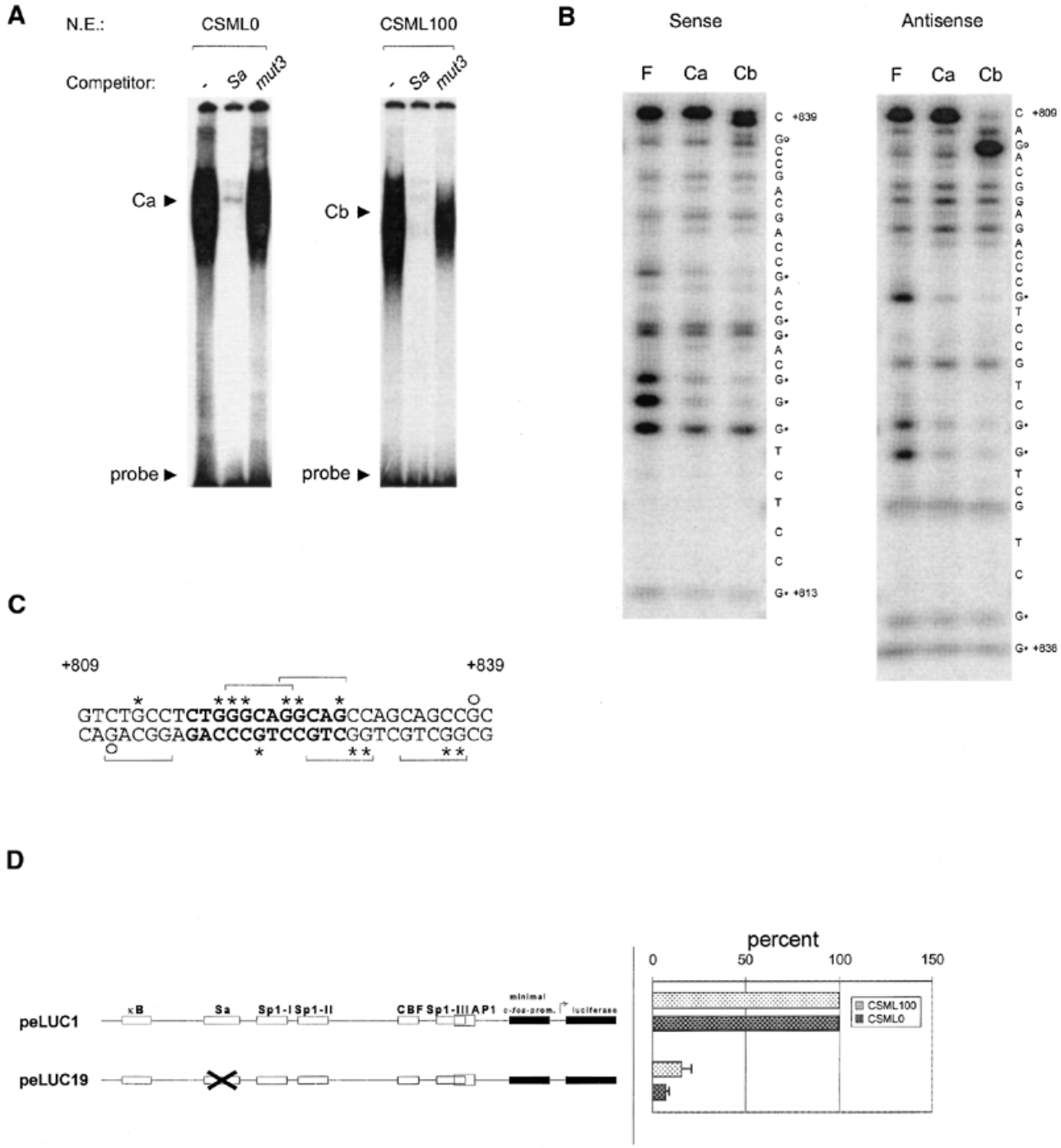


Figure 2. The minisatellite-related sequence Sa contributes to *S100A4* enhancer activity. (**A**) EMSA showing the complexes formed by Sa in CSML0 and CSML100 nuclear extracts. A Sa-containing 31mer, the *Sa* oligonucleotide, was end-labeled and analyzed by EMSA with CSML0 (5 µg) and CSML100 (10 µg) nuclear extracts. Competitor oligonucleotides *Sa* or *mut3* were added as shown. (**B**) Methylation interference analysis of the Ca and Cb complexes. F, free DNA. Guanines involved in formation of the Ca and Cb complexes are marked by asterisks. Circles indicate the hyper-reactive nucleotides revealed by analysis of the Cb complex. (**C**) Summary of the methylation interference data. The Sa core sequence is shown in bold. The 5mer motifs, GGCA/TG, are indicated by horizontal brackets. Asterisks and circles are defined in the legend to (B). (**D**) The contribution of Sa to the activity of the *S100A4* enhancer assayed in transient transfection of CSML0 and CSML100 cells with plasmid peLUC19. peLUC19 contains a mutated Sa site (*mut3*). The activity of peLUC19 in CSML0 and CSML100 cells is expressed as a percentage of the wild-type enhancer activity (peLUC1) in each cell line.

(peLUC10 and peLUC11). In CSML0 cells the Sp1-II site contributed significantly to enhancer activity, while the two other Sp1 sites were weaker. The importance of Sp1/Sp3 binding for *S100A4* enhancer function was supported by experiments in which the Sp1-deficient *Drosophila melonogaster* cell line Schneider SL-2 was co-transfected with reporters containing the *S100A4* enhancer along with Sp1 and/or Sp3 expression vectors (data not shown). The results of these experiments demonstrated that both Sp1 and Sp3 factors are potent activators of the *S100A4* enhancer.

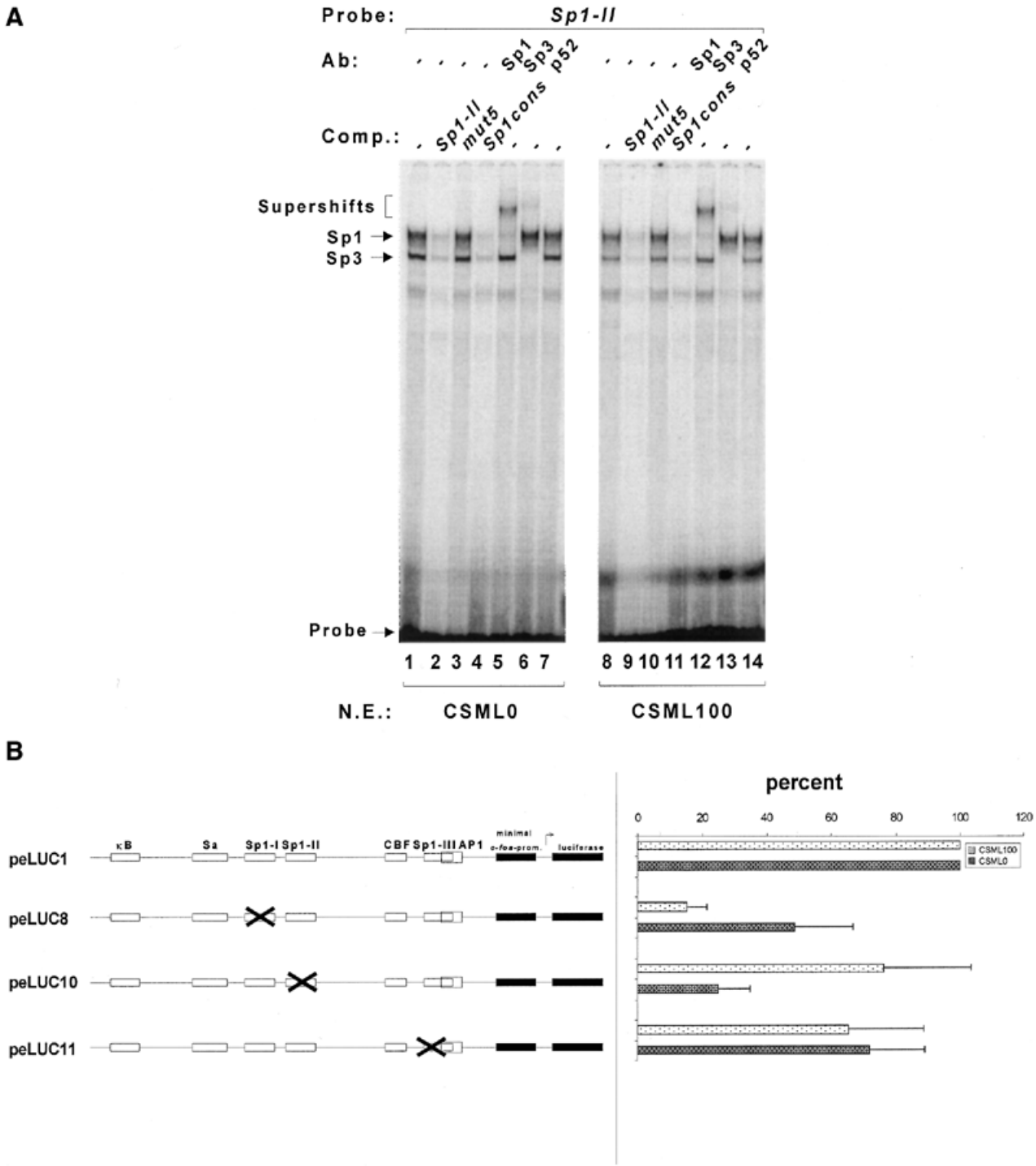


Figure 3. Sp1-like motifs bind Sp1 and Sp3 transcription factors and differentially contribute to *S100A4* enhancer function in CSML0 and CSML100 cells. (**A**) EMSA of a radiolabeled oligonucleotide containing the Sp1-II site with CSML0 or CSML100 nuclear extracts. Anti-Sp1, anti-Sp3 and control anti-p52 antibodies were added as indicated. Sp1 and Sp3 complexes and supershifted bands are indicated. A competition assay with unlabeled *Sp1-II* oligonucleotide mutant (*mut5*), as well as with an oligonucleotide containing a Sp1 consensus (Materials and Methods) binding site, is also shown. (**B**) Effect of mutating the Sp1-I, Sp1-II and Sp1-III sites on *S100A4* enhancer activity. The Sp1-I, Sp1-II or Sp1-III site was substituted in peLUC1 by the *mut4*, *mut5* or *mut6* sequence (see Fig. 1D). The activity of the resulting constructs, peLUC8, peLUC10 and peLUC11, was measured in transiently transfected CSML0 or CSML100 cells and expressed as a percentage of the peLUC1 activity.

As a CSML100 cell-specific footprint in the area 880–891 was observed (Fig. 1C), we tested whether this footprint was due to binding of CBF factors, which represent a heterodimer comprised of two subunits, α and β. Whereas the β-subunit is represented by a single ubiquitously expressed polypeptide, multiple α isoforms are encoded by three different genes,

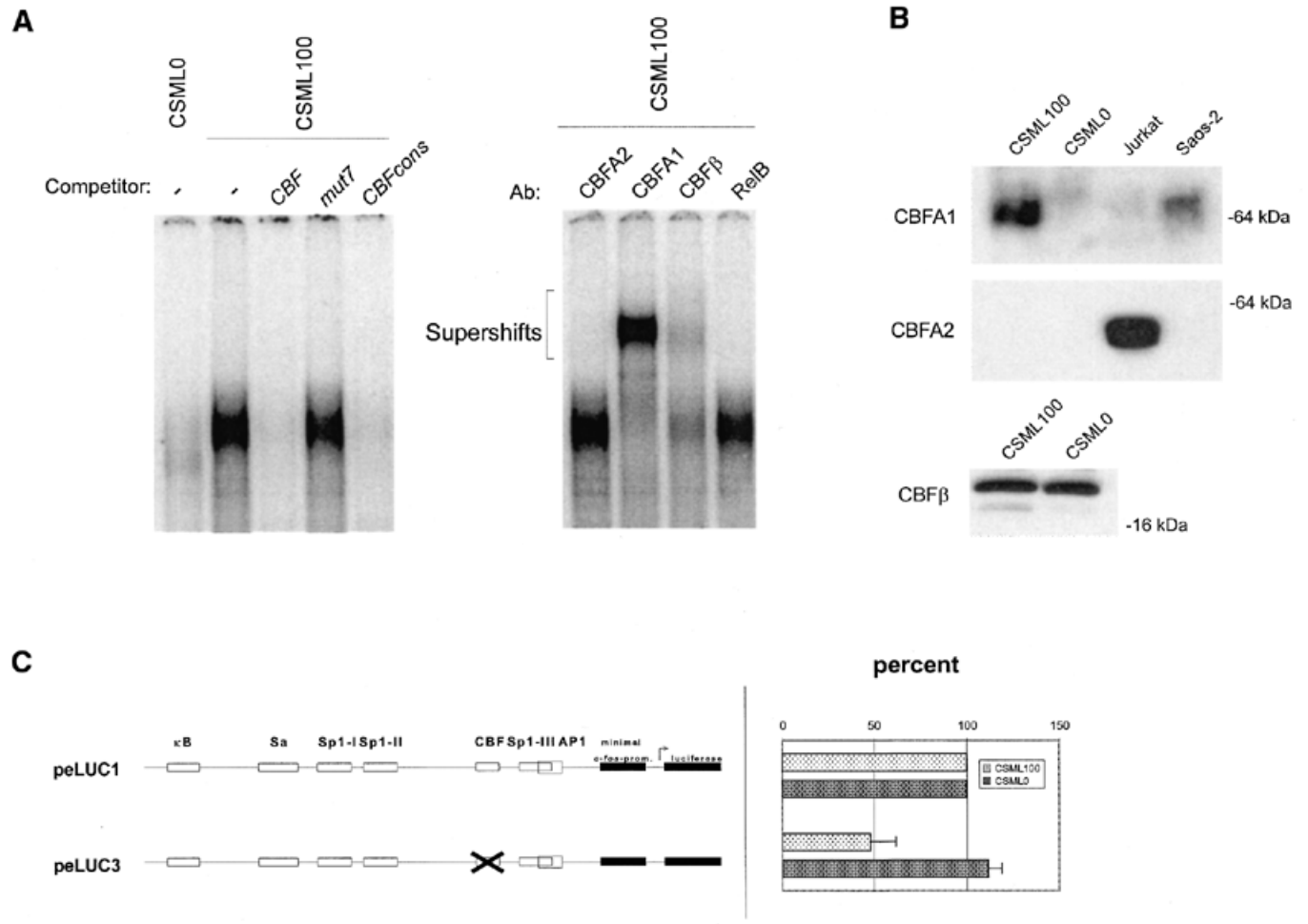


Figure 4. Structural and functional analysis of the CBF-binding site. (**A**) EMSA of complexes formed by the *CBF* oligonucleotide with CSML0 and CSML100 nuclear extracts. As competitors the *CBF* oligonucleotide, *mut7* or a CBF-containing oligonucleotide derived from the Moloney murine leukemia virus enhancer (*CBFcons*, Materials and Methods) were used. (Right) Supershift analysis of the CBF complex formed between *CBF* oligonucleotide and nuclear proteins from CSML100 cells. The competitors and antibodies used are indicated above the lanes. (**B**) Western blot analysis of proteins from CSML0 and CSML100 cells. The antibodies used for immunostaining are indicated on the left. Nuclear extracts prepared from Saos-2 and Jurkat cells expressing CBFA1 and CBFA2 proteins, respectively, were used as positive controls. (**C**) Transient transfection analysis of a reporter construct with a mutated CBF site in CSML0 and CSML100 cells.

CBFA1, *CBFA2* and *CBFA3*, mouse homologs of the *Drosophila* pair-rule gene *runt* (34).

As expected, EMSA revealed an abundant complex in CSML100 but not in CSML0 nuclear extracts incubated with the *CBF* oligonucleotide (Fig. 1D), spanning the 879–893 site (Fig. 4A). We concluded that this complex was comprised of CBF proteins because its formation was blocked by a CBF consensus oligonucleotide, *CBFcons*, but not by *mut7* (Fig. 1D), where the CBF recognition sequence was mutated (Fig. 4A). To identify the proteins forming this complex we employed a gel supershift assay with specific anti-CBFA1, anti-CBFA2 and anti-CBFβ antibodies. The complex was completely supershifted with anti-CBFA1, but not affected by anti-CBFA2 antibodies. Incubation with the anti-CBFβ antibody resulted in the appearance of a supershifted band and simultaneous inhibition of the complex (Fig. 4A). We therefore concluded that the CBF complex, which was specific for CSML100 cells, represented a CBFA1–CBFβ heterodimer. To clarify which CBF components are expressed in CSML100 but deficient in CSML0 cells we examined expression of the CBF subunits by western blot analysis. Consistent with the general view of the β-subunit as a ubiquitously expressed factor (35), the CBFB protein was detected in both CSML0 and CSML100 cells (Fig. 4B). On the other hand, western blot analysis revealed absence of both CBFA1 and CBFA2 proteins in CSML0 and presence of CBFA1 in CSML100 cells, confirming the gel supershift data.

In order to assess the functional importance of the CBF complex, the CBF site-containing sequence was replaced by *mut7* (Fig. 1D) in peLUC1 (peLUC3). Inactivation of the CBF site reduced enhancer activity to 48% in CSML100 cells, whereas no effect was seen in CSML0 cells (Fig. 4C). This is consistent with CBFα deficiency in CSML0 cells.

The AP-1 site-containing 900–916 area was found to be protected from DNase I digestion in the presence of CSML100 but not CSML0 nuclear extracts (Fig. 1C). This observation is in good agreement with our previous observation that the AP-1 complex is more abundant in CSML100 than in CSML0 cells (25). Analysis of the AP-1-binding site in the *S100A4* enhancer by EMSA revealed a low amount of c-Fos–JunD heterodimers

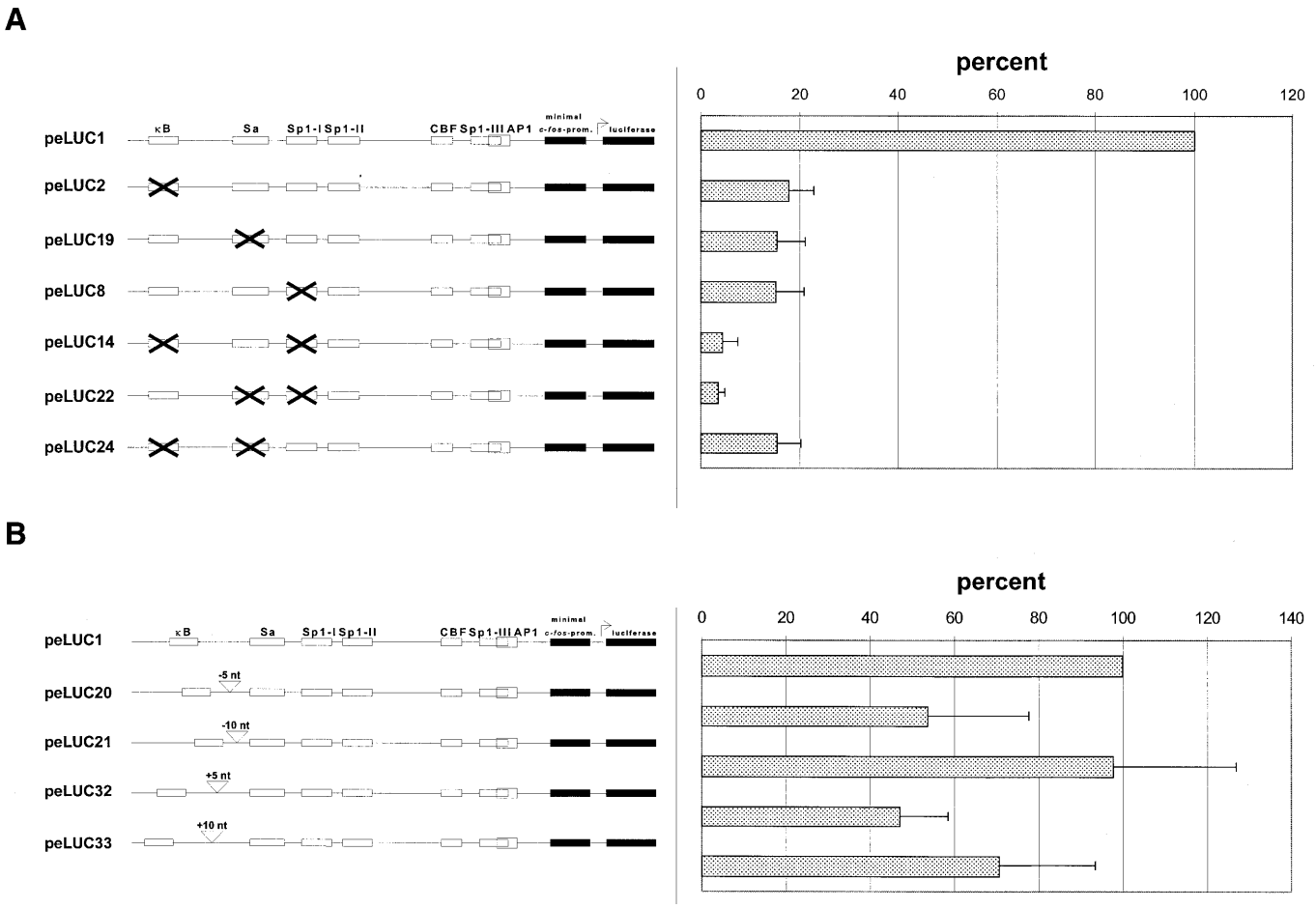


Figure 5. Summary of the functional analysis of the 5′-part of the *S100A4* enhancer in CSML100 cells. (**A**) Transient transfection analysis of reporter plasmids bearing single or double point mutations in the κB, Sa and Sp1-I sites in the *S100A4* enhancer. (**B**) Transient transfection analysis of reporter plasmids with changed phasing between the κB and Sa sites. In peLUC20 and peLUC21 5 and 10 nt are deleted, respectively. In peLUC32 and peLUC33, 5 and 10 nt are inserted, respectively.

in CSML0 cells and abundant Fra-1–JunD and Fra-2–JunD complexes in CSML100 nuclear extracts (data not shown).

Cooperative function of the κB and Sa sequences

As shown in the previous sections, we have identified seven elements comprising the *S100A4* enhancer. Interestingly, whilst the contributions of particular elements to enhancer activity differed from each other in CSML0 cells, the individual mutations of three essential 5′-sequences (κB, Sa and Sp1-I) affected enhancer function equally in CSML100 cells (summarized in Fig. 5A). This observation could reflect a cooperative way of action between the proteins interacting with two or all of these elements in CSML100 cells. To test this speculation, we examined the activity of reporters bearing double mutations in the κB, Sa and Sp1-I sites in different combinations. In CSML100 cells double mutations in the κB and Sp1-I sites (peLUC14) or in the Sa and Sp1-I sites (peLUC22) inhibited enhancer activity to 4–5% of that of the wild-type enhancer (Fig. 5A). This effect was apparently more prominent than that of any of the mutations in single sites, which lowered enhancer activity to 15–18% of peLUC1 activity (peLUC2, peLUC19 and peLUC8). On the other hand, peLUC24 (mutations in the κB and Sa sites) retained 17% of peLUC1 activity and was therefore as active as the peLUC2 and peLUC19 constructs with individual mutations in the κB or Sa sequences, thereby demonstrating functional cooperation between them. A physical interaction between the proteins binding the κB and Sa sequences may underlie the observed cooperation in CSML100 cells. If activity of KRC protein and the factors binding the Sa sequence depends on an interaction between them, it is likely that the helical phasing between their DNA binding sites is important for full activity. To test this hypothesis, 5 or 10 bp deletions (peLUC20 and peLUC21), as well as 5 or 10 bp insertions (peLUC32 and peLUC33), were introduced between the κB and Sa sites. These plasmids were examined in transient transfections of CSML100 cells (Fig. 5B). The insertion or deletion of 5 bp decreased enhancer activity by 50%. On the other hand, the mutations maintaining the helical phasing relationship either did not interfere with enhancer activity (peLUC21) or had a minor effect (peLUC33). These data suggest functional cooperation between factors binding the κB and Sa sequences in CSML100 cells. In contrast, the described cooperation was not observed on analyzing the activity of peLUC1-based double mutants in CSML0 cells (data not shown).

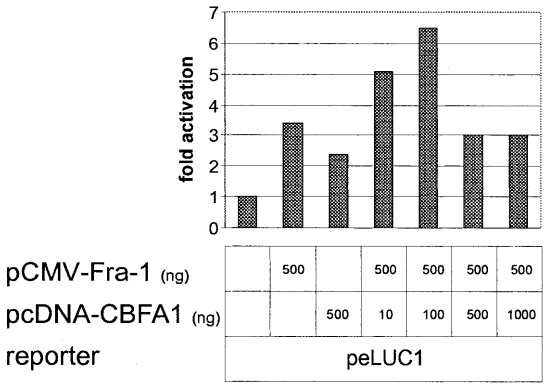


Figure 6. Overexpression of the Fra-1 and CBFA1 transcription factors leads to partial reconstitution of the *S100A4* enhancer in CSML0 cells. CSML0 cells were transfected with the peLUC1 reporter, along with various amounts of Fra-1 and CBFA1 expression vectors as indicated under the bars.

Expression of exogenous CBFA1 and Fra-1 in CSML0 cells leads to activation of the *S100A4* enhancer

Although the *S100A4* intronic enhancer is active in both CSML0 and CSML100 cells in transient transfection assays, its fine structure and function differ in expressing versus non-expressing cells. This is reflected by the fact that the enhancer was ~10-fold more active in CSML100 cells than in CSML0 cells when referred to the activities of three viral enhancers (Fig. 1B). One of the striking differences between the two cell lines was the lack of expression of CBFA1 and Fra-1 proteins in CSML0 cells (Fig. 4 and data not shown), resulting in the absence of CBF and a decreased level of AP-1 binding in the 883–889 and 902–908 regions, respectively. In order to test whether deficiency of these factors may determine the difference in enhancer activity between CSML0 and CSML100 cells, we transfected CSML0 cells with the peLUC1 reporter construct together with expression plasmids for Fra-1 and CBFA1. Expression of Fra-1 resulted in a 3-fold increase in activity of the *S100A4* enhancer, whereas expression of CBFA1 led to a 2-fold increase (Fig. 6). The CBF transcription factors play a dual role in transcriptional regulation. In addition to their ability to activate transcription, CBFα proteins may recruit the co-repressor Groucho/TLE to the promoters of target genes, thereby inducing repression (36). Bae *et al.* reported that the transactivation induced by CBFα declined at high concentrations of the expression vector (37). We therefore examined responsiveness of the *S100A4* enhancer using different amounts of CBFA1 expression plasmid. When 100 ng pcDNA-CBFA1 was applied in combination with 500 ng pCMV-Fra-1, enhancer function was stimulated >6-fold, whereas higher amounts of pCMV-CBFA1 led to a weaker activation (Fig. 6). Thus, simultaneous expression of two transcription factors absent in CSML0 cells brought *S100A4* enhancer activity nearly to the level observed in CSML100 cells.

DISCUSSION

This work includes a detailed study of the intronic enhancer of the *S100A4* gene. The functional sites within the enhancer are, in 5′→3′ order, κB, minisatellite DNA (Sa), Sp1-I, Sp1-II, CBF, Sp1-III and AP-1. The general enhancer activity in CSML0 cells was 7- to 12.5-fold lower than in CSML100 cells when referred to activities of three viral constructs (Fig. 1B). Moreover, though in CSML0 cells the *S100A4* enhancer retained a certain level of activity, mutation analysis of the enhancer revealed that its functional organization was significantly different from that in CSML100 cells.

Previously, we identified a factor activating the *S100A4* enhancer via the κB site as a member of the ZAS protein family, namely KRC protein (16,21), which has been implicated in V(D)J recombination (38). Interestingly, Sa, the functional site within the *S100A4* enhancer located 10 bp downstream from κB, contains a minisatellite DNA core sequence, which has also been implicated in recombination processes (39–40). Four proteins interacting with minisatellite DNA have previously been identified. Three of them, Msbp-1, Msbp-2 and Msbp-3, interact with the minisatellite core element and one protein (Msbp-4) binds another minisatellite-related sequence, the mo-1 6mer motif (30–33). The Msbp-1–Msbp-4 genes have never been cloned and the biological functions of the corresponding proteins have, to our knowledge, not been documented. Within the minisatellite sequence we revealed 12 guanines directly involved in DNA–protein interactions. Seven of these guanines are located inside the 10mer matching the minisatellite core element. On the other hand, 11 of the 12 detected guanines are present in the GGCA/TG motifs, which are related to mo-1 (GGCAGG) (Fig. 2C). We cannot therefore at present conclude whether the proteins interacting with the minisatellite element of the *S100A4* enhancer in CSML0 and CSML100 cells are identical to Msbp-1–Msbp-4. However, the characteristics of the proteins interacting with Sa certainly seem to differ between CSML0 and CSML100 cells, as shown in our EMSA and methylation interference experiments. Furthermore, mutating the minisatellite sequence leads to different effects on the activity of the *S100A4* enhancer in the two cells lines. These observations, as well as functional cooperation between the κB and Sa elements revealed in CSML100 but not in CSML0 cells, might reflect a difference in the composition and abundance of Sa-binding proteins expressed in these two cell lines.

At present we do not know whether a physical interaction between KRC and Msbp is a prerequisite for their functional cooperation. DNase I footprint experiments showed that mutating the Sa sequence did not affect KRC binding (data not shown). These data support a model in which binding of KRC and Msbp occurs independently and in which functional cooperation between them happens after binding to the enhancer DNA. An interaction may occur either between these two factors or between them and other components of the transcription machinery. Such a model is not unique, as the mechanism of functional synergy between c-Myb and CBF also does not involve cooperative binding to DNA (41).

Three Sp1-related sequences in the *S100A4* enhancer all interact with the Sp1 and Sp3 transcription factors and we found that mutation of these sites reduced enhancer activity to various extents dependent on the site. Though Sp3 can act as a repressor of transcription, both Sp1 and Sp3 demonstrated nearly identical abilities to transactivate the *S100A4* enhancer in SL-2 cells (data not shown). This contrasts with the data obtained, for example, with the alcohol dehydrogenase 5 (42) or *HIV-1* promoters (43), but is consistent with the results of

studies of the promoters of the integrin genes *CD11b* and *CD11c* in the same *Drosophila* cell line (44). Currently there is no mechanistic explanation of how the nucleotide context may determine whether Sp3 activates or represses transcription in a given enhancer.

Whereas KRC, Sa-binding factors and the Sp1 and Sp3 proteins are expressed in both CSML100 and CSML0 cells, expression of CBF and AP-1 was different (Fig. 4; 25). We detected an equal amount of CBFB protein in both cell lines, which is in accordance with the previously reported ubiquitous CBFB expression. The CBFα subunit CBFA1, on the other hand, was only detected in CSML100 cells, determining the cell specificity of the CBF complex detected by EMSA. Consistent with the differential expression of CBF in CSML0 and CSML100 cells, a mutation abolishing CBF binding differentially affected enhancer activity in the two cell lines. We previously demonstrated that AP-1 is more abundant in CSML100 than in CSML0 cells due to an elevated level of Fos-related antigens, in particular Fra-1 (25). Interestingly, as was shown in different *in vivo* models, both Fra-1 and CBFA1 play important roles in bone development (45,46). The importance of these factors for function of the *S100A4* enhancer was revealed in transient transfection experiments, where simultaneous expression of Fra-1 and CBFA1 in CSML0 cells led to a >6-fold increase in *S100A4* enhancer activity (Fig. 6). These data suggest that the relative invalidity of the *S100A4* enhancer in CSML0 cells is partly due to a lack of CBFA1 and Fra-1 expression.

Though the *S100A4* enhancer is 7- to 12.5-fold more active in CSML100 than in CSML0 cells, some additional mechanisms should exist to entirely inhibit enhancer function in non-expressing cells. A correlation between DNA methylation and inactivity of the *S100A4* gene was reported in mammary and colon adenocarcinoma cell lines of mice, rats and humans and in human lymphoma cell lines (15,18,20,47,48). Moreover, 5-azacytidine-mediated demethylation leads to activation of the *S100A4* gene in CSML0 cells (our unpublished results). On the other hand, methylation analysis of the *S100A4* enhancer DNA *in vivo* revealed that this sequence is unmethylated in both S100A4-expressing CSML100 and non-expressing CSML0 cells (data not shown). Taken together, these data suggest that a methyl-CpG-binding repressor complex binds elsewhere in the gene, thereby repressing transcriptional activation. Though *S100A4* belongs to a group of CpG-poor genes (~1 CpG per 100 bp), methylation-dependent DNA binding proteins, such as MeCP-2 (49), MBD2 and MBD4 (50), may bind and repress transcription by recruiting the general co-repressor complex mSin3A/HDAC (51,52). Even sparse methylation may repress transcription of transfected genes in transient transfection assays. However, this inhibition does not occur when transcription of a reporter is driven by a strong enhancer (53). The relative weakness of the *S100A4* enhancer in CSML0 cells described here may therefore cause the sensitivity of the gene to full repression mediated by DNA methylation.

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