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

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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, that 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).[4] 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 (TCRs) 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 because of 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 up-regulated 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 APC expression of CD86 (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).

[4] The abbreviations used are: APC, antigen-presenting cell; DC, dendritic cell; March, membrane-associated RING-CH; March-I v2, March-I variant 2; MEF, mouse embryonic fibroblast; puromycin, puromycin *N*-acetyltransferase; TCR, T-cell receptor; TSS, transcription start site(s); pMHC-II, peptide–MHC-II; TLR, toll-like receptor; EST, expressed sequence tag; BM DC, bone marrow–derived DC; RACE, rapid amplification of cDNA ends.

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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 down-regulation 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 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 GenBank[TM] for human RING-CH domain–containing E3 ligases (20). Both the Vega (24) and Ensembl (25) gene annotation systems 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. 1*A*. The location of the 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. 1*B*). It should be noted that *March-I* variants 1 and 3 contain a truncated form of exon 7, and variants 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 three *March-I* isoforms (our exon 5 primer sets cannot distinguish between variants 1 and 4); however, spleen DCs and bone marrow– derived DCs (BM DCs) only contained *March-I* variant 2 (*March-I v2*) mRNA (Fig. 1*C*). 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 with that present in spleen DCs (Fig. 1*D*) and that the small amount of *March-I* mRNA in brain consisted primarily of variants 1/4 and 3 (Fig. 1*E*). These variants are capable of down-regulating expression of MHC-II in transfected cells;[5] 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 pretreated with LPS for 1 h before addition of actinomycin D, and the amount of *March-I v2* mRNA present over time was determined by quantitative RT-PCR. In agreement with previous findings (12, 19), 1-h pretreatment of DCs resulted in a modest but detectable reduction in *March-I v2* mRNA present at the time of actinomycin D addition (Fig. 2*A*). 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 pretreatment of DCs (Fig. 2*B*). These data strongly suggest that down-regulation 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 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 1). 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. 3*A*). Because 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 (but still retaining the *GFP* open reading frame). Because *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* using GM-CSF. Because 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*-acetyltransferase (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 constructs expressed *GFP* mRNA in DCs well above promoterless control lentivirus levels (Fig. 3*B*). 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
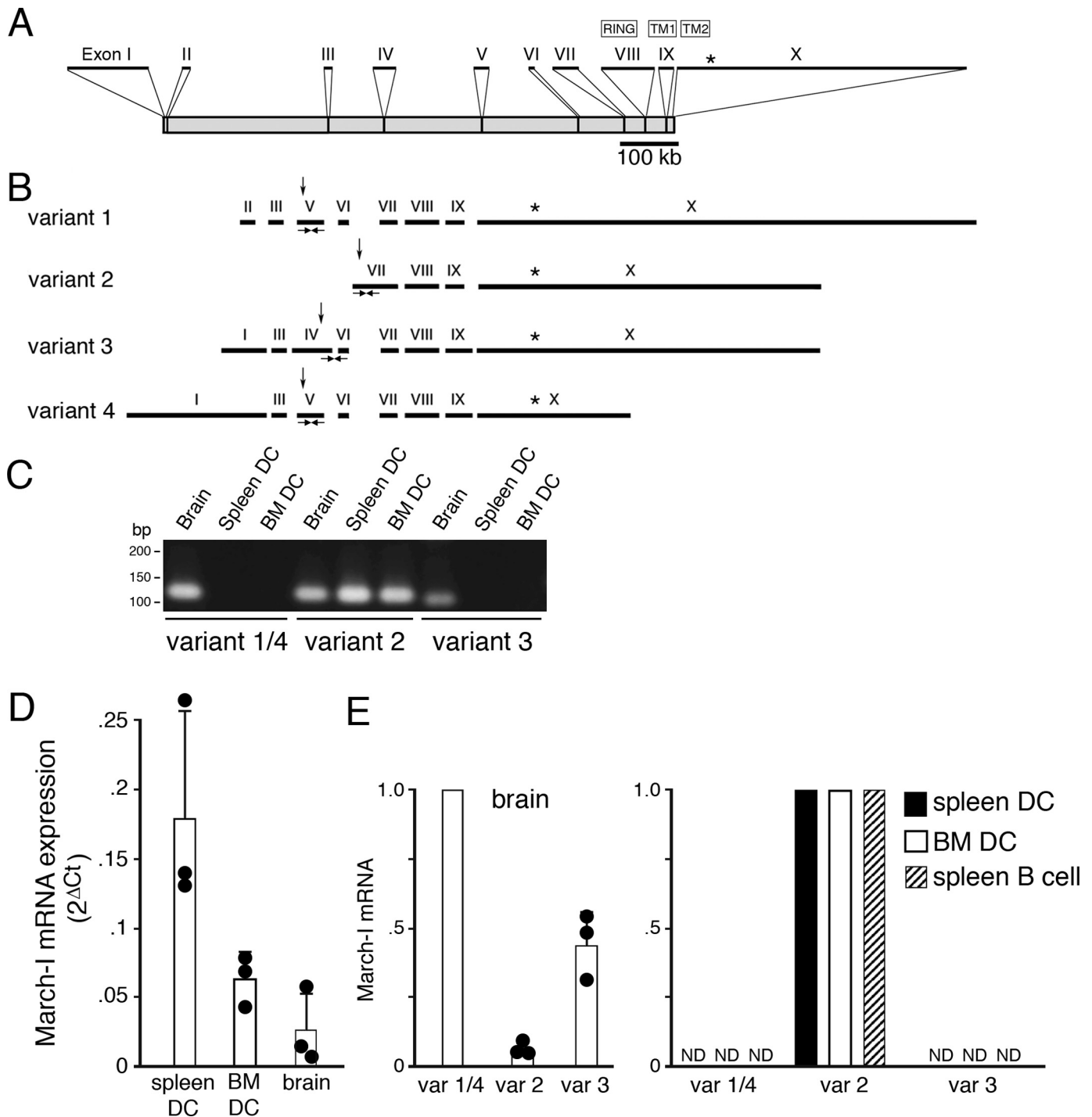
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[5] S. K. Mittal, unpublished observation.

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 (*I–X*), 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 compositions 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 variant 1/4 and variant 3 in the brain. Forty cycles of PCR amplification for each primer pair were performed, and aliquots of the PCR 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 $2^{\Delta Ct}$ ($\Delta Ct = Ct_{GAPDH} - Ct_{March-I}$) value for each condition. The results shown are the average (*error bars* represent S.D.) of three independent experiments. *E,* the relative amount of each *March-I* isoform present in spleen DCs, BM DCs, spleen B cells, and brain was determined by RT-PCR, and the $2^{\Delta Ct}$ ($\Delta Ct = Ct_{GAPDH} - Ct_{March-I}$) value for each sample was determined. The amount of mRNA for the most abundant *March-I* variant present in spleen DCs, BM DCs, B cells, and brain was arbitrarily designated a value of 1. The results shown are the average (*error bars* represent S.D.) of three independent experiments. *var,* variant; *ND,* not detectable.
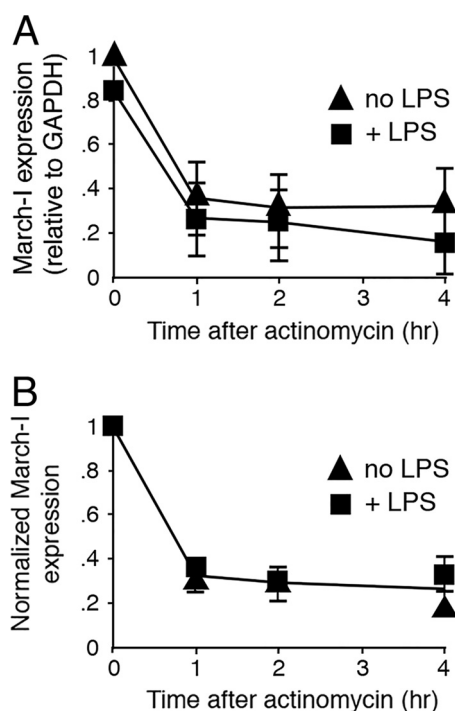
Figure 2. DC activation does not promote *March-I v2* mRNA degradation. DCs were pretreated (or not) with LPS for 1 h, washed, and then incubated in complete medium containing actinomycin D (5 μg/ml) for the indicated time. Cells were harvested, mRNA was isolated, and *March-I v2* expression was determined 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 incubated 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 (*error bars* represent S.D.) of three independent experiments.

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 nine independent clones was identified by DNA sequence analysis. B, 12 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 ± S.D. for each set of analyses is shown.

A clone #	Length of 5′ UTR (bp)	B 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
	x̄ = 8 ± 4	gil15399624	13
		gil17041423	6
		gil26385744	13
			x̄ = 12 ± 2

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 down-regulation of *March-I* expression (12, 13, 29), and we have shown that, after as little as 2 h of stimulation with LPS, *March-I* mRNA is reduced by ~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 TLR4 ligand LPS or the TLR9 ligand CpG for 2 h prior to isolating mRNA and measuring expression of *GFP* in our *March-I v2* promoter-*GFP* constructs. Both LPS and CpG down-regulated expression of the *March-I v2* (−663 to +125), *March-I v2* (−385 to +125), and *March-I v2* (−175 to +125) promoter constructs (Fig. 4*A*), 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. 4*B*). 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 whether 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 with *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 with 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

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

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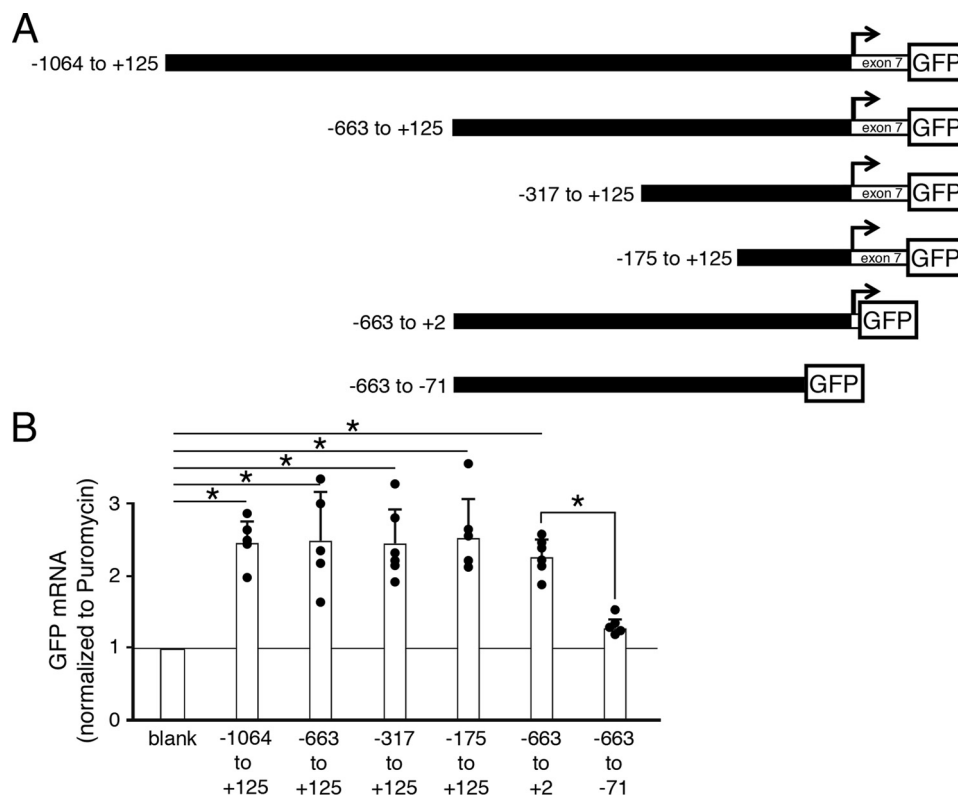*J. Biol. Chem.* (2018) 293(12) 4478–4485 **4481**

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 was 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 under "Experimental procedures") and expressed relative to the amount of mRNA detected in the promoterless blank construct. The results shown are the average (*error bars* represent S.D.) of multiple independent experiments. *, $p < 0.05$.

in three independent spleen DC samples there is ~1 molecule of *March-I v2* mRNA per cell.[6] Such a finding is consistent with the nearly undetectable expression of endogenous March-I protein in APCs reported by others (8, 12, 23).

Because *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 was also down-regulated in LPS- and CpG-activated DCs. The sequences responsible for down-regulation 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 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 subcultured every 2nd or 3rd day and maintained at subconfluent 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 1640 medium containing 10% fetal bovine serum, 50 μM β-mercaptoethanol, 10 mM Hepes, pH 7.4, 25 μg/ml gentamycin, and 20 ng/ml GM-CSF) as described previously (29).

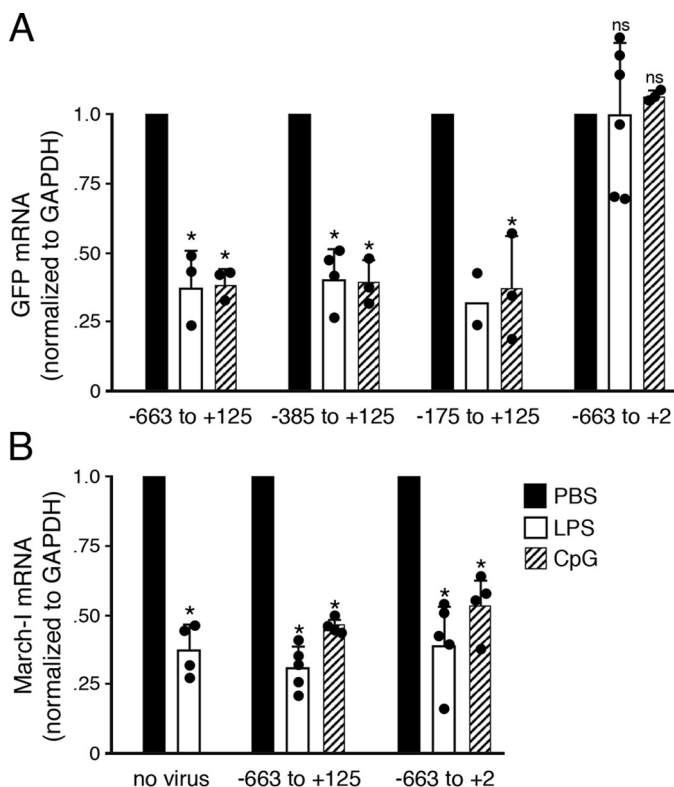[6] S. Kaul, unpublished observation.

A

B

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 h. Cells were harvested, and expression of endogenous *March-I v2* or *GFP* mRNA and *GAPDH* was determined by RT-PCR. *GFP* mRNA expression (*A*) or endogenous *March-I v2* expression (*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 under "Experimental procedures." *March-I* RING domain primers were used to detect endogenous *March-I*. The results shown are the average (*error bars* represent S.D.) of multiple independent experiments. *, $p < 0.05$; *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 DCs, 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 with the *March-I v2* (−663 to +125) promoter inserted into the expression vector in the backward orientation. In each sample, the amount of *GPF* 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 MEFs, HEK-293 cells, HeLa cells, 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 $2^{\Delta Ct}$ ($\Delta Ct = Ct_{\text{plus RT}} − Ct_{\text{minus RT}}$). The results shown are the average (*error bars* represent S.D.) of three independent experiments. The expression of *GFP* driven by the *March-I v2* promoter in each cell type was compared with that using the control promoter. *, $p < 0.05$; *ns*, not significant.

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 and 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 sequences of all *March-I* promoter lentiviral constructs were confirmed by DNA sequence analysis.

Promoter construct primer sequences

The promoter construct primer sequences used were: BIG-S-F, gagagactcgagccacattttggacaccagcgttttgag; SM-S-F, gagagactcgaggccacaggataagtccaatagtg; SMSF-400, gagagactcgaggggccttgttgctaactcaaggtg; SMSF-300, gagagactcgagcccaggcaaattgagctattccac; P4S-R, gagagaggatcccaagttagataaatttggcatcttg; SM-AS-F, gagagaggatccgccacaggataagtccaatagtg; P4AS-R, gagagactcgagcaagttagataaatttggcatcttg; PR1-F3, ggtataacatggcttctac; PR4-R1, caagttagataaatttggcatc; P4SR-120, gagagaggatcctgcactgccctgctgttgcttcac; P4SR-ATG, gagagaggatcctgaatctggggaagtcaggggggcttg.

RT-PCR primer sequences

The oligonucleotides used for measuring relative transcript levels in this study were: *GFP*, 5′-caacagccacaacgtctatatcat-3′ and 5′-atgttgtggcggatcttgaag-3′; puromycin, 5′-gttcgccgactacc-ccg-3′ and 5′-agagttcttgcagctcggtg-3′; and *March-I* RING domain, 5′-aagagagcccactcatcacacc-3′ and 5′-atctggagcttttccc-

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, SMSF-400, and SMSF-300, respectively, and the common reverse primer P4S-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
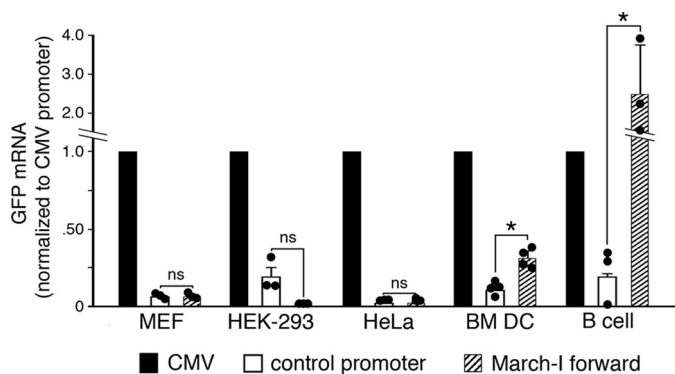
acttcc-3′. The oligonucleotides used selectively in the RT-PCR assays for *March-I* variants were: variant 1/4, 5′-gatgtggccgat-gcctctcaa-3′ and 5′-tcgttgggctgcttgcttttg-3′; variant 2, 5′-actt-gtttctccaggcaagc-3′ and 5′-agtgggctctcttcatctcct-3′; and variant 3, 5′-gcaatgggagaagttcaatggg-3′ and 5′-gttgggctgct-tgctttgata-3′.

Generation of lentiviral stocks

Lentiviral stocks were generated by transfecting subconflu-ent HEK-293T cells with lentiviral promoter plasmid, EZ-LentiPACK Packaging plasmid, and EZ-Transfx reagent as described in the EZ-LentiPACK Packaging System kit (Cello-mics Technology). Lentiviral supernatants were harvested 48 and 72 h after transfection. Viral supernatants were centrifuged briefly ($500 \times g$, 10 min) to remove cellular debris. The approx-imate titer of viral supernatants was quantitated using Lentivi-rus Titration XpressCards (Cellomics Technology).

Lentiviral transduction

Lentivirus ($10 – 100$ μl of HEK-293 viral supernatant) was added to bone marrow cultures on day 2 of culture in BM DC medium containing 10 μg/ml protamine sulfate (Sigma). BM DC medium was changed every other day. Day 7 BM DC cultures were cultured in the presence or absence of 1 μg/ml LPS (Sigma) and 2 μg/ml CpG-1668 (Invivogen) for 2 h. BM DCs were then washed and 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) was added to 8×10^6 B cells in 2 ml of RPMI 1640 medium 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 $1100 \times g$ for 90 min and then placed in a 37 °C, 5% CO_2 incubator. After 2 days, cells were harvested at 4 °C and 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 cul-tured in RPMI 1640 medium 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 contain-ing 10 μg/ml protamine sulfate, and the medium was changed after 1 day. The next day cells were harvested at 4 °C and 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 accord-ing to the manufacturer's instructions. cDNA was synthesized using $1 – 2$ μg of total RNA and the Superscript III First-Strand Synthesis kit (Invitrogen). RT-PCR was performed using a QuantiStudio6 Flex sequence detection system (Applied Bio-systems) and the Quantitect SYBR Green PCR kit (Qiagen).

Quantitation of March-I v2 promoter activity

In experiments monitoring expression of *March-I v2* pro-moter activity in lentivirus-transduced BM DCs, RT-PCR was used to quantitate the amount of *GFP* and puromycin mRNA present in the sample. Ct values for *GFP* and puro-mycin were determined, and data are shown as $2^{\Delta Ct}$ ($\Delta Ct = Ct_{puromycin} − Ct_{GFP}$). $2^{\Delta Ct}$ for the promoterless (blank) vec-tor control was arbitrarily set at 1.0, and all values are expressed relative to the blank vector control.

In experiments monitoring regulation of *March-I v2* pro-moter activity in transduced BM DCs 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 $2^{\Delta Ct}$ ($\Delta Ct = Ct_{GAPDH} − Ct_{GFP}$). In experiments using the *March-I v2* (−663 to +125) and *March-I v2* (−663 to +2) sense con-structs, 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 *March-I v2* (−175 to +125) sense constructs, the control virus was a *March-I v2* (+125 to −385) antisense construct. The data shown are $2^{\Delta Ct}$ for the sense construct/$2^{\Delta Ct}$ for the antisense construct. The amount of *GFP* mRNA present after treatment of each sam-ple 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)[7] and Vega Genome Browser Release 68 (http://vega.archive.ensembl.org).[7] 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 OTTMUST00000040256 (variant 1), OTTMUST00000040267 (variant 2), OTTMUST00000040269 (variant 3), and OTTMUST00000040271 (variant 4). The Vega Genome Browser transcript identifiers for human *March-I* are ENST00000503008 (variant 1) and ENST00000339875 (variant 2). 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 analyzed using two-tailed Student's *t* tests. *p* values <0.05 were considered statistically significant. *p* values are indicated by * for $p < 0.05$, and non-significant differences are indicated by ns.

[7] Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party-hosted site.

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