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Title: Characterization of the Mouse Myeloid-Associated Differentiation Marker (MYADM) Gene: Promoter Analysis and Protein Localization

Subtitle: MYADM - promoter analysis and protein localization

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Abstract

Hematopoietic differentiation is a complex process involving many genes inducing functional changes and characteristics of different cell lineages. To understand this process, it is important to identify genes involved in lineage commitment and maturation of hematopoietic progenitor cells. Recently we isolated the novel gene MYADM which is strongly up-regulated as multipotent progenitor cells differentiate towards myeloid cells. Because it is not expressed in lymphocytes, understanding the transcriptional control of MYADM could further explain differences in gene expression between myeloid and lymphoid cells. To identify regulatory elements controlling its restricted expression, we have analyzed the 5´-flanking region of the MYADM gene. The proximal promoter was found to lack both TATA and CCAAT boxes, but contained several potential binding sites for both ubiquitous and myeloid-specific transcription factors. Maximal promoter activity was contained within 800 bp from the tentative transcription initiation site, which was reduced as portions of the 5´-end were deleted, and completely abolished when the transcription initiation site was deleted. This promoter sequence had higher activity in myeloid cells compared to B cells, and activity was enhanced during myeloid differentiation, suggesting that we have identified the MYADM core promoter. Computer predictions had suggested MYADM to encode a protein with multiple transmembrane domains. By immunofluorescence and confocal microscopy we demonstrate that the protein is localized to the nuclear envelope and to intracytoplasmic membranes, indicating that MYADM constitutes an integral membrane protein.

Introduction

In the hematopoietic system, stem cells undergo a commitment process to become multipotent progenitor cells, which give rise to all different types of mature blood cells. The molecular mechanisms underlying commitment and differentiation of hematopoietic stem cells remains largely unknown. Hematopoietic commitment is driven by alterered expression of specific combinations of transcription factors, which activate lineage-specific gene programs inducing differentiation towards specific blood cell lineages [1]. The transcriptional regulation of genes is controlled by the binding of sequence-specific DNA-binding proteins to regulatory promoters and enhancers [2]. One of the key issues is to understand how transcriptional activation of genes restricted to specific cell types is accomplished. Tissue-specific regulation of genes is likely to be complex and to involve combinations of transcription factors, not exclusively tissue-specific, acting together to generate a tissue-specific promoter [3].

To identify novel genes involved in differentiation of hematopoietic progenitor cells, we applied differential display analysis during differentiation towards mature granulocytes and monocytes of an IL-3 dependent multipotent progenitor cell line, FDCP-mix [4]. This approach led to the identification of a novel gene that was strongly upregulated during myeloid differentiation, termed MYADM for myeloid-associated differentiation marker gene [5]. Interestingely, MYADM was shown to be expressed exclusively in the myeloid lineage of hematopoietic cells. Subsequently the human MYADM gene has been cloned and shown to be regulated in a similar manner as the mouse gene [6]. The restricted expression pattern of the MYADM gene provides a system to identify regulatory elements and transcription factors controlling the differential activation of genes as progenitor cells commit to the myeloid lineage.

The aim of the present study was to identify cis-acting elements responsible for its myeloid-restricted expression. We report the characterization of a potential promoter to the MYADM gene. By primer extension analysis, we estimated the position of a major transcription initiation site. The promoter region was found to lack both TATA and CCAAT-boxes, a characteristic feature of many myeloid-specific promoters [3], but contained several putative binding sites for both ubiquitous (Sp1) and tissue-specific (MZF-1, C/EBPß, AML1, PU.1) transcription factors.

We also show by immunofluorescent staining of epitope-tagged MYADM that the protein localizes to intracytoplasmic membranes and in particular to the nuclear envelope, further supporting computer predictions that MYADM contains multiple transmembrane domains.

Materials and Methods

Cell lines and cell culture. The FDC-P1 cell line was cultured in IMDM supplemented with 10% fetal calf serum (FCS), 10% IL-3 containing supernatant, 2 mM L-glutamine, and antibiotics. The B lymphoid cell lines 70Z/3 and A20 were cultured in RPMI 1640 supplemented with 10% FCS, 25 mM Hepes, 5 X 10^{-5} M β-mercaptoethanol, and antibiotics. COS cells were maintained in DMEM supplemented with 10% FCS and antibiotics. FDCP-mix cells were maintained in IMDM supplemented with 20% horse serum and 10% IL-3 supernatant. All cell lines were kept at 37°C in a humidified atmosphere containing 5% CO_2. To induce differentiation, cells were washed 3x in PBS and grown for 4 days in 20% FCS, 0.01% IL-3 supernatant, huG-CSF (1,000 U/ml; Genetics Institute), mGM-CSF (20 ng/ml; Peprotech), and huM-CSF (50 ng/ml; Peprotech).

Cloning of the MYADM promoter region. A genomic BAC library from mouse ES-129/SvJ cells was screened by Genome Systems Inc. (St. Louis, MI) with a 995-bp fragment derived from the 5´-end of the MYADM cDNA. High molecular weight inserts from three positive BAC clones, identified by hybridization, were purified on high yield columns (Genome Systems Inc.) and used to identify the adjacent upstream sequence of the full length cDNA by direct sequencing. In parallell, DNA from one BAC clone was used to construct a GenomeWalker library of adaptor-ligated fragments (CLONTECH, Palo Alto, CA). After two rounds of PCR amplifications with gene-specific and adaptor-specific primers, several PCR products were isolated and sequenced. The two procedures together resulted in a DNA fragment of 2.2 kb of which a part was cloned via XhoI (3´-end) and HindIII (5´-end) sites. Searching for putative transcription-factor binding sites was performed by the MatInspector v2.2 program using TRANSFAC 4.0 matrices [7-9].

Primer extension analysis. Primer extension was performed using a 30-mer oligonucelotide complementary to the non-coding sequence 119 bp downstream of the transcriptional start site. The oligonucleotide, end-labeled with $\gamma[^{32}P]$-ATP, was hybridized at 65°C for 90 min with 2 μg of poly(A)$^+$RNA from FDC-P1 cells or 50 μg of total RNA from 70Z/3 cells in a buffer containing 0.15M KCl, 10mM Tris-HCl, pH 8.3, and 1mM EDTA. Annealed primer was extended with Superscript II reverse transcriptase (Invitrogen) at 42°C for 60 min. The reaction product was treated with DNase-free RNase (Invitrogen) at 37°C for 15 min to remove the RNA templates. The cDNA product was phenol-chloroform extracted, ethanol precipitated, and separated on a 9% denaturing acrylamide gel, transferred onto filter paper, dried and exposed to X-ray film. The oligonucleotide used for primer extension was also used to initiate sequencing reactions, using a plasmid containing a genomic DNA fragment as template. Basic dideoxy sequencing was performed using T7 Sequenase version 2.0 DNA Polymerase kit (Amersham Pharmacia Biotech) according to the manufacturer´s protocol.

Northern blot. Fifteen μg of total RNA, isolated utilizing TRIZOL reagent (Invitrogen), was separated by formaldehyde agarose gel electrophoresis, and transferred onto a Hybond-N nylon filter (Amersham Pharmacia Biotech). The filter was hybridized in 50% formamide at 42°C with a 600-bp ApaI-BamHI fragment of MYADM cDNA as probe. As control, the filter was dehybridized and reprobed with a probe specific for mouse 28S rRNA.

Cloning of luciferase constructs and epitope-tagged MYADM. To assay for promoter activity, the 5´-flanking region, spanning from –1372 bp to +99 bp, was cloned via the XhoI and HindIII into the promoterless plasmid pGL3:Basic (Promega, Madison, USA), containing the

luciferase gene as a reporter. Promoter deletion constructs were made by using convenient restriction sites within the promoter region (PstI, EcoRI, SpeI, XbaI, and NheI), as shown in Fig 3, and cloned into the pGL3:Basic vector. As control for the luciferase assay we used CMV-Luc, a plasmid containing the cytomegalovirus promoter driving the luciferase gene.

Luciferase reporter assay. Cells growing exponentially were harvested, washed twice in PBS, and resuspended in medium at room temperature. Ten to twenty million cells in a volume of 0.5 ml were cotransfected by electroporation in a Gene Pulser II (Bio-Rad, Hercules, CA) with 10 μg of luciferase reporter plasmid and 2 μg of CMV-β-galactosidase plasmid. FDC-P1 cells were pulsed at 950 μF, 260 V, FDCP-mix cells at 1050 μF, 280 V, and 70Z/3 and A20 cells at 950μF, 190 V. The transfected cells were transferred to 37°C complete medium and incubated for 24 h. Cell extracts were prepared, and luciferase and β-galactosidase activity was analyzed using a commercial reporter gene assay system (Dual-light; TROPIX, Bedford, MA). Ten μl of each cell extract was used for quantification, and reporter gene activity was measured for 5 s with a 2 s delay in a TD-20/20 luminometer (Turner designs).

Subcellular localization. Epitope-tagged MYADM was generated by PCR introducing a nine amino acid peptide from hemagglutinin (HA) of influenza virus in the N-terminal end of MYADM and cloned into the expression vector pCI-neo (Promega). COS cells were plated on cover slips in 35 mm culture dishes and transiently transfected with 5 μg plasmid using the calcium phosphate precipitate method. Eighteen hr after transfection, cells were fixed with 4% paraformaldehyde in PBS. Blocking and permeabilization was performed with 0.3% TritonX-100 in TBS (10mM Tris-HCl, pH8; 150mM NaCl) and 1% BSA. Cells were stained

with 0.2 μg/ml anti-HA rat antibody (Boehringer Mannheim, Germany) in TBS-1% BSA and washed 3X in TBS, followed by incubation with a secondary fluorescein (FITC)-conjugated goat-anti-rat IgG (Jackson Immuno Research, West Grove, PN), diluted 1:1000. Cells were washed 3 X in TBS, mounted on glass slides and analyzed by fluorescent microscopy or confocal laser scanning microscopy.

Results

Sequence analysis of the 5´-flanking region of the MYADM gene

To isolate the MYADM promoter region, a mouse genomic BAC library from an embryonic stem cell line (ES-129/SvJ) was screened with a 5´-probe of the MYADM cDNA. The screening procedure resulted in three positive BAC clones which were used to identify the adjacent upstream sequence of the full length MYADM cDNA. A 5´-Rapid Amplification of cDNA Ends (RACE) analysis with a commercial mouse brain cDNA library was performed to clone the full length cDNA sequence. The 5´-end of the longest cDNA clone obtained from the 5´-RACE was located 187 bp upstrem the translational start codon (ATG), and was assumed to be the putative site for initiation of transcription. Sequencing upstream the potential promoter in the 5´-flanking region was performed by using an antisense primer complementary to the 5´-end of MYADM cDNA, and one of the BAC clones as template. After several rounds of sequential sequencing, a region of approximately 1.5 kb upstream the hypothetical transcription initiation site was analyzed for the presence of regulatory elements responsible for transcriptional activation of the MYADM gene. Unexpectedly, sequence analysis revealed that this DNA region contains neither a canonical TATA-like element nor a CCAAT motif close to the transcription start site. A computer scan, however, disclosed a series of putative binding sites for known transcription factors that are expressed in myeloid cells and could hypothetically confer the promoter a tissue-specific expression. As shown in Fig. 1, there was several GC-rich regions indicative for potential binding sites for Sp1, of which one was located only 40 bp upstreams of the putative transcription initiation site. The most GC-rich region was from -1350 to -1100, which has a 68% GC content, and contains binding sites for PU.1 and Sp1. In addition, three sites for myeloid zinc finger (MZF)-1 were identified between -380 and -295. This is of interest since MZF-1 is highly expressed in a

restricted manner in differentiated myeloid cells [10]. Further upstreams at position -758 to -734, two sites for AML1 and C/EBPß, respectively, were found. These two transcription factors are known to affect myelopoiesis. Finally, additional sites for GATA-1, NF-KB, and E-box-binding proteins were found.

Identification of the transcription initiation site

To confirm the transcriptional start site identified by the 5´-RACE study we performed a primer extension analysis (Fig. 2), using a 30-mer oligonucleotide positioned 61 bp upstream the hypothetical 5´-end. The primer, complementary to the non-coding strand, was hybridized to poly(A)+RNA from FDC-P1 cells (lane1) or total RNA from 70Z/3 cells (lane 2) as negative control, and was extended by reverse transcription. The primer-extended product was run on a sequencing gel together with sequence reactions using the same primer and a 1-kb genomic DNA fragment encompassing the 5´-end as a template. As determined by sequencing and computer analysis, this fragment consists of 155 nt of exon 1 followed by approximately 850 nt of intron 1 (not shown). Although we were not able to exactly map the transcription initiation site, it was estimated to be positioned within a region 55-60 bp upstream the 5´-end of the cDNA. The G nucleotide in the middle of this region was designated to be the +1 transcription start site (Fig. 2). Multiple bands of weaker intensity indicated the presence of additional start sites, used at lower frequencies.

Functional analysis of the putative MYADM promoter

To determine whether the genomic DNA flanking exon 1 contains a functional promoter, a 1.5 kb fragment was cloned into the promoter-less vector pGL3:Basic with luciferase as a reporter gene, and a series of deletion constructs were generated as shown in Fig. 3B-C. The

luciferase constructs were electroporated into the myeloid cell line FDC-P1 expressing the MYADM gene, and the B lymphoid cell lines 70Z/3 and A20, which do not express MYADM (Fig. 3A). Reporter assays showed a 6-fold increase in luciferase activity in FDC-P1 cells when the whole 1.5 kb fragment was used, compared to empty pGL3:Basic vector. Maximal activity was seen with the shorter (–806/+99) construct giving a 12-fold increase in promoter activity. Interestingly, activity was reduced several-fold when portions of the 5′-end were deleted in constructs (-806/+99) and (-523/+99). The (–835/-71) construct, in which the 3′-end containing the transcription initiation site (+1) was deleted, showed a drop in promoter activity to background levels. These data indicate that the region –71 to + 99, -806 to -524, and -523 to -206 are important for high promoter activity. Surprisingly, the 1.5-kb fragment as well as some of the 5′-deletion constructs showed activity when transfected to the B lymphoid cell lines, although the region between -806 and -206 had significantly higher activity in FDC-P1 cells.

Because the region between -1372 and -806 contains sites for both PU.1 and Sp1, we also generated two constructs with deleted 3′-ends, (–1372/-806) and (–1372/-1166). Both were able to activate transcription of the reporter gene to nearly the same extent as the 1.5-kb fragment despite the lack of the major transcription initiation site. The activity with the (–1372/-806) construct was similar in 70Z/3 as in FDC-P1.

We next decided to compare the promoter activity of the 1.5 kb fragment and the 5′-deletion constructs in undifferentiated versus differentiating FDCP-mix cells. To this end, FDCP-mix cells were induced towards myeloid differentiation in low concentration of IL-3 together with G-CSF, GM-CSF, and M-CSF for 4 days, after which luciferase assays were performed. Overall the levels of luciferase activity was similar in undifferentiated and differentiating cells (not shown). However, the activity of (-806/+99) and (-523/+99) was

higher in differentiating cells (Fig. 4), indicating that the DNA sequence contains part of the MYADM promoter.

Localization of epitope tagged MYADM as an integral membrane protein

To gain more information about the possible function of the MYADM protein, we next decided to determine its subcellular localization. Due to difficulties in generating MYADM-specific antibodies, we epitope-tagged the protein by introducing the nine amino acid hemagglutinin (HA) peptide in the N-terminal end. HA-tagged MYADM was transiently transfected in COS cells and localization was performed by indirect immunofluorescent staining using an anti-HA antibody. The MYADM protein was expressed in a heterogenous pattern with high expression in the nuclear membrane (Fig. 5). No expression was detected in the cytoplasm or within the nucleus. Similar staining pattern was seen in the macrophage cell line P388 and in 3T3 mouse fibroblasts (not shown). To localize the MYADM more precisely confocal microscopy was also performed. Consistently, the protein was found predominantly in the nuclear membrane (Fig. 5b-d), thus establishing the MYADM protein as an integral membrane protein.

Discussion

The genes for both human and mouse MYADM have been described and show high sequence homology and similar genomic structure [5, 6]. In both species the gene is divided into three exons, of which exon 3 contains the complete coding sequence (not shown). In this study, we have identified a putative transcription initiation site for the mouse MYADM gene and analyzed the 5´-flanking region for promoter activity. We were not able to map the precise nucleotide at which transcription is initiated, although the nucleotide at position -273 upstream of the translational start site is likely to initiate transcription. Promoter activity studies showed that a 900-bp sequence (-806/+99) enhances transcription up to 12-fold in transfected FDC-P1 cells (Fig. 3). Step-wise deletion from the 5´-end resulted in decreased promoter activity, indicating the presence of regulatory sequences enhancing transcription. This sequence contains several binding sites for myeloid-associated transcription factors such as MZF-1, AML1, and C/EBPβ. These sites were mainly dispersed at position -380 to -295 containing three sites for MZF-1, and at position -758 to -734 with two sites for AML1 and C/EBPß, respectively. Since this portion also showed higher activity in FDCP-mix cells allowed to differentiate towards granulocytes and monocytes compared to undifferentiated cells, we strongly believe that the sequence (-806/+99) constitutes an important part of the MYADM promoter. However, since this construct also had some activity in lymphoid cells, further analysis are necessary before defining this region as the core promoter.

Deletion of the 3´-end containing the putative initiation site abolished promoter activity, suggesting this region to be crucial for transcriptional activation. The promoter region was found to lack both TATA or CCAAT motifs, but contained several potential binding sites for the ubiquitous transcription factor Sp1. Extensive studies have shown that these features are common for a number of inducible and tissue-specific genes [11-14]. Many myeloid

promoters that direct lineage specific expression generally lack a TATA box or defined initiator sequence [15-19]. Consistently, these promoters often depend on a functional Sp1 site, which can interact with TATA-binding protein, TBP, as well as TBP-associated factor, TAF110, to help mediate recruitment of the basal transcription machinery [20]. Several studies have shown that elimination of Sp1 binding sites destroys promoter activity [21-27], implicating an important function of Sp1 sites in TATA-less promoters. It has been demonstrated that Sp1 can act as an initiator of transcription in genes lacking a TATA-box by directing the preinitiation complex to a region 40-100 bp downstream its binding site [28], and therefore our finding of a consensus Sp1 binding site 40 bp uptream the putative initiation site suggests that it may function as an initiator in the MYADM promoter.

The reason that two of the promoter constructs with deleted 3´-ends (-1372/-1166) and (-1372/-806) showed activity in FDC-P1 despite the lack of transcription initiation site is unclear. However, since both Sp1 and PU.1 have been shown to interact with the TATA-binding protein TBP [20, 29], it is possible that these may direct the assembly of an initiation complex. The fragment (-1372/-1166) showed promoter activity in 70Z/3 cells, suggesting the presence of some regulatory sequences active in both myeloid and lymphoid cells. A plausible explanation for this is that PU.1 is an important transcription factor in both myeloid cells and B lymphocytes [30]. It is also possible that the sequence acts as a promoter by chance.

Since the full-length promoter sequence had similar activity in myeloid and lymphoid cells, additional regulatory elements positioned outside the sequence analyzed could be involved in the myeloid-directed expression. Further analysis is required to complete the descripiton of positive and negative regulatory DNA elements that control MYADM gene

expression, and will include additional cell lines as well as foot printing and conformational analysis of potential binding sites by mutagenesis and electromobility shift assays.

To gain further information on a possible function of MYADM, we epitope-tagged the protein and identified its subcellular localization by immunofluorescence and confocal microscopy. Our intention was first to detect native MYADM protein inside cells, however, we have not been able to generate antibodies neither to the whole protein, nor to peptides corresponding to the N- and C-terminal ends. This is likely explained by the fact that MYADM is an highly hydrophobic protein with multiple transmembrane protein difficult to dissolve for protein studies. Indeed, HA-tagged MYADM appeared mainly as an integral membrane protein in the nuclear membrane and in intracytoplasmic membrane locations. This is in agreement with the reports suggesting the MYADM gene to encode a protein containing multiple putative transmembrane domains [5, 6]. In addition, based on analysis of conserved domain involved in membarne apposition events, it has been proposed that MYADM is a distant member of the MARVEL gene family [31]. These proteins are continously shuttled between the trans-Golgi network, plasma membranes, and endosomes and show a localization pattern similar to the MYADM protein [32-34]. Thus, the localization of MYADM to intracytoplasmic membranes may indicate an involvement of MYADM in the sorting of specific proteins during the maturation of myeloid cells. While the biological function of MYADM remains unknown, the information reported here will provide the basis for further studies into its expression and function.

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Figure legends

Fig. 1. Nucleotide sequence of the 5´-flanking region of the MYADM gene. The tentative transcription initiation site (designated +1), indicated by an *arrow*, was determined by primer extension analysis as shown in Fig. 2. Multiple minor transcription start sites are indicated by *asterisks.* Location of the oligo used for primer extension is *underlined*. Potential binding sites for transcription factors are *boxed*, and *grey colour* indicate that the orientation of the consensus sequence is reversed.

Fig. 2. Primer extension analysis. A [^{32}P]-labelled 30-mer oligonucleotide complementary to the 5´-end of MYADM cDNA was hybridized to either 5 µg of poly(A)$^{+}$RNA from FDC-P1 cells (lane 1) or 50 µg of total RNA from 70Z/3 cells (lane 2) as a negative control. The primer was extended with Superscript II reverse transcriptase and the generated product was separated on a 9% sequencing gel, transferred to filter paper, dried, and exposed to x-ray film. Sequence reactions carried out with the same primer, using a 1-kb genomic fragment cloned into a plasmid as template, were analyzed in the same gel (lanes A, C, G, T). The *arrow* indicates the estimated major transcription initiation site, and the sequence surrounding the start site is indicated on the left. Additional minor transcription initiation sites are indicated by *asterisks*.

Fig. 3. Deletion analysis of MYADM promoter activity. (**A**) MYADM mRNA expression in cell lines FDC-P1, 70Z/3, and A20 used for promoter activity analysis. The probe to 28S rRNA was used as a control. (**B-C**) The indicated luciferase constructs were electroporated into the cell lines FDCP-1, 70Z/3, and A20. Cotransfection with

CMV-β-gal plasmid was used to normalize for transfection efficiency. After a 24 h incubation, cell extracts were assayed for luciferase activity and determined as relative luciferase activity. Activity values are relative to empty pGL3:Basic vector. The data are presented as the mean ± SD for four independent electroporations performed in triplicates.

Fig. 4. Analysis of MYADM promoter activity during myeloid differentiation. FDCP-mix cells were transfected with the promoter constructs as indicated and then incubated in either 10% IL-3 supernatant (day 0) or differentiation cytokines (0.01% IL-3 supernatant, G-CSF (1,000 U/ml), GM-CSF (20 ng/ml), and M-CSF (50 ng/ml) for 4 days (day 4), after which luciferase assays were performed. The data are presented as the mean ± SD for three independent tranfections performed in triplicates.

Fig. 5. Localization of MYADM protein to intracytoplasmic membranes and the nuclear membrane. COS cells were transiently transfected with plasmid containing HA-tagged MYADM. Eighteen hours after transfection, cells were fixed and analyzed by indirect immunofluorescence (a), and confocal microscopy (b-d) after staining with an anti-HA antibody and FITC-conjugated secondary goat-anti-rat IgG. Optical sections taken at 1-μm intervals through a single COS cell (b-d) show a heterogenous staining pattern with a predominant expression at the nuclear membrane. Bars correspond to 10μm.

Figure 1

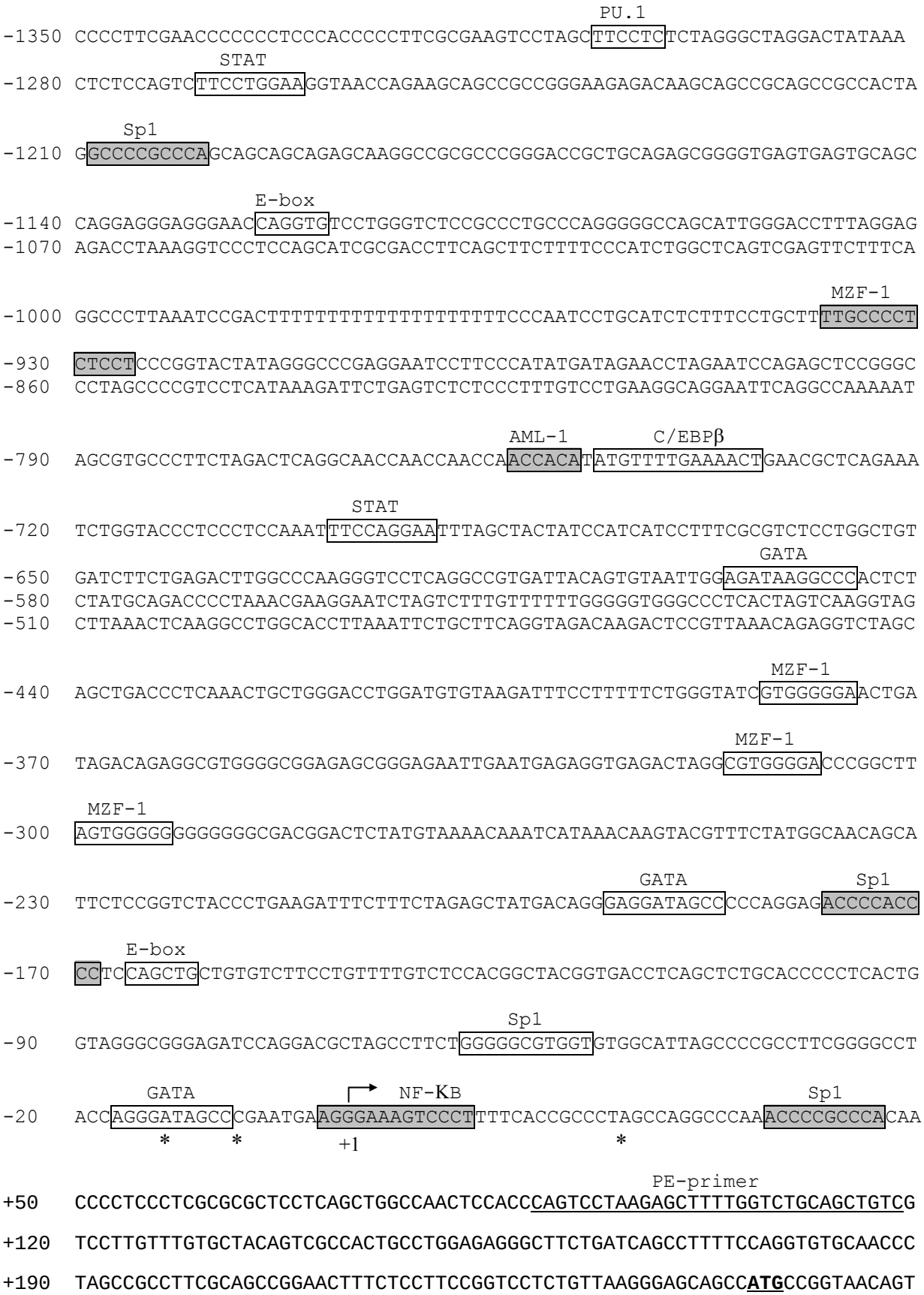
PU.1
-1350 CCCCTTCGAACCCCCCCTCCCACCCCCTTCGCGAAGTCCTAGCTTCCTCTCTAGGGCTAGGACTATAAA
STAT
-1280 CTCTCCAGTCTTCCTGGAAGGTAACCAGAAGCAGCCGCCGGGAAGAGACAAGCAGCCGCAGCCGCCACTA
Sp1
-1210 GGCCCCGCCCAGCAGCAGCAGAGCAAGGCCGCGCCCGGGACCGCTGCAGAGCGGGGTGAGTGAGTGCAGC
E-box
-1140 CAGGAGGGAGGGAACCAGGTGTCCTGGGTCTCCGCCCTGCCCAGGGGGCCAGCATTGGGACCTTTAGGAG
-1070 AGACCTAAAGGTCCCTCCAGCATCGCGACCTTCAGCTTCTTTTCCCATCTGGCTCAGTCGAGTTCTTTCA
MZF-1
-1000 GGCCCTTAAATCCGACTTTTTTTTTTTTTTTTTTTCCCAATCCTGCATCTCTTTCCTGCTTTTGCCCCT
-930 CTCCTCCCGGTACTATAGGGCCCGAGGAATCCTTCCCATATGATAGAACCTAGAATCCAGAGCTCCGGGC
-860 CCTAGCCCCGTCCTCATAAAGATTCTGAGTCTCTCCCTTTGTCCTGAAGGCAGGAATTCAGGCCAAAAAT
AML-1
C/EBPβ
-790 AGCGTGCCCTTCTAGACTCAGGCAACCAACCAACCAACCACACATATGTTTTGAAAACTGAACGCTCAGAAA
STAT
-720 TCTGGTACCCTCCCTCCAAATTTCCAGGAATTTAGCTACTATCCATCATCCTTTCGCGTCTCCTGGCTGT
GATA
-650 GATCTTCTGAGACTTGGCCCAAGGGTCCTCAGGCCGTGATTACAGTGTAATTGGAGATAAGGCCCACTCT
-580 CTATGCAGACCCCTAAACGAAGGAATCTAGTCTTTGTTTTTTGGGGGTGGGCCCTCACTAGTCAAGGTAG
-510 CTTAAACTCAAGGCCTGGCACCTTAAATTCTGCTTCAGGTAGACAAGACTCCGTTAAACAGAGGTCTAGC
MZF-1
-440 AGCTGACCCTCAAACTGCTGGGACCTGGATGTGTAAGATTTCCTTTTTCTGGGTATCGTGGGGGAACTGA
MZF-1
-370 TAGACAGAGGCGTGGGGCGGAGAGCGGGAGAATTGAATGAGAGGTGAGACTAGGCGTGGGGACCCGGCTT
MZF-1
-300 AGTGGGGGGGGGGGGGCGACGGACTCTATGTAAAACAAATCATAAACAAGTACGTTTCTATGGCAACAGCA
GATA
Sp1
-230 TTCTCCGGTCTACCCTGAAGATTTCTTTCTAGAGCTATGACAGGGAGGATAGCCCCCAGGAGACCCCACC
E-box
-170 CCTCCAGCTGCTGTGTCTTCCTGTTTTGTCTCCACGGCTACGGTGACCTCAGCTCTGCACCCCCTCACTG
Sp1
-90 GTAGGGCGGGAGATCCAGGACGCTAGCCTTCTGGGGGCGTGGTGTGGCATTAGCCCCGCCTTCGGGGCCT
GATA
NF-KB
Sp1
-20 ACCAGGGATAGCCCGAATGAAGGGAAAGTCCCTTTTCACCGCCCTAGCCAGGCCCAAACCCCGCCCACAA
* * +1 *
PE-primer
+50 CCCCTCCCTCGCGCGCTCCTCAGCTGGCCAACTCCACCCAGTCCTAAGAGCTTTTGGTCTGCAGCTGTCG
+120 TCCTTGTTTGTGCTACAGTCGCCACTGCCTGGAGAGGGCTTCTGATCAGCCTTTTCCAGGTGTGCAACCC
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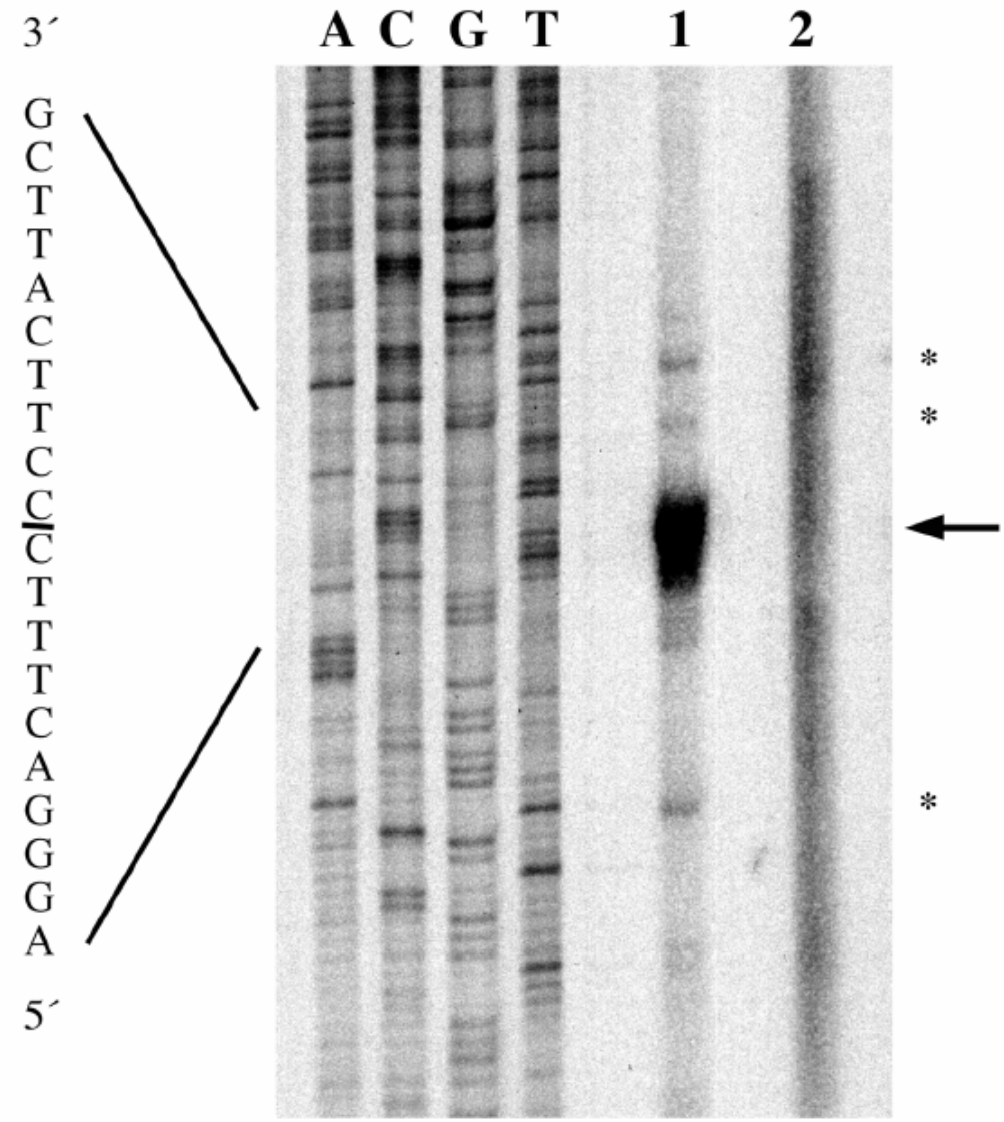
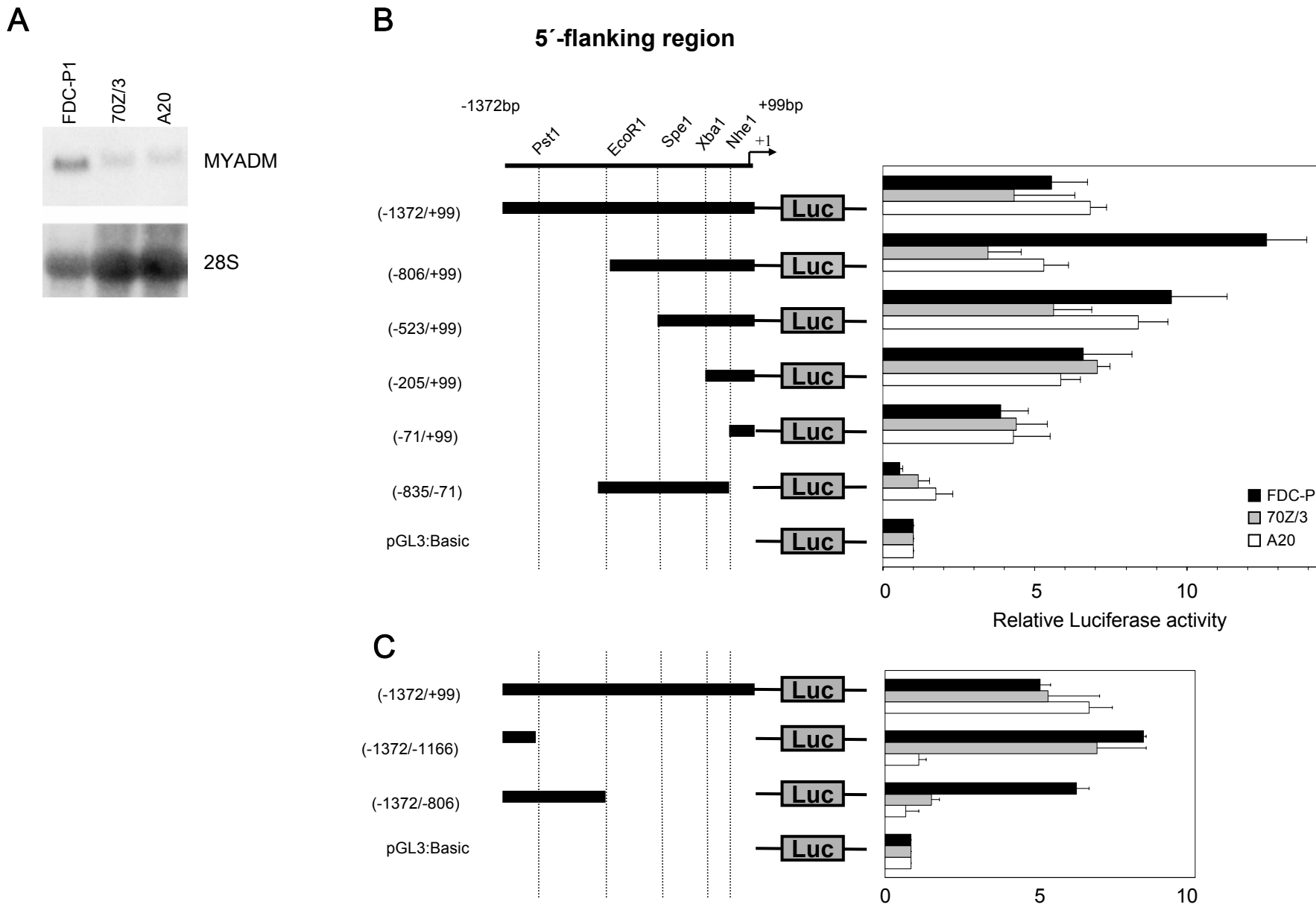
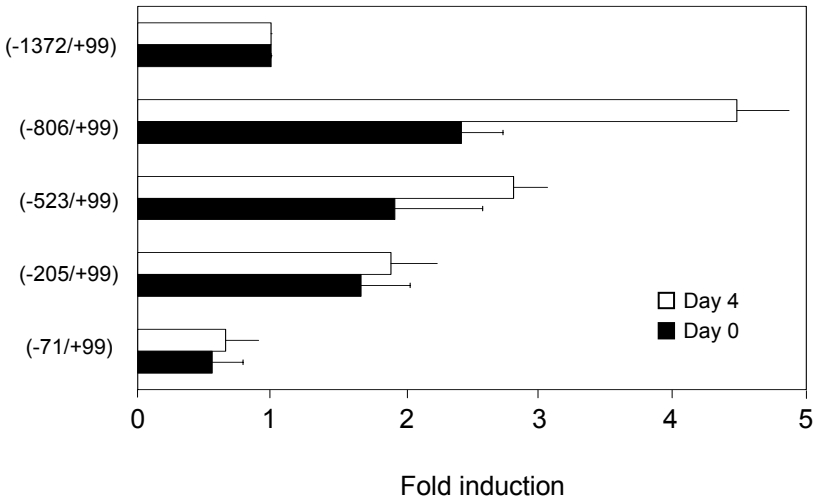
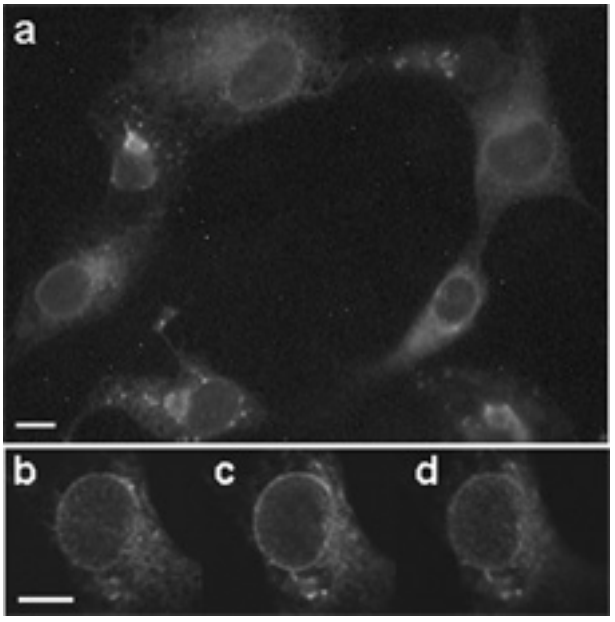
Figure 2




A
FDC-P1
70Z/3
A20
MYADM
28S
B
5´-flanking region
-1372bp
+99bp
Pst1
EcoR1
Spe1
Xba1
Nhe1
+1
(-1372/+99)
(-806/+99)
(-523/+99)
(-205/+99)
(-71/+99)
(-835/-71)
pGL3:Basic
Luc
FDC-P
70Z/3
A20
0
5
10
Relative Luciferase activity
C
(-1372/+99)
(-1372/-1166)
(-1372/-806)
pGL3:Basic
Luc
0
5
10


(-1372/+99)
(-806/+99)
(-523/+99)
(-205/+99)
(-71/+99)
Day 4
Day 0
0
1
2
3
4
5
Fold induction


a
b
c
d