



Transcriptional regulation of mouse α -selectin

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ABSTRACT

α -selectin mediates the initial tethering and rolling of lymphocytes in high endothelial venules. To study the transcriptional regulation of mouse α -selectin, we cloned 4.5 kb 5'-flanking sequences of the mouse *sell*. Luciferase analysis of serial 5'-deletion mutants showed that the first 285 bp was sufficient to drive high promoter activity in EL4 cells, but not in Sell-negative HeLa cells, suggesting that this fragment harbors the minimal mouse *sell* promoter and contains cis-elements for lymphocyte-specific expression. Site-directed mutagenesis and chromatin immunoprecipitation showed that Mzf1, Klf2, Sp1, Ets1, and Irf1 bind to and activate the mouse *sell* promoter. Over expression of these transcription factors in EL4 cells increased expression of *sell* mRNA. Silencing the expression of Sp1 by siRNA significantly decreased *sell* promoter activity in EL4 cells. We conclude that *sell* transcription is regulated by Mzf1, Klf2, Sp1, Ets1, and Irf1.

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1. Introduction

α -selectin (CD62L) is an adhesion molecule enabling naive and central memory lymphocytes to home to lymph nodes and to transmigrate into tissues at sites of inflammation [1]. Sell is expressed on all myeloid cells, naive T cells, and some activated and memory T cells [2]. Naive T cells express high level of Sell, upon activation Sell is rapidly cleaved from cell surface by membrane metalloproteases, followed by 3- to 4-fold up-regulation over the resting level by 48 h after activation. This increased Sell expression gradually wanes by 2 to 3 days, with a total loss of expression by day 6 to 7 [1]. Effector memory T cells express low level of Sell, whereas central memory T cells re-gain the capacity to express high level of Sell [3]. Aberrant leukocyte recruitment resulting from dysregulated Sell expression has been associated with diseases such as diabetes, ischemia/reperfusion injuries and pulmonary inflammation [4]. Using a dual adoptive transfer approach, Roberts et al. showed that CD8⁺CD62L^{high} central memory T cells had the better ability to recall antigen compared to CD62L^{low} effector memory cells after secondary pathogen challenge [5]. This suggests the involvement of Sell in acquisition and maintenance of memory.

The regulation of Sell expression has been studied extensively at the protein level, but accumulating evidence indicates that expression of Sell is also regulated at the transcriptional level. Cleavage of cell surface Sell after T cell activation accompanied up-regulation of *sell* transcription and the rapid degradation of the corresponding mRNA [6]. Interferon α up-regulated steady state *sell* mRNA levels between 3 and 72 h in IFN α -

sensitive Daudi B cells [7]. Transcriptional regulation of *sell* has also been reported in pathological conditions. Mice exposed to live scabies mites exhibited decreased *sell* mRNA expression in the periarteriolar lymphoid sheath, primary follicle, and marginal zone of the spleen [8].

Studies on the genomic structure of human *sell* suggested [9] that the promoter of human *sell* was at least 10 kb upstream of the translation start codon ATG. However, Tatewaki et al. showed that a reporter construct containing -885 bp of the human *sell* could be transactivated by human T-cell lymphotropic virus type 1 Tax in Jurkat cells [10], suggesting that the promoter region of human *sell* is located immediately upstream of the ATG. Mouse *sell* was cloned in 1989 [11], but there have been no reports addressing the mechanism of its transcriptional regulation. Using a luciferase reporter assay, Bai et al. showed that a construct containing -3.7 kb of the mouse *sell* could be transactivated by Klf2 in HeLa cells [12], suggesting that -3.7 kb contains mouse *sell* promoter. As a first step to understand how *sell* is regulated transcriptionally, we cloned 4.5 kb of the 5'-flanking sequence of the mouse *sell* and characterized the core promoter in lymphocytes.

2. Materials and methods

2.1. Reagents

FBS, RPMI1640, DMEM, and penicillin/streptomycin were from Invitrogen (Carlsbad, CA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) unless specified otherwise. All chemicals were purchased from Sigma (St. Louis, MO). The transcription factor expression plasmids, pBK-CMV-mKlf2-HA was a gift from Dr. Lingrel (University of Cincinnati, Cincinnati, Ohio); pSG5-Ets1 from

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Dr. Watson (Hollings Cancer Center, Charleston, SC); pCB6-Mzf1 from Dr. Sukhatme (University of Chicago, IL); pN3-Sp1 from Dr. Suske (Institute for Molecular Biology, Marburg, Germany); and pRc/RSV-Irf1 from Dr. van den Elsen (LUMC, Leiden, Netherlands). All expression plasmids were then subcloned into pcDNA3.1 to produce pcDNA3.1-Ets1, -Sp1, -Mzf1, -Klf2, and -Irf1 respectively. A BAC clone, RP24-99A3, containing the mouse *sell*, was purchased from CHORI (Oakland, CA). All primers were synthesized by Invitrogen and the sequences are available upon request.

2.2. Cell culture

EL4, Jurkat, and HeLa cells obtained from ATCC (Manassas, VA) were cultured as recommended by vendor.

2.3. 5' Rapid Amplification of cDNA Ends (RACE)

mRNAs were prepared from EL4 cells using Genelute Direct mRNA Miniprep Kit (Sigma). 5' RACE was conducted using SMART™ RACE cDNA Amplification Kit as suggested by the manufacturer (Invitrogen). Briefly, 1 µg of mRNA was used as the start material, 5' RACE PCR was performed by using the universal primer and a mouse *sell*-specific primer complementary to nucleotides from +77 to +105. PCR products were then cloned into pCR2.1 vector (Invitrogen), transformed into DH5α, and 5' ends sequenced from 20 randomly picked transformants.

2.4. Promoter analysis

The BAC clone was used to clone the longest promoter, which contains 4561 bp spanning from the last 47 bp of *sele* to the ATG of *sell*. This fragment was amplified by PCR and cloned into pGL3-Basic, designated as mSell4500. The other 5' serial deletion mutants, which contain about 3500, 1500, 1085, 485, 285, 185, and 105 bp of the 5' sequence of mouse *sell* were amplified by PCR using mSell4500 as template and also cloned into pGL3-Basic and were designated as mSell3500, mSell1500, mSell1085, mSell485, mSell285, mSell185, and mSell105 accordingly. The sequence identity of each construct was confirmed by sequencing (Retrogen). Putative transcription factor binding sites were searched using Genomatix (www.genomatix.de) and TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html).

2.5. Transient transfections and luciferase assay

EL4 cells were seeded 2 h before transfection in 12-well plates containing 1 ml of medium at 8×10^5 cells per well. HeLa cells were seeded at 1×10^5 cells per well in 12-well plates the day before transfection. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 1.5 µl of Lipofectamine 2000 was mixed with 50 µl of serum-free medium and incubated at RT for 5 min, and then 50 µl of serum-free medium containing 20 ng of pRL-CMV expressing renilla luciferase and 1 µg of luciferase reporter constructs, in case of co-transfection with transcription factor-expressing constructs 1 µg of transcription factor expressing constructs or pcDNA3.1 (Vec), was added to the mixture and incubated for another 20 min at RT. The solution was then added to the seeded cells and luciferase activity was measured 36 h after transfection on Autolumate Plus LB 953 (Berthold) using Dual-Luciferase Reporter Assay System (Promega). The luciferase activity was normalized to that of Renilla activity. Data were presented as percentage of pGL3-promoter (a SV40 promoter-driven luciferase) from at least three independent experiments.

2.6. Site-directed mutagenesis

Mutagenesis of the putative transcription factor binding sites in the context of mSell285 was performed using the GeneEditor In vitro

Site-directed Mutagenesis System (Promega). All mutations were designed not to introduce alternative putative transcription factor binding sites. The putative MZF1/KLF2 binding site was mutated GGTGG>AACAA (designated as 285M); the Sp1 binding site GGCG>CATA (285S); the Ets1 binding site GGATG>ACGCA (285E); and the IRF1 binding site AGAAATGAAAG>CACTGTAGCGA (285I). All mutations were confirmed by sequencing (Retrogen).

2.7. Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using the EZ-ChIP Assay Kit (Upstate Cell Signaling Solutions) according to the manufacturer's instructions. Briefly, 2×10^7 EL4 cells were fixed with 1% formaldehyde at RT for 10 min. Following cell lysis DNA was sonicated to shear the genomic DNA to the average sizes of 400 bp on a Branson 450 Sonifier O2. DNA-protein complexes were immunoprecipitated with 3 µg of antibodies against Sp1 (clone PEP2), Ets-1 (clone N-276), Klf2 (Abcam), Irf1 (H-8), and Mzf1 (H-45) respectively, overnight at 4 °C. Normal rabbit IgG and no antibody were always included as controls. The input DNAs were taken from sheared DNAs before immunoprecipitation. After reverse cross-linking and protein digestion DNA was recovered and subjected to 30 cycles of PCR using primers amplifying -285/-58 fragment. The PCR products were resolved on a 1% agarose gel and visualized with ethidium bromide.

2.8. RNA interference of Sp1

EL4 cells were transiently transfected with 0.4 and 0.8 µg of Sp1 siRNA or 0.8 µg of siRNA-A, a non-targeting 20–25 nt siRNA (Santa Cruz) using Lipofectamine 2000 as described. Cells were lysed 36 h after transfection. Ten µg of the lysate was resolved on 10% SDS-PAGE and analyzed by Western blot. Since 0.8 µg of Sp1 siRNA significantly decreased Sp1 expression, EL4 cells were then co-transfected with 0.8 µg of Sp1 siRNA or 0.8 µg of siRNA-A with mSell285 and luciferase activity was analyzed as described.

2.9. Western blot analysis

Cells were lysed in lysis buffer containing 1% (w/v) SDS, 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, and 1× protease inhibitor cocktail. The cell lysates were sonicated, boiled for 5 min, and quantitated using BioRad Protein Assay. Ten µg of each sample was resolved on 10% SDS-PAGE and then transferred to PVDF membrane. The membrane was first probed with anti-Sp1 antibody (clone PEP2). To show equal loading of each sample, the same membrane was stripped and re-probed with mouse anti β-actin (Abcam).

2.10. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from EL4 cells transiently transfected with Ets1, Irf1, Klf2, Mzf1, and Sp1 for 36 h using the RNeasy kit (QIAGEN). One µg total RNA from each sample was then reverse transcribed according to the manufacturer's instructions and the PCR was performed to amplify an 85 bp fragment of *sell* cDNA. The PCR products were resolved on a 2% agarose gel and visualized with ethidium bromide. Mouse GAPDH was used as a loading control.

3. Results and discussion

3.1. Identification of transcription initiation sites

To determine the transcription initiation sites (TIS) of the mouse *sell*, 5'-RACE was performed on mRNA from EL4 cells, a murine lymphoma cell line. The PCR products were cloned into pCR2.1 and sequencing analysis of twenty transformed colonies was performed. The results showed that mouse *sell* has multiple TISs, however one



Fig. 1. Mapping of the transcription initiation sites. The sequence shown is from nucleotide -131 to +3. The translation start codon ATG is shown in italic. The -50 and -100 positions are labeled with a star underneath each. Identified transcription initiation sites indicated by arrows on top, numbers represent the frequency of the given end in twenty sequenced transformants.

major TIS at the position -53 (Adenine of the translation start codon ATG is designated +1) was identified in seven colonies, whereas other TISs appeared only once or twice (Fig. 1).

3.2. Transcriptional analysis of the mouse *sell* 5' regulatory sequence

To test whether the 5'-flanking sequence had promoter activity, mSell4500 and serial 5' to 3' deletion mutants mSell3500, mSell1500, mSell1085, mSellSell, mSell285, mSell185, and mSell105 (Fig. 2A) were transfected into EL4 and HeLa cells. Luciferase activity was analyzed. Of all deletion mutants tested (Fig. 2B), the mSell285 construct had the highest promoter activity, which reached almost a

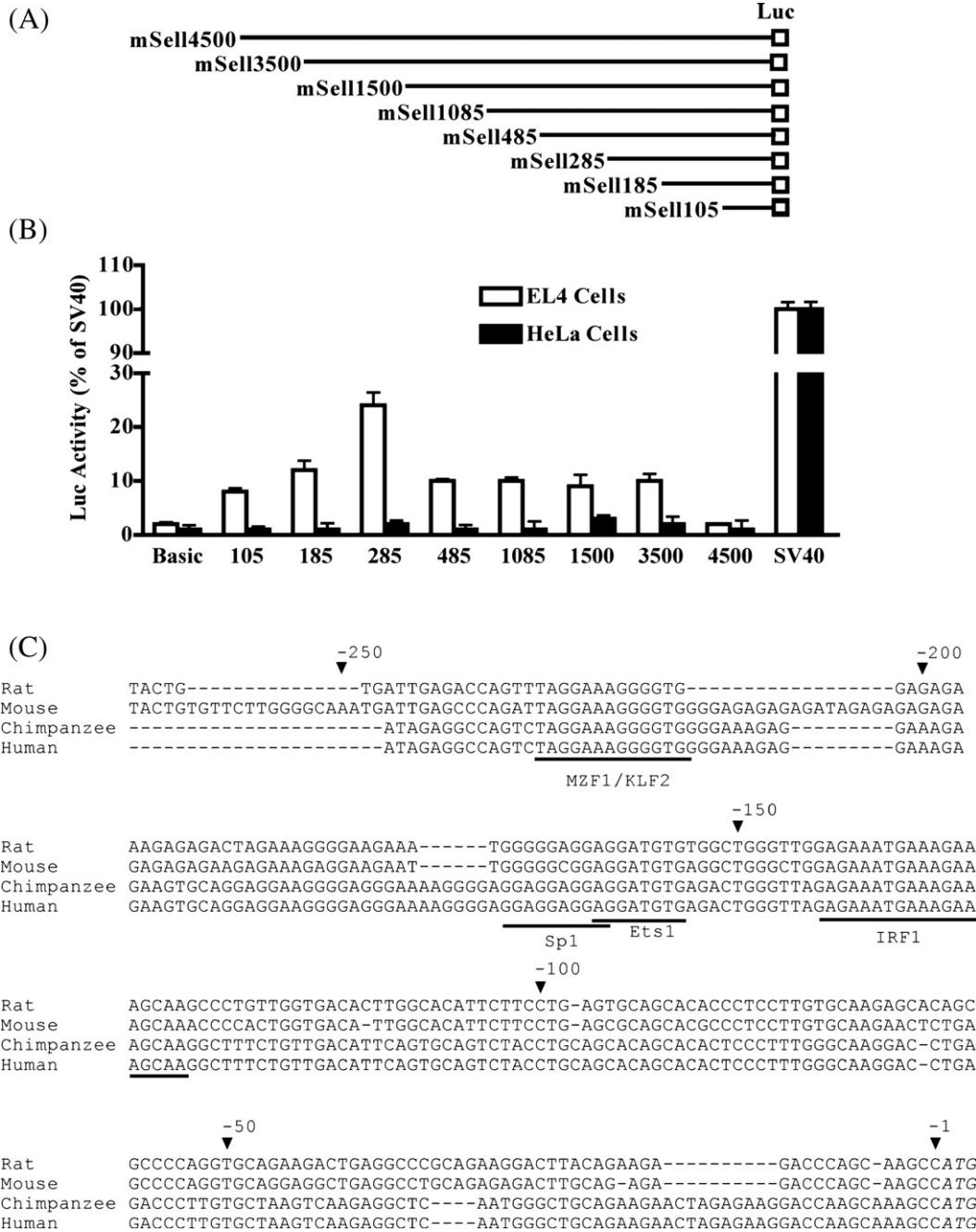


Fig. 2. Promoter activity of the 5' flanking sequence of mouse *sell*. (A) Schematic representation of the serial 5' deletion reporter constructs. (B) Luciferase activity from transiently transfected mouse EL4 (open bars) or HeLa (black bars) cells. Luciferase activity expressed as percentage of pGL3-Promoter (a SV40 promoter driven reporter represented as SV40) transfected EL4 or HeLa cells. Data presented are mean±SD of at least three independent experiments in triplicate. (C) Alignment of 5'-flanking sequences of mouse, rat, chimpanzee, and human *sell*. Annotated sequences of the first ~260 bp of 5'-flanking sequences. The numbers shown on the top of the arrowheads are based on the mouse sequence. Putative transcription factor binding sites are underlined.

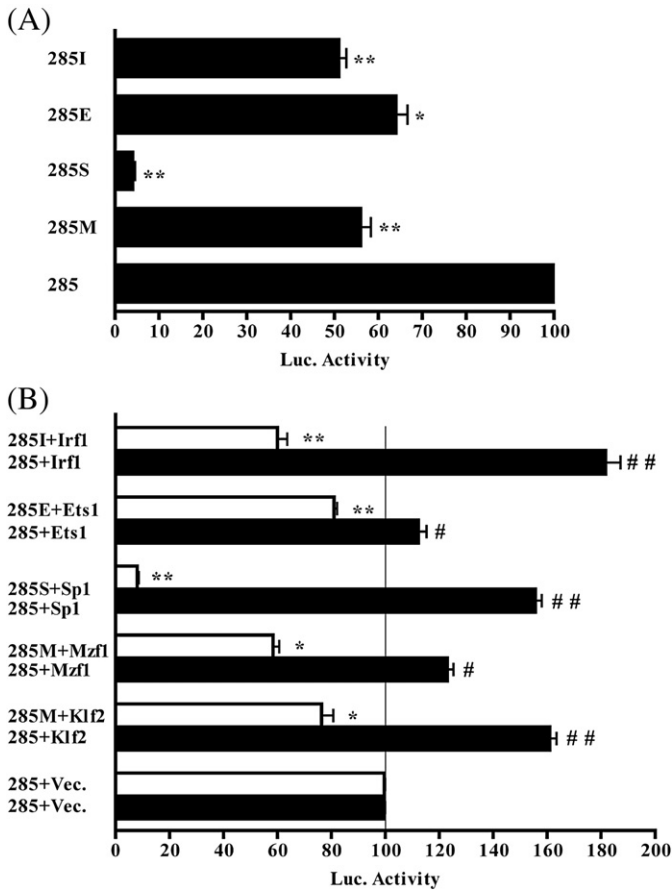


Fig. 3. Ets1, Mzf1, Klf2, Sp1, and Irf1 activate mouse *sell* promoter activity. (A) Luciferase activity from transiently transfected EL4 cells with mSell285 (285), 285E, 285M, 285S, 285I. (B) EL4 cells were co-transfected with mSell285 and pcDNA3.1-Ets1, -Mzf1, -Klf2, -Sp1, and -Irf1, respectively, as shown in black bars or co-transfected with mSell285 or 285E, 285M, 285S, and 285I and its corresponding transcription factor expression plasmids (open bars). pcDNA3.1 (shown as Vec) was used as a control to exclude the promoter effects of the vector backbone. Luciferase activity of wild-type mSell285 alone or cotransfected with Vec was set at 100. Data presented are mean \pm SD of at least three independent experiments in triplicate; mean values are compared by unpaired *t*-test. $P < 0.05$ is considered significant.

quarter of the transcriptional activity of the SV40 promoter-driven control (shown as SV40), in EL4 cells, whereas the same construct in HeLa cells showed only 1% transcriptional activity of the SV40 promoter. Transfection of the deletion mutants into Jurkat cells, an immortalized line of human T lymphocytes similar to murine EL4 cells, produced similar results (data not shown). These results suggest that this 285 bp region harbors the core promoter of *sell* and also contains the tissue-specific cis-elements that contribute to lymphocyte-specific transcriptional activation. The transcriptional activity observed with longer constructs was lower, indicating that repressors exist upstream of -285. Further deletion to -185 and to -105 decreased the promoter activity by 50% and 67%, respectively.

To identify potential transcription factor binding sites, alignment of the 5' sequences of mouse, rat, chimpanzee, and human showed that they are GC-rich and contain several highly conserved regions in the first 268 bp (numbering based on mouse 5' sequence). Using TFSEARCH and Genomatix suite, we identified four highly conserved regions (Fig. 3C), which are labeled as MZF1/KLF2, a composite potential binding site for Mzf1 (Myeloid zinc finger 1) on the sense strand and Klf2 (Kruppel-like factor 2) on the anti-sense strand; Sp1 (Specificity protein 1); Ets1 (E26 transformation specific sequence); and IRF1 (Interferon response factor 1). No TATA-box or CCAAT-box was found.

3.3. Characterization of the core promoter of *sell*

Since the first 285 bp contained the core promoter of *sell*, the functional contribution by each potential binding site in the region was analyzed by mutational analysis combined without or with over-expression of the corresponding transcription factor. Mutation of the potential Sp1 binding site decreased the promoter activity by 96% and mutations of Ets1, MZF1/KLF2, and IRF1 binding sites decreased the promoter activity by 35%, 44% and 49% of that of intact mSell285, respectively (Fig. 3A).

To analyze whether the transcription factors Sp1 Mzf1, Ets1, Klf2, and Irf1, can activate the *sell* promoter, each transcription factor expression plasmid was co-transfected with mSell285 and the transcriptional activity was analyzed. Over-expression of Klf2, Mzf1, Sp1, Ets1, and Irf1 each increased the promoter activity by 59%, 27%, 55%, 11% and 82%, respectively, compared to the mSell285 co-transfected with empty vector, pcDNA3.1 (solid bars in Fig. 3B). In contrast, co-transfection of 285S, 285M, 285E, and 285I with the corresponding transcription factor-expressing plasmids (285M was co-transfected with Mzf1 and Klf2 expression plasmids separately) failed to bring the transcriptional activity up to the level of that of

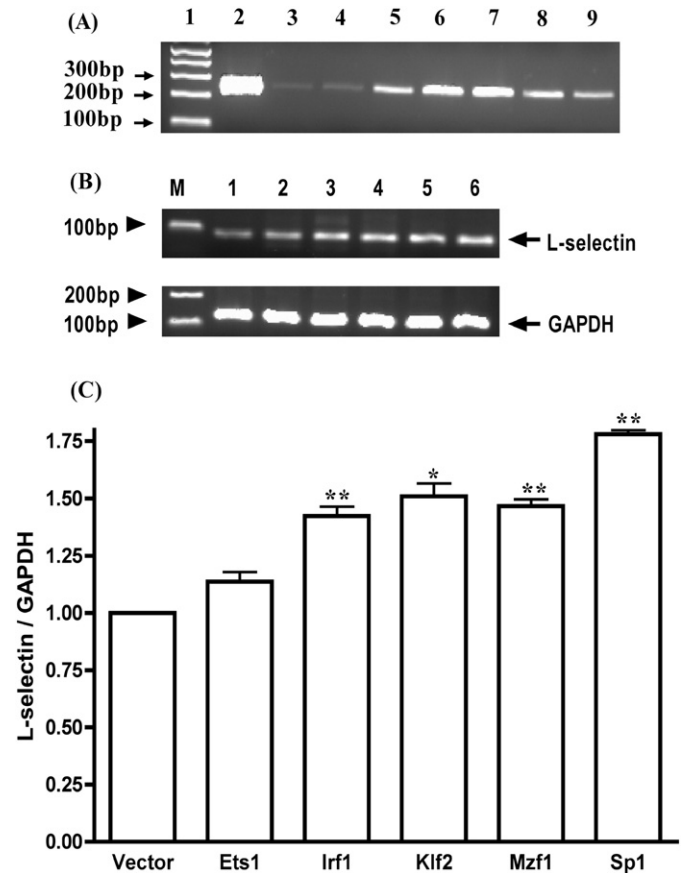


Fig. 4. Ets1, Irf1, Klf2, Mzf1, and Sp1 bind L-selectin promoter in vivo, and upregulate *sell* expression. (A) Representative gel of ChIP analysis showing the in vivo binding of transcription factors to the *sell* promoter. The eluted genomic DNA was amplified by PCR and visualized with ethidium bromide on a 1.5% agarose gel. Lane 1, 1 kb plus ladder; lane 2, 1% input; lane 3, no antibody; lane 4, normal rabbit IgG; lane 5, Ets1 antibody; lane 6, Irf1 antibody; lane 7, Klf2 antibody; lane 8, Mzf1 antibody; and lane 9, Sp1 antibody. (B) Representative gel of RT-PCR showing Ets1, Irf1, Klf2, Mzf1, and Sp1 upregulate *sell* mRNA expression. Lane M, 1.0 kb plus ladder; lane 1, basal level of L-selectin expression, lane 2 to lane 7 are L-selectin expression in EL4 cells transfected with Ets1, Irf1, Klf2, Mzf1, and Sp1 expressing plasmids, respectively. (C) Ratio of *sell* to GAPDH mRNA expression in each group. Data presented are mean \pm SD of at least three independent experiments in triplicate; mean values are compared by unpaired *t*-test. $P < 0.05$ is considered significant.

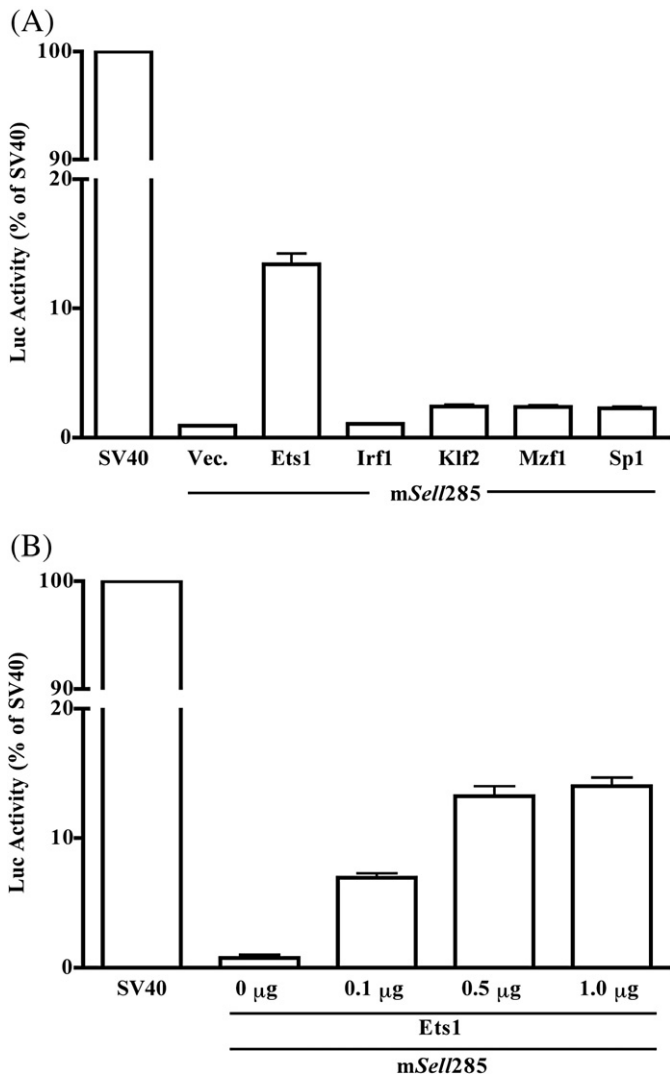


Fig. 5. Ets1 contributes to lymphocyte-specific *sell* promoter activity. (A) Luciferase activity from HeLa cells transiently co-transfected with mSell285 and one of the transcription factor expression plasmids were analyzed and graphed as percentage of SV40. (B) Luciferase activity from HeLa cells transiently co-transfected with mSell285 and an increasing amount of Ets1 expression plasmid shown in the figure. Luciferase activity of SV40 was set at 100. Data presented are mean \pm SD of at least three independent experiments in triplicate.

mSell285 (open bars in Fig. 3B). Taken together, the four potential transcription factor binding sites in the core promoter region are all functional and transcription factors, Ets1, Mzf1, Sp1, Klf2, and Irf1 most likely bind to the core promoter region and activate *sell* transcription in EL4 cells.

3.4. Transcription factors, Ets1, Mzf1, Sp1, Klf2, and Irf1 bind to the *sell* promoter in vivo, and upregulate *sell* mRNA in EL4 cells.

To test whether Ets1, Mzf1, Sp1, Klf2, and Irf1 bind to the promoter of mouse *sell* in EL4 cells, ChIP was performed and followed by PCR amplification of the *sell* core promoter fragment -285/-58. As shown in Fig. 4A, antibodies against Ets1 (lane 5), Irf1 (lane 6), and Sp1 (lane 9), enriched the promoter fragment significantly compared to the no antibody control (lane 3), indicating that Ets1, Irf1, and Sp1 bind to the *sell* promoter in vivo. Antibodies against Klf2 and Mzf1 failed to enrich the *sell* promoter fragments (data not shown) in EL4 cells under normal culture conditions, but enriched the fragment markedly when Klf2 and Mzf1 were over-expressed in EL4 cells (Fig. 4A, lanes 7 and 8).

To test whether Ets1, Irf1, Klf2, Mzf1, and Sp1 could activate *sell*, EL4 cells were transiently transfected with Ets1-, Irf1-, Klf2-, Mzf1-, and Sp1-expressing plasmids, or pcDNA3.1. RT-PCR was performed to analyze the expression of *sell* mRNA 36 h after the transfection. As shown in Fig. 4B, 4C, over-expression of Ets1 increased the expression of *sell* by 8% (lane 3), Irf1 by 36% (lane 4), Klf2 by 44% (lane 5), Mzf1 by 48% (lane 6), and Sp1 by 78% (lane 7), respectively. These results suggest that Ets1, Mzf1, Sp1, Klf2, and Irf1 each increase mouse *sell* mRNA expression.

3.5. Tissue-specific transcriptional activation of *sell* in T lymphocytes by Ets1

The mSell285 showed more than 11-fold more transcriptional activity in EL4 than in HeLa cells, indicating that the 285 bp contained at least some cis-elements through which corresponding endogenous lymphocyte-specific transcription factor(s) bind to and activate lymphocyte-specific transcription. To identify the transcription factor(s) responsible for the lymphocyte-specific transcriptional activation of *sell* promoter, HeLa cells were transiently co-transfected with mSell285 and one of the transcription factor expression plasmids, Ets1, Mzf1, Sp1, Klf2, and Irf1. Compared to vector co-transfected with mSell285, over-expression of Ets1 increased the promoter activity 12.6-fold, whereas over-expression of Mzf1, Klf2, Sp1, and Irf1 showed only 2.4-, 2.4-, 2.3-, and 1.1-fold increases (Fig. 5A). As shown in Fig. 5B, the transactivation by Ets1 displayed a dose-dependent response, suggesting that endogenous Ets1 is limiting for lymphocyte-specific activation of *sell* promoter.

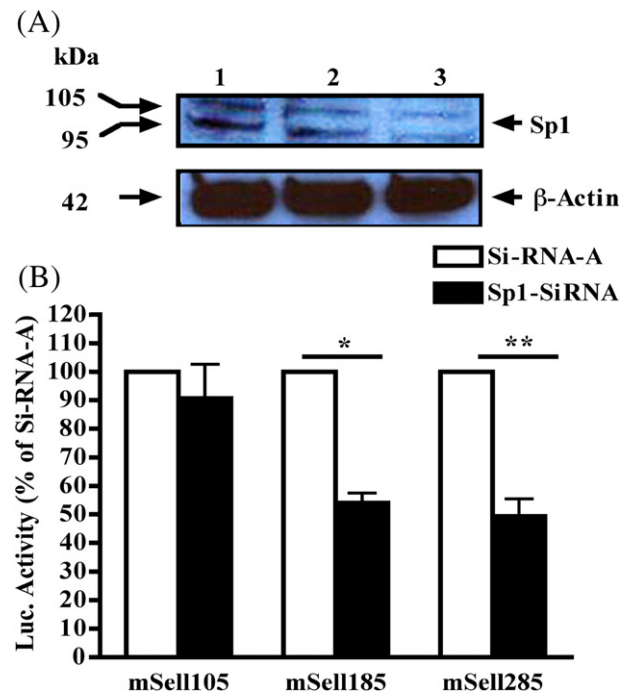


Fig. 6. Silencing of Sp1 down-regulates mouse *sell* promoter activity. (A) EL4 cells were transiently transfected with 0.8 μ g (lane 1) of Si-RNA-A (a non-targeting 20–25 nt siRNA designated as a negative control), or 0.4 or 0.8 μ g of Sp1-SiRNA (lanes 2 and 3) respectively and incubated for 36 h. Ten μ g of cell lysates was resolved on 10% SDS-PAGE, and Sp1 was detected by Western blot. Molecular weight markers indicated with numbers. β -actin was used as loading control (bottom panel). (B) EL4 cells were transiently co-transfected with mSell105, mSell185, and mSell285 and either 0.8 μ g of Sp1-SiRNA or 0.8 μ g of Si-RNA-A respectively. Luciferase activity was analyzed 36 h after co-transfection and the luciferase activity from Si-RNA-A co-transfected group was set at 100. Data presented are mean \pm SD of at least three independent experiments in triplicate; mean values are compared by unpaired *t*-test. $P < 0.05$ is considered significant.

3.6. Silencing the expression of Sp1 reduces *sell* promoter activity

We showed that mutation of the potential Sp1 binding site abolished the *sell* promoter activity, suggesting that Sp1 was essential for *sell* transcriptional regulation. To address the functional role of Sp1 in vivo, small interference RNA (siRNA) was used to silencing the endogenous Sp1 in EL4 cells. The expression level of Sp1 was markedly decreased 36 h after transfecting 0.8 μ g Sp1-siRNA compared to a Si-RNA-A, a non-targeting 20–25 nucleotides SiRNA (Fig. 6A). EL4 cells were then co-transfected with mSell105, mSell185, and mSell285 and Sp1-siRNA or Si-RNA-A. Compared to control Si-RNA-A transfection, silencing Sp1 expression significantly decreased the luciferase activity of mSell185 (46%) and mSell285 (51%) that both contain the Sp1 site. In contrast, silencing Sp1 did not significantly affect the luciferase activity of mSell105 that does not contain the Sp1 site (Fig. 6B). These results suggest that Sp1 plays an important role in transactivating mSell promoter.

4. Discussion

Of the five transcription factors identified in this report, Ets1 is expressed predominantly in lymphocytes, where it has been implicated in regulating transcription of lymphocyte-specific genes [13–17]. T cells from adult mice express high level of Ets1 that exists mainly as an unphosphorylated form that can bind to and transactivate Ets-dependent promoters in the resting state [18]. Upon T cell activation, Ets1 is down regulated at the mRNA level and Ets1 becomes phosphorylated [19]. The phosphorylated Ets1 cannot bind to the targeted promoters and at the same time will be degraded at a much higher rate [18]. This may explain the relatively low L-selectin promoter activity despite the relatively high level expression of Ets1 in EL4 cells. The binding affinity of Ets1 binds to its consensus sequence largely depends on the sequences flanking the core GGAA/T motif and on other transcription factors/cofactors with which it interacts [16, 20]. In the present study, mutation of the potential Ets1 binding site decreased the *sell* promoter activity to 65% of its wild-type counterpart, but over-expression of Ets1 in EL4 cells only increased the promoter activity 11% over that in mSell285. These observations suggest that the basal level of Ets1 expression in EL4 cells is already relatively high, and all the available binding sites for Ets1 may be saturated. Even though forced expression increased the level of Ets1, it did not increase the promoter activity much further. Alternatively the availability of other Ets1 interacting partners in EL4 cells limited the further increase of the L-selectin promoter activity.

The interaction of Ets family transcription factors with Sp1 has been shown to synergistically trans-activate many genes including human CD18 [14], Tenascin-C [21], Fc receptor γ -chain [22], and interleukin-12 p40 [23]. In the mouse *sell* promoter, an Sp1 binding site overlaps with an Ets1 binding site. Over-expression of both Sp1 and Ets1 in EL4 cells increased the mSell 285 promoter activity more than the sum of over-expression of Ets1 and Sp1 respectively, suggesting a synergistic effect of the two transcription factors (data not shown). Mutation of the Sp1 binding site abolished promoter activity (Fig. 3A), likely because this mutation not only disrupted the Sp1 binding site but the sequence flanking the core Ets1 motif, disrupting the binding of Ets-domain containing transcription factors.

Klf2 is highly expressed in naive and memory T cells, and its expression is rapidly down regulated in activated T cells. Based on the similar expression patterns of L-selectin and Klf2, Klf2 has been proposed to play a role in the transition from naive to activated and from activated to memory T cells [24]. In the mouse L-selectin promoter, a highly conserved region at –227 to –217 (AGGGGTGG-GGA) contains a potential Mzf1 binding site on the sense-strand and a Klf2 binding site on the anti-sense strand. Overexpression of Klf2 and Mzf1 in EL4 cells increased the *sell* promoter activity and upregulated *sell* mRNA expression. Mutation of the potential binding site abolished

the transactivating effect of Klf2 and Mzf1. These results suggest that both Klf2 and Mzf1 trans-activate the promoter through binding to the potential MZF1/KLF2 site. Interestingly we did not detect binding of endogenous Klf2 and Mzf1 to the L-selectin promoter by ChIP assay unless they were over-expressed in EL4 cells. These results are in agreement with the expression pattern of Mzf1, a member of the Krüppel family, which is mainly found in myeloid progenitors and precursors [25]. The expression of Klf2 is down-regulated in activated T cells such as EL4 cells [26], and the resulting low-level DNA-protein complex is beyond the detection limit of our ChIP assay.

In this report, we provided evidences that the mouse *sell* promoter lies immediately upstream of translation start codon ATG, which resolved a long-standing ambiguity suggesting that the promoter of the mouse L-selectin gene may reside more than 10 kb away from ATG as reported earlier for the human *sell* gene [9]. We further identified that Sp1, a ubiquitously expressed transcription factor that has been shown to contribute to the precise initiation of TATA-less promoters and to the tissue-specific expression of many genes, plays an essential role in the mouse L-selectin expression.

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References

- [1] C.C. Chao, R. Jensen, M.O. Dailey, Mechanisms of L-selectin regulation by activated T cells, *J. Immunol.* 159 (1997) 1686–1694.
- [2] K. Ley, G.S. Kansas, Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation, *Nat. Rev., Immunol.* 4 (2004) 325–335.
- [3] K. Kedzierska, V. Venturi, K. Field, M.P. Davenport, S.J. Turner, P.C. Doherty, Early establishment of diverse T cell receptor profiles for influenza-specific CD8(+) CD62L(hi) memory T cells, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 9184–9189.
- [4] T.H. Rainer, L-selectin in health and disease, *Resuscitation* 52 (2002) 127–141.
- [5] A.D. Roberts, K.H. Ely, D.L. Woodland, Differential contributions of central and effector memory T cells to recall responses, *J. Exp. Med.* 202 (2005) 123–133.
- [6] L. Mascarel, P. Truffa-Bachi, T lymphocyte activation initiates the degradation of the CD62 L encoding mRNA and increases the transcription of the corresponding gene, *Immunol. Lett.* 94 (2004) 115–122.
- [7] S.S. Evans, R.P. Collea, M.M. Appenheimer, S.O. Gollnick, Interferon-alpha induces the expression of the L-selectin homing receptor in human B lymphoid cells, *J. Cell Biol.* 123 (1993) 1889–1898.
- [8] L.G. Arlian, N. Fall, M.S. Morgan, In vivo evidence that *Sarcoptes scabiei* (Acari: Sarcoptidae) is the source of molecules that modulate splenic gene expression, *J. Med. Entomol.* 44 (2007) 1054–1063.
- [9] D.C. Ord, T.J. Ernst, L.J. Zhou, A. Rambaldi, O. Spertini, J. Griffin, T.F. Tedder, Structure of the gene encoding the human leukocyte adhesion molecule-1 (TQ1, Leu-8) of lymphocytes and neutrophils, *J. Biol. Chem.* 265 (1990) 7760–7767.
- [10] M. Tatewaki, K. Yamaguchi, M. Matsuoka, T. Ishii, M. Miyasaka, S. Mori, K. Takatsuki, T. Watanabe, Constitutive overexpression of the L-selectin gene in fresh leukemic cells of adult T-cell leukemia that can be transactivated by human T-cell lymphotropic virus type 1 Tax, *Blood* 86 (1995) 3109–3117.
- [11] L.A. Lasky, M.S. Singer, T.A. Yednock, D. Dowbenko, C. Fennie, H. Rodriguez, T. Nguyen, S. Stachel, S.D. Rosen, Cloning of a lymphocyte homing receptor reveals a lectin domain, *Cell* 56 (1989) 1045–1055.
- [12] A. Bai, H. Hu, M. Yeung, J. Chen, Kruppel-like factor 2 controls T cell trafficking by activating L-selectin (CD62L) and sphingosine-1-phosphate receptor 1 transcription, *J. Immunol.* 178 (2007) 7632–7639.
- [13] B.R. Szymczyna, C.H. Arrowsmith, DNA binding specificity studies of four ETS proteins support an indirect read-out mechanism of protein-DNA recognition, *J. Biol. Chem.* 275 (2000) 28363–28370.
- [14] A.G. Rosmarin, M. Luo, D.G. Caprio, J. Shang, C.P. Simkevich, Sp1 cooperates with the ets transcription factor, GABP, to activate the CD18 (beta2 leukocyte integrin) promoter, *J. Biol. Chem.* 273 (1998) 13097–13103.
- [15] M. Arman, J. Calvo, M.E. Trojanowska, P.N. Cockerill, M. Santana, M. Lopez-Cabrera, J. Vives, F. Lozano, Transcriptional regulation of human CD5: important role of Ets transcription factors in CD5 expression in T cells, *J. Immunol.* 172 (2004) 7519–7529.
- [16] J.W. Tung, S.S. Kunnavatana, L.A. Herzenberg, The regulation of CD5 expression in murine T cells, *BMC Mol. Biol.* 2 (2001) 5.
- [17] P. Salmon, A. Giovane, B. Wasyluk, D. Klatzmann, Characterization of the human CD4 gene promoter: transcription from the CD4 gene core promoter is tissue-specific and is activated by Ets proteins, *Proc. Natl. Acad. Sci. U. S. A.* 90 (1993) 7739–7743.
- [18] B. Rabault, J. Ghysdael, Calcium-induced phosphorylation of ETS1 inhibits its specific DNA binding activity, *J. Biol. Chem.* 269 (1994) 28143–28151.
- [19] N.K. Bhat, C.B. Thompson, T. Lindsten, C.H. June, S. Fujiwara, S. Koizumi, R.J. Fisher, T.S. Papas, Reciprocal expression of human ETS1 and ETS2 genes during T-cell

- activation: regulatory role for the protooncogene ETS1, *Proc. Natl. Acad. Sci. U. S. A.* 87 (1990) 3723–3727.
- [20] F. Shirasaki, H.A. Makhluf, C. LeRoy, D.K. Watson, M. Trojanowska, Ets transcription factors cooperate with Sp1 to activate the human tenascin-C promoter, *Oncogene* 18 (1999) 7755–7764.
- [21] M. Jinnin, H. Ihn, Y. Asano, K. Yamane, M. Trojanowska, K. Tamaki, Tenascin-C upregulation by transforming growth factor-beta in human dermal fibroblasts involves Smad3, Sp1, and Ets1, *Oncogene* 23 (2004) 1656–1667.
- [22] K. Takahashi, N. Hayashi, T. Shimokawa, N. Umehara, S. Kaminogawa, C. Ra, Cooperative regulation of Fc receptor {gamma}-chain gene expression by multiple transcription factors, including Sp1, GABP, and Elf-1, *J. Biol. Chem.* 283 (2008) 15134–15141.
- [23] H.J. Sun, X. Xu, X.L. Wang, L. Wei, F. Li, J. Lu, B.Q. Huang, Transcription factors Ets2 and Sp1 act synergistically with histone acetyltransferase p300 in activating human interleukin-12 p40 promoter, *Acta Biochim. Biophys. Sin. (Shanghai)* 38 (2006) 194–200.
- [24] R.E. Haaland, W. Yu, A.P. Rice, Identification of LKLF-regulated genes in quiescent CD4+ T lymphocytes, *Mol. Immunol.* 42 (2005) 627–641.
- [25] M. Gaboli, P.A. Kotsi, C. Gurrieri, G. Cattoretti, S. Ronchetti, C. Cordon-Cardo, H.E. Broxmeyer, R. Hromas, P.P. Pandolfi, Mzf1 controls cell proliferation and tumorigenesis, *Genes Dev.* 15 (2001) 1625–1630.
- [26] C.T. Kuo, M.L. Veselits, J.M. Leiden, LKLF: a transcriptional regulator of single-positive T cell quiescence and survival, *Science* 277 (1997) 1986–1990.