A major isoform of the E3 ubiquitin ligase March-I in antigen presenting cells has regulatory sequences within its gene

Sunil Kaul1#, Sharad K. Mittal12#, and Paul A. Roche1*

1Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland USA

Running title: Expression of March-I in antigen presenting cells

*To whom correspondence should be addressed: Paul A. Roche, National Institutes of Health, Bldg. 10, Room 3N103, Bethesda, MD 20892. Telephone: (301) 594-2595; E-mail: paul.roche@nih.gov

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ABSTRACT

Regulation of major histocompatibility complex class II (MHC-II) expression is important not only to maintain a diverse pool of MHC-II-peptide complexes but also to prevent development of autoimmunity. The membrane-associated RING-CH (March) E3 ubiquitin ligase March-I regulates ubiquitination and turnover of MHC-II-peptide complexes in resting dendritic cells (DCs) and B cells. However, activation of either cell type terminates March-I expression, thereby stabilizing MHC-II-peptide complexes. Despite March-I’s important role in the biology of antigen presenting cells (APCs), how expression of March-I mRNA is regulated remains unknown. We now show that both DCs and B cells possess a distinct isoform of March-I whose expression is regulated by a promoter located within the March-I gene. Using March-I promoter fragments to drive expression of GFP, we also identified a core promoter for expression of March-I in DCs and B cells, but not in fibroblasts, kidney cells, or epithelial cells, and contains regulatory regions that down-regulate March-I expression upon activation of DCs. Curiously, we found downstream sequence elements, present in the first coding exon of March-I in APCs, that confer regulation of March-I expression in activated APCs. In summary, our study identifies regulatory regions of the March-I gene that confer APC-specific expression and activation-induced modulation of March-I expression in DCs and B cells.

Activation of naïve CD4 T cells requires effective antigen processing and presentation by MHC class II-positive (MHC-II+) antigen presenting cells (APCs). APCs, including dendritic cells (DCs) and B cells, internalize potential antigens and degrade them into peptide fragments that bind to MHC-II molecules in endo/lysosomal compartments (1). These peptide-MHC-II (pMHC-II) complexes then move to the APC surface where they can be surveyed by T cell receptors (TCR) expressed on CD4 T cells (2,3). Resting DCs are continually generating new and different pMHC-II complexes as part of their “sentinel” function in the immune system and under steady-state conditions pMHC-II generation and degradation rates are identical (4). Not only must the pMHC-II complexes on the APC surface present the appropriate pMHC-II to the TCR on an antigen-specific T cell, but sustained engagement by pMHC-II with the TCR is necessary for complete T cell activation (5). DC activation suppresses the process of pMHC-II degradation, effectively “fixing” the pMHC-II repertoire on the DC surface and increasing the likelihood of effective pMHC-II-TCR interactions necessary for T cell activation (6,7).

MHC-II turnover in APCs including DCs and B cells is regulated by ubiquitination of MHC-II by the E3 ubiquitin ligase March-I (8). March-I is a transmembrane ubiquitin ligase that preferentially targets internalized pMHC-II for
lysosomal degradation (9). Not only is degradation of "old" pMHC-II important to allow T cells to sample APCs for "new" pMHC-II, but the kinetics of pMHC-II turnover can affect affinity maturation of germinal center B cells (10), negative-selection of CD4 T cells in the thymus (11), and generation of Foxp3-expressing regulatory T cells (11).

March-I is unusual among ubiquitin ligases due to its unique tissue distribution and expression pattern. March-I is expressed in resting DCs and B cells and activation of either cell type by a variety of TLR ligands terminates expression of March-I, reduces pMHC-II ubiquitination, and prolongs the half-life of pMHC-II (12,13). March-I is not expressed in non-hematopoietic APCs including thymic epithelial cells, highlighting lineage-specific regulation of March-I expression in hematopoietic APCs (14,15). Even among APCs March-I expression can be upregulated, as monocytes and macrophages express almost no March-I unless the cells are treated with interferon-γ and IL-10 (16-19). In addition to controlling pMHC-II expression, March-I also regulates the expression of CD86 in APCs (12,20,21), a costimulatory protein that is important for the generation of immunogenic, and not tolerogenic, T cells. However, it is likely that most of the biologically-significant effects of March-I reside in its ability to regulate pMHC-II turnover, as mutant mice expressing non-ubiquitinatable forms of MHC-II (but still containing endogenous March-I and CD86) show phenotypes that are nearly indistinguishable from those of March-I-deficient mice (22).

Despite the well-documented role of March-I in controlling pMHC-II and CD86 expression in APCs (and thereby influencing immunological consequences of pMHC-II/CD86 dysregulation), the mechanisms regulating tissue-specific expression of March-I and TLR-mediated downregulation of March-I mRNA expression have not been addressed. March-I protein has a very short half-life (23), and for this reason it is likely that March-I expression is regulated primarily at the transcriptional level. In this study we have examined the March-I gene, identified the March-I isoform present in APCs, and have identified the regulatory sequences within the March-I gene that confer tissue-specific expression and activation-induced repression of March-I transcription in DCs.

RESULTS AND DISCUSSION

March-I variant 2 is the primary form of March-I found in DCs- March-I was originally identified using a BLAST search of the GenBank database for human RING-CH domain-containing E3 ligases (20). Both the Vega (24) and Ensembl (25) gene annotation system indicate that two variants of human March-I and four variants of mouse March-I exist, however the relative abundance of these March-I variants in professional APCs has not been determined. The organization of the March-I gene as annotated in the Ensembl database is shown in Fig. 1A. The location of E3 ligase RING domain and transmembrane domains 1 and 2 (present in exons 8, 9, and 10, respectively) are indicated and are common to each variant of March-I.

In an attempt to quantitate March-I mRNA expression in DCs and in mouse brain (a tissue in which ESTs for each March-I variant have been identified) we designed PCR primers that selectively amplify March-I variants 1/4, 2, and 3 (Fig. 1B). It should be noted that March-I variants 1 and 3 contain a truncated form of exon 7 and variant 1 and 2 contain a truncated form of exon 9, most likely due to the recognition of internal splice-acceptor sequences in these exons (26). Mouse brain contained transcripts encoding at least 3 March-I isoforms (our exon 5 primer sets cannot distinguish between variant 1 and 4), however spleen DC and bone marrow-derived DC (BM DC) only contained March-I variant 2 (March-I v2) mRNA (Fig. 1C). Quantitative RT-PCR using RING-domain primers common to each variant demonstrated that the absolute amount of March-I in brain was quite low as compared to that present in spleen DCs (Fig. 1D) and that the small amount of March-I mRNA in brain consisted primarily of variants 1/4 and 3 (Fig. 1E). These variants are capable of downregulating expression of MHC-II in transfected cells3, however whether their function is regulation of MHC-II expression in the brain remains to be determined. Unlike the results in brain, RT-PCR revealed that March-I v2 was the only form of March-I detected in DCs and B cells. These data demonstrate that March-I v2 is the primary, if not the only, isoform of March-I present in DCs and B cells. It is important to note that March-I v2 was the isoform originally identified by Bartee et al. (20) and is the variant that has been used in all March-I overexpression studies published to date.
LPS-signaling does not affect March-I v2 mRNA stability- March-I mRNA expression in a variety of APCs is rapidly reduced upon exposure of the cells to the TLR4 ligand LPS (12,13). We explored the possibility that reduced expression of March-I v2 mRNA in DCs exposed to LPS was a consequence of enhanced degradation of pre-existing March-I v2 mRNA. Actinomycin D is an inhibitor of RNA synthesis when used at low concentrations (27), and for this reason actinomycin D-treatment has been used as a method to measure mRNA half-life. DCs were left untreated or pre-treated with LPS for 1 hr before addition of actinomycin D and the amount of March-I v2 mRNA present over time was determined by qRT-PCR. In agreement with previous findings {De Gassart, 2008 #944; Mittal, 2015 #1606}, 1 hr pre-treatment of DCs resulted in a modest but detectable reduction in March-I v2 mRNA present at the time of actinomycin D addition (Fig. 2A). However, when normalizing the amount of March-I v2 mRNA present in each sample to that present at time 0, the rate of March-I v2 mRNA degradation was unaffected by LPS pre-treatment of DCs (Fig. 2B). These data strongly suggest that downregulation of March-I v2 mRNA expression is regulated transcriptionally and not by post-transcriptional degradation of March-I v2 mRNA.

Analysis of the March-I v2 promoter- We performed 5′RACE and performed EST database searches to identify the transcription start site(s) (TSS) of March-I v2. 5′RACE analysis revealed that the 5′ UTR of mouse March-I v2 was extremely short and was located only 8.9 ± 4.2 bp upstream of the translation start site in exon 7 (Table I). Examination of the mouse EST database confirmed our 5′RACE results and confirmed that the 5′ UTR of mouse March-I v2 was less than 20 bp upstream of the translation start site.

Based on our identification of the March-I v2 TSS, we generated March-I v2 promoter constructs containing up to 1 kb upstream of the TSS driving expression of GFP in lentiviral vectors (Fig. 3A). Since regulatory elements are often present in the first coding exon of genes (28), we included the first 125 bp of exon 7 in some of our promoter constructs. As a negative control we used the lentiviral vector containing no promoter elements (while still retaining the GFP open reading frame). Since March-I v2 is only expressed in professional APCs (including DCs) and March-I v2 expression is down-regulated in activated APC lines, bone marrow cells were transduced with lentivirus prior to differentiation of the cells into DCs in vitro by using GM-CSF. Since we were primarily interested in monitoring March-I v2 promoter activity in our studies we simultaneously monitored expression of mRNA for endogenous March-I v2 and March-I v2 promoter-dependent expression of GFP in each sample. Each lentivirus encoded puromycin N-acetyl-transferase (puromycin, whose expression was dependent on the phosphoglycerate kinase promoter), and for that reason transduction efficiency in each condition was normalized to the amount of puromycin mRNA present in the sample. Each of the promoter construct expressed GFP mRNA in DCs well above promoterless control lentivirus levels (Fig. 3B). Sequences in the first 125 bp of exon 7 were not required for basal expression of GFP, as expression of the March-I v2 (-663 to +125) GFP construct was nearly indistinguishable from that of the shorter March-I v2 (-663 to +2) GFP construct. Importantly, deletion of 71 bp upstream of the TSS almost completely prevented March-I v2 promoter activity, highlighting an important role for sequences present within this region for March-I v2 core promoter activity. From these data we conclude that March-I v2 promoter constructs containing as little as 175 bp upstream of the TSS allow March-I v2 expression in DCs and that sequences present in exon 7 are not required for basal March-I v2 promoter activity.

TLR-signaling regulates March-I v2 promoter activity- Activation of APCs leads to downregulation of March-I expression (12,13,29) and we have shown that after as little as 2 hr of stimulation with LPS March-I mRNA is reduced by approximately 50% (19). To directly examine whether activation of DCs affects the function of our March-I v2 promoter GFP reporter constructs, we stimulated immature DCs transduced with March-I v2 promoter-GFP lentivirus with the TLR-4 ligand LPS or the TLR-9 ligand CpG for 2 hr prior to isolating mRNA and measuring expression of GFP in our March-I v2 promoter-GFP constructs. Both LPS and CpG downregulated expression of the March-I v2 (-663 to +125), (-385 to +125), and (-175 to +125) promoter constructs (Fig. 4A), demonstrating that these promoter constructs...
contained sequences that respond to DC activation signals. Curiously, GFP expression was not altered by LPS- or CpG-treatment of cells expressing the March-I v2 (-663 to +2) promoter construct. Monitoring endogenous March-I v2 expression in these transduced cells confirmed that the cells responded to these activation stimuli like non-transduced cells (Fig. 4B). These data reveal a role for sequences present in March-I v2 exon 7 in the response of the March-I v2 promoter to DC activation by LPS or CpG.

The March-I v2 promoter is only active in APCs-March-I v2 is expressed primarily in APCs (14,15), so we set out to determine if the March-I v2 (-663 to +125) promoter construct we identified resulted in cell type-specific expression of our GFP reporter. Whereas we could reliably detect CMV-promoter driven expression of GFP in epithelial cells, kidney cells, and fibroblasts, we were unable to detect any activity of the March-I v2 promoter construct in these cells types above background levels (Fig. 5). By contrast, both the CMV and March-I v2 promoter constructs resulted in expression of March-I v2 mRNA in DCs, although the March-I v2 promoter was significantly weaker than the CMV promoter. The March-I v2 promoter construct was also expressed well in spleen B cells. (Human CMV promoter activity in lentivirus is weak in mouse B cells (30) and for this reason March-I v2 promoter activity seems high as compared to CMV promoter activity.) Although it is not possible to examine March-I v2 promoter activity in every cell type in the mouse, these data strongly suggest that the March-I v2 promoter identified in this study contains the DNA elements necessary for cell-specific expression of March-I v2 in APCs.

In this report we show that of the isoforms of March-I annotated in Vega and Ensembl databases, only March-I v2 is expressed in DCs and B cells. Whereas other March-I variants are expressed in brain, RT-PCR demonstrated that the overall expression of March-I in brain is quite low as compared to spleen DCs. Having said that, the amount of March-I v2 mRNA expressed in spleen DCs is also quite small, as we have quantitated the amount of March-I v2 mRNA present in resting spleen DCs (using plasmid DNA as a standard) and found that in three independent spleen DC samples there is approximately 1 molecule of March-I v2 mRNA per cell4. Such a finding is consistent with the nearly undetectable expression of endogenous March-I protein in APCs reported by others (8,12,23).

Since March-I v2 cannot, by definition, be the product of alternative splicing, we reasoned that nucleotide sequences regulating expression of March-I in APCs would be present upstream of the variant 2 TSS. Within only a few hundred bp of the TSS were intragenic promoter sequences that could drive expression of a GFP reporter in DCs but not in kidney cells, epithelial cells, or fibroblasts, suggesting that this region of the March-I v2 promoter contained the sequences necessary for cell type-specific expression of March-I.

DC activation by a variety of TLR ligands leads to down-regulation of March-I expression (12,13). Analysis of March-I v2 mRNA half-life in DCs demonstrated that DC activation did not destabilize existing March-I v2 mRNA, suggesting there are regulatory sequences present in the March-I v2 promoter that directly regulate March-I v2 mRNA expression. Like endogenous March-I v2, expression of March-I v2 promoter/GFP reporter constructs were also downregulated in LPS and CpG-activated DCs. The sequences responsible for downregulation of March-I v2 promoter activity were present in March-I exon 7. Regulatory regions are often present in exons of genes (28) and exon 7 contains numerous DNA elements, including downstream control elements and downstream promoter elements (28) that could be responsible for the regulation of March-I v2 expression in APCs. Future studies using mutant mice lacking these regulatory regions could reveal the importance of the terminating March-I v2 expression in APCs and how dysregulation of March-I v2 expression affects immunity.

EXPERIMENTAL PROCEDURES

Cell isolation and culture-C57BL/6 mice were bred and maintained in-house at the NCI-Frederick animal facility. All mice were cared for in accordance with National Institutes of Health guidelines and approved by the National Cancer Institute Animal Care and Use Committee. HEK-293 cells, HeLa cells and mouse embryonic fibroblasts (MEFs) were cultured in DMEM containing 10% fetal bovine serum, and 10 mM Hepes, pH 7.4. Cells were sub-cultured every 2nd or 3rd day and maintained at sub-confluent levels. DCs
and B cells from mouse spleens were purified by positive selection or negative selection, respectively, using Miltenyi Biotec Kits. BM DCs were generated by differentiating mouse bone marrow cells in BM DC medium (RPMI containing 10% fetal bovine serum, 50 µM β-mercaptoethanol, 10 mM Heps, pH 7.4, 25 µg/ml gentamycin containing 20 ng/ml GM-CSF) as described previously (29).

**Lentiviral promoter constructs**- The various DNA fragments spanning the putative March-I v2 promoter were cloned into the promoterless lentiviral plasmid pLV-unsGFP-PGK-Puro (Cellomics). This plasmid encodes destabilized GFP (whose expression is regulated by an experimentally-determined promoter) as well as puromycin (whose expression is regulated by the phosphoglycerate kinase promoter). The bp numbering for each promoter construct is shown relative to the March-I v2 transcription start site identified by 5’ RACE (as described in this report). LV-102 [-1064 to +125], LV-115 [-663 to +125], LV-309 [-317 to +125] and LV-314 [-175 to +125] were constructed first by generating the PCR fragments using C57BL/6 mouse genomic DNA as template and different forward primers, BIG-S-F, SM-S-F, SMFS-400 and SMFS-300 respectively and the common reverse primer P4SR-R. The antisense construct LV-123 [+125 to -663] was constructed in a similar fashion by amplifying the DNA fragment using the forward primer SM-AS-F and the reverse primer P4AS-R. The resultant PCR fragment was digested with BamHI and XhoI and cloned into pLV-unsGFP-PGK-Puro. LV-26 [-385 to +125] and LV-27 [-125 to -385] were constructed by first PCR amplifying a 758 bp fragment using PR4-F3 and PR4-R1 as primers and mouse genomic DNA as template, and then cloning the PCR product in TA cloning vector pCR4-TOPO (Invitrogen). This plasmid was digested with EcoRI and a 513 bp EcoRI fragment from this digestion was cloned into pLV-unsGFP-PGK-Puro. LV-306 [-663 to -71] and LV-665 [-663 to +7] were constructed using the common forward primer SM-S-F and reverse primers, P4SR-120 or P4SR-ATG respectively. The resultant PCR fragments were digested with XhoI and BamHI and cloned in these sites in pLV-unsGFP-PGK-Puro. The nucleotide sequence of all March-I promoter lentiviral constructs was confirmed by DNA sequence analysis.

**Promoter construct primer sequences**- BIG-S-F: gagagacctgacagccacattttggacacaggctggttgag SM-S-F: gagagacctgagcccagcataatgtaagttgtgag SMSF-400: gagagacctgacaggggctttgtgcctcaactaagttg SMSF-300: gagagacctgacagccacaaattgatagttccac P4S-R: gagagagagatcagacagttggaatccttg SM-AS-F: gagagaggatcctgacacagatactcagaatagtgag P4AS-R: gagagagagttagataatctctctggtggttgac PR1-F3: ggttaaccttctctc P4R4-R1: caagtttagataatcttgcctac P4SR-120: gagagagagatcctgacagttggtggttgac P4SR-ATG: gagagagatctctgacagatcgagctc gagcggtg 

**RT-PCR primer sequences**- The oligonucleotides used for measuring relative transcript levels used in this study were: GFP (5’-caagagccacagaagctatcat-3’ and 5’-aattggtggaggtttcaagag-3’), puromycin (5’-gagagggcagtcctaatct-3’ and 5’-aattggtggaggtttcaagag-3’). The oligonucleotides used selectively in the RT-PCR assays for March-I variants were: variant 1/4 (5’-aattggtggaggtttcaagag-3’ and 5’-aattggtggaggtttcaagag-3’), variant 2 (5’-aattggtggaggtttcaagag-3’ and 5’-aattggtggaggtttcaagag-3’), and variant 3 (5’-aattggtggaggtttcaagag-3’ and 5’-aattggtggaggtttcaagag-3’).

**Generation of lentiviral stocks**- Lentiviral stocks were generated by transfecting sub-confluent HEK-293T cells with lentiviral promoter plasmid, EZ-LentiPack Packaging plasmid, and EZ-transfix reagent as described in the EZ-LentiPACK Packaging System Kit (Cellomics Technology). Lentiviral supernatants were harvested 48 hr and 72 hr after transfection. Viral supernatants were centrifuged briefly (500xg x 10 min) to remove cellular debris. The approximate titer of viral supernatants was quantitated using Lentivirus Titration XpressCards (Cellomics Technology).

**Lentiviral transduction**- Lentivirus (10-100 µl of HEK-293 viral supernatant) were added to bone marrow cultures on day 2 of culture in BM DC media containing 10 µg/ml protamine sulfate (Sigma). BM DC medium was changed every other day. Day 7 BM DC cultures were cultured in
presence or absence of 1 µg/ml LPS (Sigma), 2 µg/ml CpG-1668 (Invivogen) for 2 hr. BM DCs were then washed, harvested at 4°C, and the cell pellet was immediately resuspended in TRIzol (Ambion) and stored at -80°C.

Primary spleen B cells were isolated by negative selection using Miltenyi Biotec Kits and transduced with lentivirus by spinoculation. Lentivirus (250 µl of HEK-293 viral supernatant) were added to 8 x 10^6 B cells in 2 ml RPMI-1640 media containing 10% fetal bovine serum, non-essential amino acids, the B cell activating factor BAFF (10 ng/ml), 10 mM Hepes, pH 7.4 and 10 µg/ml polybrene (Sigma) in a tissue culture plate. After 15 min incubation at room temperature, the cultures were subjected to centrifugation at 1000g for 90 min and then placed in a 37°C 5% CO₂ incubator. After 2 days cells were harvested at 4°C, washed, and the cell pellet was immediately resuspended in TRIzol (Ambion) and stored at -80°C.

Adherent MEFs, HEK-293 cells, and HeLa cells were cultured in RPMI containing 10% fetal bovine serum, and 10 mM Hepes, pH 7.4. Cells were transduced using (10-100 µl of HEK-293 viral supernatant) in the above medium containing 10 µg/ml protamine sulfate, the medium was changed after 1 day. The next day cells were harvested at 4°C, washed, and the cell pellet was immediately resuspended in TRIzol (Ambion) and stored at -80°C.

RNA Isolation, cDNA Synthesis and RT-PCR- Total RNA was isolated from the TRIzol suspensions according to manufacturer’s instructions. cDNA was synthesized using 1-2 µg of total RNA and Superscript III First-Strand Synthesis Kit (Invitrogen). RT-PCR was performed using a QuantStudio6 Flex sequence detection system (Applied Biosystems) and the Quantitect SYBR Green PCR kit (Qiagen).

Quantitation of March-I v2 promoter activity- In experiments monitoring expression of March-I v2 promoter activity in lentivirus transduced BM DCs, RT-PCR was used to quantify the amount of GFP and puromycin mRNA present in the sample. Ct values for GFP and puromycin were determined and data are shown as 2^ΔCt (ΔCt= Ct_{puromycin}−Ct_{GFP}). 2^ΔCt for the promoterless (blank) vector control was arbitrarily set at 1.0 and all values expressed relative to the blank vector control.

In experiments monitoring regulation of March-I v2 promoter activity in transduced BM DC treated with either PBS, LPS, or CpG, RT-PCR was used to quantitate the amount of GFP and GAPDH mRNA present in the sample. Ct values for GFP and GAPDH were determined and data are shown as 2^ΔCt (ΔCt= Ct_{GAPDH}−Ct_{GFP}). In experiments using the March-I v2 (-663 to +125) and (-663 to +2) sense constructs the control virus was a March-I v2 (+125 to -663) antisense construct and in experiments using the March-I v2 (-385 to +125) and (-175 to +125) sense constructs the control virus was a March-I v2 (+125 to -385) antisense construct. The data shown are 2^ΔCt for the sense construct/2^ΔCt for the antisense construct. The amount of GFP mRNA present after treatment of each sample with LPS or CpG was expressed as a fraction of that present in the PBS-treated control sample.

Bioinformatics- Human and mouse March-I variant annotations are described in Ensembl Genome Browser 90 (http://ensembl.org) and Vega Genome Browser Release 68 (http://vega.archive.ensembl.org). The entire 10 exon mouse March-I gene is located on chromosome 8 between bp 65,618,086 and 66,471,637 (Vega Genome Browser numbering). The Vega Genome Browser transcript identifiers for mouse March-I are variant 1 (OTTMUST0000040256), variant 2 (OTTMUST0000040267), variant 3 (OTTMUST0000040269), and variant 4 (OTTMUST0000040271). The Vega Genome Browser transcript identifiers for human March-I are variant 1 (ENST00000503008) and variant 2 (ENST00000339875). The mouse EST database in GenBank (NCBI) was screened using a Basic Local Alignment Search Tool (BLAST) search of nucleotide sequences present in mouse March-I exon 7 that are not present in the 5’truncated forms of exon 7 present in March-I variants 1, 3, and 4.

Statistical Analyses- Results were analysed using two-tailed Student’s t-tests. P values <0.05 were considered statistically significant. P values are indicated by *p<0.05 and non-significant differences are indicated by ns.
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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: SK designed and performed experiments, analyzed results, and wrote the paper. SM designed and performed experiments, analyzed results, and wrote the paper. PAR designed experiments, analyzed data, and wrote the paper.
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FOOTNOTES
This work was supported the Intramural Research Program of the National Institutes of Health. 2Present address: Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts USA; 3S.M, unpublished observation; 4S.K., unpublished observation.

The abbreviations used are: APC, antigen presenting cell; DC, dendritic cell; March, membrane-associated RING-CH E3 ubiquitin ligase; March-I v2, March-I variant 2; major histocompatibility complex class II, MHC-II; MEF, mouse embryonic fibroblast; puromycin, puromycin N-acetyltransferase; TCR, T cell antigen receptor; TSS, transcription start site

LEGENDS

Table 1. March-I v2 has a very short 5' UTR.
A. 5'RACE was performed using mRNA isolated from mouse spleen DCs. The length of the 5'UTR (relative to the initiation codon) for 9 independent clones was identified by DNA sequence analysis. B. Twelve different March-I ESTs were aligned to the March-I v2 coding sequence and the length of the 5'UTR (relative to the initiation codon) was determined. The Genbank ID for each EST is indicated. The average (± SD) for each set of analyses is shown.

Figure 1. March-I v2 is the predominant March-I variant expressed in DCs. A. Schematic representation of the March-I gene as described in UCSC Genome Browser. The positions of each exon, their relative size, and their location within the March-I gene is indicated. The location of the translation stop codon in exon X is indicated by an asterisk. B. The composition of March-I variant 1, variant 2, variant 3, and variant 4 (as described in Ensembl) are indicated. The position of the E3 ligase RING domain, transmembrane domain 1(TM1), and transmembrane domain 2 (TM2) are indicated. The relative position of the translation initiation codon in each variant is indicated by an arrow and the common translation stop codon in exon X is indicated by an asterisk. The locations of specific PCR primers that specifically amplify March-I fragments specific to variant 1/4, variant 2, and variant 3 are indicated. C-E. PCR primers specific for March-I variant 1/4, variant 2, or variant 3 were used to amplify mRNA from spleen DCs, BM DCs, and brain. C. Representative gel showing the presence of March-I variant 2 in all tissues and only small amounts of variants 1/4 and variant 3 in the brain. Forty cycles of PCR amplification for each primer pair was performed and aliquots of the PCR reaction were analyzed on an agarose gel. D. Quantitation of the amount of total March-I mRNA (using a primer set common to all March-I variants) was performed by RT-PCR and data were normalized to expression of GAPDH in each sample. Data are shown as the 2^(-ΔCt) (ΔCt= CtGAPDH - CtMarch-I) value for each condition. The results shown are the average (± SD) of three independent experiments. E. The relative amount of each March-I isoform present in spleen DC, BM DC, spleen B cell, and brain was determined by RT-PCR and the 2^(-ΔCt) (ΔCt= CtGAPDH - CtMarch-I) value for each sample was determined. The amount of mRNA for the most abundant March-I variant present in spleen DC, BM DC, B cell, and brain was arbitrarily designated a value of 1. The results shown are the average (± SD) of three independent experiments. ND=not detectable.

Figure 2. DC activation does not promote March-I v2 mRNA degradation.
DCs were pre-treated (or not) with LPS for 1 hr, washed, and then incubated in complete medium containing actinomycin D (5 μg/ml) for the indicated time. Cells were harvested, mRNA was isolated, and March-I v2 expression was determined by RT-PCR. The data from each condition were normalized to the expression of GAPDH at each time point. A. March-I v2 mRNA expression in each condition was plotted as a function of time in actinomycin. B. March-I v2 mRNA expression in either untreated or LPS-treated DCs at each time point was normalized to March-I v2 expression at t=0 (time of addition of actinomycin D). The results shown are the average (± SD) of three independent experiments.
Figure 3. Identification of an internal March-I v2 promoter in DCs.
A. The transcription start site of March-I v2 is indicated by an arrow, and promoter constructs consisting of DNA sequences 5’ of the TSS with (or without) 125 bp of exon 7 fused to GFP were generated. B. DCs were transduced with lentivirus encoding the indicated promoter construct and expression of GFP mRNA and lentivirus-encoded puromycin were determined by RT-PCR. The “blank” viral construct encoded GFP but did not contain any March-I promoter sequences. GFP mRNA expression was normalized to the amount of puromycin mRNA present in the sample (as described in Experimental Procedures) and expressed relative to the amount of mRNA detected in the promoterless blank construct.
* p<0.05.

Figure 4. The LPS- and CpG-responsive element in the March-I v2 promoter resides in exon 7.
DCs were left untreated or were transduced with lentivirus encoding the indicated promoter construct and incubated in the presence of PBS (as a control), LPS, or CpG for 2 hr. Cells were harvested expression of endogenous March-I v2 or GFP mRNA and GAPDH were determined by RT-PCR. A. GFP mRNA expression (panel A) or endogenous March-I v2 expression (panel B) in each sample was normalized to the amount of GAPDH mRNA present in the sample and expressed relative to the amount of mRNA detected in the PBS-treated control cells for each lentiviral construct as described in Experimental Procedures. March-I RING domain primers were used to detect endogenous March-I. *, p<0.05; ns=not significant.

Figure 5. The March-I v2 promoter is inactive in non-APCs.
Epithelial (HeLa) cells, kidney (HEK-293) cells, mouse embryonic fibroblasts, and BM DCs were transduced with lentivirus encoding GFP using the CMV promoter, the March-I v2 (-663 to +125) “forward” promoter, or a negative “control promoter”. Expression of GFP, puromycin, and GAPDH mRNA was determined by RT-PCR. Data using HEK-293 cells, HeLa cells, BM DC, and spleen B cells were normalized to the amount of puromycin mRNA present in the sample and the control was the promoterless lentivirus. Data using MEFs were normalized to the amount of GAPDH mRNA present in the sample and the control was lentivirus using the March-I v2 (-663 to +125) promoter inserted into expression vector in the backwards orientation. In each sample the amount of GFP mRNA was expressed relative to the amount of mRNA detected using the CMV promoter construct (arbitrarily designated a value of 1). Control experiments using RNA from MEF, HEK-293, HeLa, or BM DCs that were not reverse-transcribed revealed negligible contamination of cDNA with host cell genomic DNA or lentivirus DNA. Spleen B cell cDNA did contain contaminating lentivirus DNA, and therefore RT-PCR data were calculated as $2^{-\Delta\Delta C_t} (\Delta C_t=C_{\text{plus RT}} - C_{\text{minus RT}})$. The results shown are the average (± SD) of three independent experiments. The expression GFP driven by the March-I v2 promoter in each cell type was compared to that using the control promoter. *, p<0.05; ns=not significant.
March-I has a very short 5’ UTR

| clone # | Length of 5’ UTR (bp) | EST ID                | Length of 5’ UTR (bp) |
|---------|------------------------|-----------------------|-----------------------|
| 10RR    | 6                      | gil11271443           | 14                    |
| 8R      | 9                      | gil27175666           | 14                    |
| 1RR     | 4                      | gil27172195           | 12                    |
| 3R      | 14                     | gil27169797           | 12                    |
| 4RR     | 9                      | gil26309935           | 12                    |
| 13R     | 5                      | gil26306254           | 12                    |
| G4      | 6                      | gil26303246           | 12                    |
| H4      | 6                      | gil26297059           | 12                    |
| A5      | 16                     | gil15401008           | 13                    |
|         |                        | gil15399624           | 13                    |
|         |                        | gil17041423           | 6                     |
|         |                        | gil26385744           | 13                    |

$\bar{x} = 8 \pm 4$

$\bar{x} = 12 \pm 2$

Table 1
Fig. 1

A: Genetic map showing exons (I-X) and introns (II-VIII) with RING, TM1, and TM2 domains.

B: Variant maps

- Variant 1
- Variant 2
- Variant 3
- Variant 4

C: Reverse transcription PCR (RT-PCR) gel showing bands for variants 1/4, 2, and 3.

D: Graph showing March-I mRNA expression in spleen, BM, and brain.

E: Graph showing March-I mRNA expression in brain for variants 1/4, 2, and 3.
Fig. 3
**Fig. 4**

(A) GFP mRNA (normalized to GAPDH) for different regions:
-663 to +125
-385 to +125
-175 to +125
-663 to +2

(B) March-I mRNA (normalized to GAPDH) for different treatments:
-663 to +125
-663 to +2

Legend:
- **PBS**
- **LPS**
- **CpG**

Note: ns denotes non-significant differences.
A major isoform of the E3 ubiquitin ligase March-I in antigen presenting cells has regulatory sequences within its gene
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