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Molecular Mechanisms for Synchronized Transcription of Three Complement C1q Subunit Genes in Dendritic Cells and Macrophages*[S]

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Background: C1q is assembled in a 1:1:1 ratio from three subunits demanding synchronized expression of three genes in a cluster; C1q deficiency causes lupus.
Results: A PU.1/IRF8-stimulated "core promoter" appears to exist in this gene cluster.
Conclusion: A core promoter helps synchronize transcription of three C1q genes.
Significance: This helps explain the highly conserved clustering of three C1q genes in all animal species, including zebrafish.

Hereditary homozygous C1q deficiency is rare, but it almost certainly causes systemic lupus erythematosus. On the other hand, C1q levels can decline in systemic lupus erythematosus patients without apparent C1q gene defects and the versatility in C1q production is a likely cause. As an 18-subunit protein, C1q is assembled in a 1:1:1 ratio from three different subunits. The three human C1q genes are closely bundled on chromosome 1 (C1qA-C1qC-C1qB) and their basal and IFNγ-stimulated expression, largely restricted to macrophages and dendritic cells, is apparently synchronized. We cloned the three gene promoters and observed that although the C1qB promoter exhibited basal and IFNγ-stimulated activities consistent with the endogenous C1qB gene, the activities of the cloned C1qA and C1qC promoters were suppressed by IFNγ. To certain extents, these were corrected when the C1qB promoter was cloned at the 3′ end across the luciferase reporter gene. A 53-bp element is essential to the activities of the C1qB promoter and the transcription factors PU.1 and IRF8 bound to this region. By chromatin immunoprecipitation, the C1qB promoter was co-precipitated with PU.1 and IRF8. shRNA knockdown of PU.1 and IRF8 diminished C1qB promoter response to IFNγ. STAT1 instead regulated C1qB promoter through IRF8 induction. Collectively, our results reveal a novel transcriptional mechanism by which the expression of the three C1q genes is synchronized.

The complement system is an enzymatic cascade, consisting of >20 abundant plasma proteins, and its activation on microbial pathogens leads to killing and clearance through the formation of membrane lytic complexes, opsonins, and anaphylatoxins (1–3). These complement proteins are mostly of hepatic origin (4), but C1q is extrahepatically produced by tissue macrophages and dendritic cells (DCs)[2] (5–8). These are potent phagocytes and major antigen-presenting cells instrumental to the induction of adaptive immunity and tolerance (9–11). The evolutionary advantages and transcriptional mechanisms that sustain this distinct mode of C1q production by antigen-presenting cells are not understood.

C1q recognizes antibody-bound pathogens to trigger the complement classical pathway (1–3). In addition, C1q also recognizes apoptotic cells and thus augments phagocytosis (12–14). It binds to membrane blebs on apoptotic cells and, on the molecular level, C1q can bind to surface-exposed calreticulin, which is otherwise a resident protein of the endoplasmic reticulum in live cells (12, 13). Apoptotic cells absorb polyclonal IgM, which then recruits C1q (14). Apoptotic cells are sources of self-antigens, and their impaired clearance by phagocytes, which can occur in C1q deficiency, can elicit autoantibody production (15). The dominant manifestation in genetic C1q deficiency is the development of systemic lupus erythematosus (SLE) (16–18).

In addition to these ligand-dependent C1q functions, which can involve complement activation and phagocytosis, ligand-independent C1q functions have also been reported (19). For example, both soluble and solid phase C1q were shown to affect monocyte differentiation into DCs so that these cells were less immunostimulatory or more tolerogenic (20, 21). Second, $C1q^{-/-}$ mouse DCs produced IL-12 abnormally, which affected IFNγ induction from T cells (22, 23). These can impact on the induction of immunity or autoimmunity.

Homozygous hereditary C1q deficiency is rare but it represents the strongest known genetic risk for SLE pathogenesis (24). Without known gene defect, acquired C1q deficiency can occur (25, 26). C1q consumption by immune complexes and anti-C1q autoantibodies are likely causes (27, 28). Besides, diverse microbial structures, cytokines, hormones, and drugs

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[2] The abbreviations used are: DC, dendritic cell; SLE, systemic lupus erythematosus; RLM-RACE, RNA ligase-mediated rapid amplification of 5′ cDNA ends; TSS, transcription start site; GAS, IFNγ-activated site; ISRE, interferon-stimulated response element.

affect C1q production (19), which can contribute to the decline of C1q levels in SLE patients.

C1q is a delicate 18-subunit assembly in 1:1:1 ratio from three types of subunits (29). The C1q genes are highly clustered (C1qA-C1qC-C1qB) within a genomic region of ~25 kb (30). How the transcription of these three genes is synchronized in macrophages and DCs is relevant to SLE pathogenesis but remains to be investigated. Here, we report a 53-bp region in the C1q gene cluster that responds to IFNγ through PU.1 and IRF8, two DC/macrophage-associated transcription factors, and potentially regulates all three genes from the center of this gene cluster.

EXPERIMENTAL PROCEDURES

RNA Ligase-mediated Rapid Amplification of 5′ cDNA Ends (RLM-RACE)—The transcription start site (TSS) for each C1q gene was determined using the RLM-RACE kit (Invitrogen) with RNA isolated from cultured human macrophages (1–5 μg) (21). After dephosphorylation with calf intestinal phosphatase, RNA was decapped at the 5′ end with tobacco acid pyrophosphatase and a 44-bp GeneRacer RNA oligo was then ligated to the 5′ ends. cDNA was synthesized from the RNA using C1q gene-specific primers (supplemental data). 5′ end sequences were PCR-amplified using common forward primers embedded in the GeneRacer oligonucleotides and respective reverse gene-specific primers (supplemental data). The PCR products were cloned using the TOPO cloning reaction kit (Invitrogen) and sequenced using the BigDye Terminator v3.1 cycle sequencing reagents (Applied Biosystems).

Cloning of C1q Gene Promoters—Putative promoter for each C1q gene was PCR-amplified from genomic DNA to include the TSS, an extensive 5′ region (>2 kb) and a short 3′ region. The primers were flanked by restriction sites: C1qA (5′-gcggtaccaggcagcactcagctcacattac-3′/5′-gcctcgagttgtcaactccaactggatgct-3′, KpnI/XhoI), C1qB (5′-ccgctagcaggtgagggggtgtaggtttctt-3′/5′-ccgctagcaggtgagggggtgtaggtttctt-3′, NheI/HindIII), and C1qC (5′-ccgctagccttcacacccacagacacactca-3′/5′-ccaagcttgcaggtgggcggtttctgc-3′, NheI/HindIII). The PCR products were cloned into the pGL3-basic vector in conjunction with the 3′ firefly luciferase reporter gene (Promega). This yields the pGL3-C1qA-Luc, pGL3-C1qB-Luc, and pGL3-C1qC-Luc plasmids with 2407, 2477, and 2594 bp promoter sequences, respectively.

Transfection—These cloned C1q gene promoters were transfected into the mouse RAW264.7 macrophage cells (ATCC) following a published protocol (31). Briefly, RAW264.7 cells were cultured in DMEM supplemented with 10% (v/v) bovine calf serum (HyClone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine at 37 °C, and 5% CO_2. For transfection, cells were harvested and resuspended in serum-free RPMI 1640 medium (2.5×10^7 cells/ml). The C1q promoter plasmid (10 μg) was mixed in a 40-μl volume with the pRL-β-actin promoter plasmid (1 μg). In a 4-mm cuvette, the plasmid mix was further mixed with RAW264.7 cells (0.4 ml), which was then pulsed in the Gene Pulser Xcell system (Bio-Rad) at 300 V with 975 μF capacitance and infinite resistance. The cells were left stand for 5 min and then resuspended in RPMI 1640 medium (8.5 ml) containing 10% (v/v) bovine calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10 μM chloroquine. Cells were cultured in 48-well plates (0.5 ml/well) overnight at 37 °C and 5% CO_2 and then changed to fresh RPMI 1640 medium (1 ml/well) where chloroquine was replaced by 1.2% (v/v) dimethyl sulfoxide. After 24 h, luciferase activities were measured. Where cells were stimulated with mouse IFNγ (10 ng/ml), it was added 4 h after the change of medium.

Luciferase Assay—The Dual-Luciferase reporter assay kit (Promega) was used to determine firefly luciferase expression resulting from the C1q gene promoters and constitutive *Renilla* luciferase expression under a β-actin promoter. The transfected RAW264.7 cells, with or without IFNγ treatments, were washed in PBS and lysed in the passive lysis buffer (65 μl/well) for 45 min with shaking. The cell lysate (2 μl) was first mixed with the firefly luciferase substrate (luciferase assay reagent II, 25 μl) and then measured in the TD-20/20 luminometer (Turner Designs). The *Renilla* luciferase substrate was then added (Stop & Glo, 25 μl) to measure the control β-actin promoter activity. Relative luciferase activity was calculated for each experiment (firefly luciferase reading/*Renilla* luciferase reading × 100), and data were presented as mean ± S.D. of triple experiments.

Mutagenesis—Potential *cis*-acting regions in the cloned C1q gene promoters were initially mapped by 5′ and 3′ deletions of the promoters. Specified regions were PCR-amplified using primers listed in supplemental data. 5′ deletion of the pGL-C1qB-Luc vector to −273 bp yielded a basic C1qB gene promoter (B273). Point mutations were introduced to the B273 promoter using the QuikChange® site-directed mutagenesis kit (Stratagene) and primers listed in supplemental Table S4. In some experiments, the B273 promoter was cloned at the 3′ end of the firefly luciferase gene with C1qA or C1qC promoters at the 5′ end.

shRNA Knockdown—Oligonucleotides representing PU.1, IRF1, IRF8, and STAT1 target sequences (supplemental data) were synthesized, and double-stranded DNA was generated by annealing complementary oligonucleotides. These were cloned into the pSUPER.basic vector (OligoEngine, Seattle, WA). To transfect RAW264.7 cells, each shRNA plasmid (10 μg) was mixed with the B273 plasmid (1 μg) and the control β-actin promoter plasmid (1 μg) in a 40-μl volume for electroporation. Cells were cultured to measure luciferase activities. To evaluate shRNA knockdown of the transcription factors, cell lysates were subjected to Western blotting.

Western Blotting—To detect PU.1, IRF1, IRF8, and STAT1, shRNA-transfected RAW264.7 cells were lysed for 30 min at 4 °C in radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet-40, 0.25% (w/v) sodium deoxycholate, and 1 mM EDTA) supplemented with a protease inhibitor mixture (Sigma). After clearing by centrifugation, proteins were separated on 10% (w/v) SDS-PAGE gels and electroblotted onto PVDF membranes. The blots were blocked for 1 h in TBS (50 mM Tris and 150 mM NaCl, pH 7.4) containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat milk and then incubated overnight at 4 °C with antibodies specific for mouse PU.1, IRF1, IRF8, and STAT1 (#9172, rabbit; Cell Signaling Tech. Inc., Danvers, MA) and, as a control, with a β-actin antibody (Sigma-Aldrich). The PU.1 (H-135, rabbit), IRF1 (C-20, rabbit),

and IRF8 (C-19, goat) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). After washing, the blots were incubated for 2 h with alkaline phosphatase-conjugated secondary antibodies and developed using the Immuno-Star AP substrate (Bio-Rad).

DNA Binding Assay—A double-stranded, 53-bp DNA fragment (−133 to −81 bp) was generated by annealing a sense oligonucleotide (5′-ctcatttacagtaaatccagtgggttgcagaaataggacctgaaactgcctga-3′) and a biotinylated antisense oligonucleotide (5′-biotin-tcaggcagtttcaggtcctatttctgcaacccactggatttactgtaaatgag-3′). The two oligonucleotides were mixed at 1 pmol/μl each in an annealing buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl) and after boiling for 5 min were cooled to room temperature. On the other hand, nuclear extracts were prepared from RAW264.7 cells, with or without IFNγ treatment, using the NE-PER nuclear extraction reagents (Thermo Scientific, Rockfold, IL).

Streptavidin-agarose resins (50 μl of packed volume) were washed and resuspended in 100 μl of TEN buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and 100 mM NaCl). Half of the resins were mixed with the 53-bp probe (30 μl) overnight at 4 °C and, as a control, the other half of the resins were mixed with the annealing buffer. After washing once with TEN buffer and twice with TGEDN buffer (20 mM Tris, pH 8.0, 10% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, and 0.1% (v/v) Triton X-100), the resins were resuspended in 0.5 ml of TGEDN buffer and incubated for 4 h at 4 °C with the nuclear extract (200 μg proteins) and sonicated salmon sperm DNA (25 μg). The resins were washed five times each with 0.5 ml of 0.5× TGEDN buffer and resuspended in 50 μl of elution buffer (0.5% (w/v) SDS and 1 M NaCl). Proteins were eluted by heating at 50 °C for 20 min and then separated on 10% (w/v) SDS-PAGE gels. As controls, total nuclear extracts were loaded on the gels. Precipitated PU.1, IRF1, IRF8, and STAT1 were detected by Western blotting.

ChIP—Macrophages (~1×10^7) were cultured in a 100-mm plate with macrophage colony-stimulating factor (20 ng/ml) (21). On day 6, cells were fixed by adding formaldehyde to 1% (w/v). After gently mixing for 10 min, reactions were stopped by adding glycine to 150 mM. After 5 min, cells were washed three times with PBS, harvested, and resuspended in 1 ml of L1 buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, and protease inhibitors). After incubation on ice for 5 min, the insoluble nuclear fraction was pelleted and resuspended in 1 ml of L2 buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 1% (w/v) SDS, and protease inhibitors), and 0.2-ml aliquots were made in 1.5-ml Eppendorf tubes. Sonication was performed in the Bioruptor at its high setting (Diagenode, Inc., Liege, Belgium) for 15 cycles (30 s on/30 s off) in an ice bath. Soluble nuclear lysate was obtained by centrifugation for 30 s at 13,000 rpm and 4 °C.

The nuclear lysate (50 μg) was diluted 1:10 with a dilution buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 0.2 M NaCl, and 1% (v/v) Triton X-100). A 1:10 dilution of the L2 buffer was used in further incubation and washing steps. Protein A-Sepharose resins (20 μl) were washed and mixed for 3 h at 4 °C with the PU.1, IRF1, IRF8, or STAT1 antibody (10 μg/ml). The resins were similarly incubated with non-immune rabbit IgG (Bio-Rad) and used to absorb the nuclear lysate for 3 h at 4 °C. After addition of sonicated salmon sperm DNA (25 μg), the preabsorbed lysate was then incubated overnight at 4 °C with resins loaded with the PU.1, IRF8, STAT1, or IRF1 antibodies or, as controls, resins loaded with the anti-His antibody or resins without antibody.

The resins were washed five times with buffer A (20 mM Tris, pH 8.0, 2 mM EDTA, 0.5 M NaCl, 1% (v/v) Triton X-100, and 0.1% (w/v) SDS) and three times with buffer B (buffer A with an additional 0.5 M NaCl). Bound nuclear materials were eluted for 4 h at 65 °C in 0.1 ml of elution buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 10 mM dithiolthreitol, and 1% (w/v) SDS). Eluted DNA was enriched using the GFX PCR and gel band purification kit (GE Healthcare). The presence of C1qA, C1qB, and C1qC promoter sequences was determined by PCR using specific primers: C1qA (5′-cgcccaatgtcccagtctt-3′/5′-gttgtcaactccaactggat-3′), C1qB (5′-tgagttggcagaaccaaa-3′/5′-agcaggctggccccttttc-3′), C1qC (5′-aaagtgtcaagtcagggaaa-3′/5′-tgcaggtgggcggtttct-3′). PCR products were visualized on 1% agarose gels.

RESULTS

Coordinated Transcription of Three Endogenous C1q Genes—The fact that C1q is assembled in a 1:1:1 ratio from its C1qA, C1qB, and C1qC subunits (29) demands synchronized transcription of the three C1q genes. Furthermore, these mechanisms apparently also ensure that C1q gene transcription is largely restricted to DCs and macrophages. As shown in Fig. 1*A*, DCs constitutively express all three mRNA species that were, after IFNγ treatment, all up-regulated (Fig. 1*A*). Similar basal and IFNγ-stimulated expression of the three C1q mRNA species was observed in macrophages (Fig. 1*B*). The magnitudes of up-regulation were the highest with the C1qB mRNA and the lowest for C1qA mRNA. Overall, the three C1q genes showed largely coordinated basal and IFNγ-stimulated transcription. Mechanistically, this could be achieved by having similar relevant *cis*-acting elements in all three gene promoters. The fact that the three genes are in a rare but highly conserved chromosomal cluster (Fig. 2) implies that synchronization may also be achieved through a core element inside this gene cluster (30, 32).

Cloned C1qA and C1qC Gene Promoters Lack Basal and IFNγ-stimulated Activities of Endogenous Genes—To examine the first hypothesis, *i.e.* that the three C1q gene promoters function independently and synchronization is achieved by having the relevant *cis*-acting elements duplicated in all three promoters, these promoters were each cloned into a firefly luciferase reporter plasmid (pGL3-basic) and transfected into the mouse RAW264.7 macrophage cells. Cloning was performed after validation of the TSS for each C1q gene by RLM-RACE (supplemental data). In the transfected RAW264.7 cells, the three promoters displayed varying degrees of basal activity with the C1qB promoter being many times stronger than the C1qC promoter (Fig. 2). Similar to its endogenous counterpart (Fig. 1), the cloned C1qB promoter was highly inducible by IFNγ (Fig. 2*C*). In contrast, the cloned C1qC promoter dramatically departed from the activity of its endogenous counterpart by exhibiting weak basal activity, which was further suppressed by IFNγ (Fig. 2). The C1qA promoter was also suppressed by

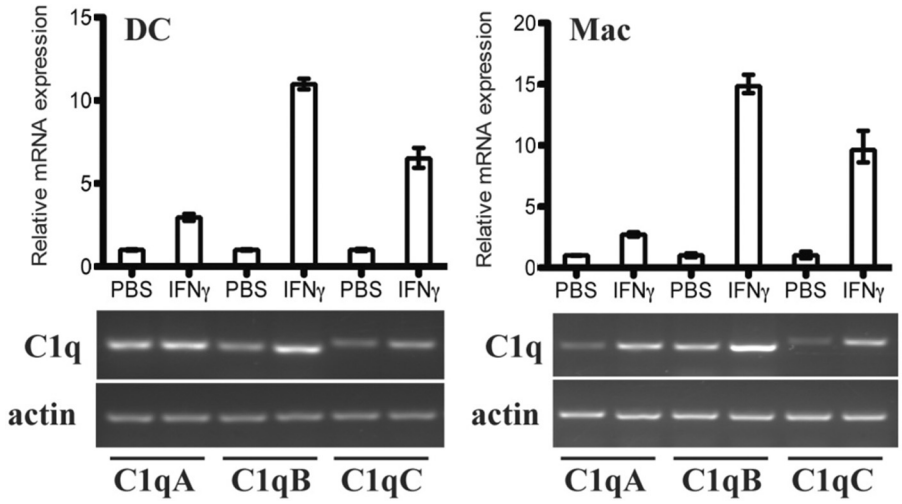

FIGURE 1. **Basal and IFNγ-induced expression of C1qA, C1qB, and C1qC mRNA in cultured DCs (*left*) and macrophages (*right*).** DCs and macrophages were cultured from blood monocytes and were, at day 6, cultured for a further 24 h either untreated (PBS) or treated with human IFNγ (100 ng/ml). RNA was isolated for real-time PCR using primers specific for C1qA, C1qB, and C1qC. Results were normalized to the levels of β-actin mRNA in each experiment, and data were presented as folds of C1q mRNA induction by IFNγ taking that detected in untreated cells as 1. Conventional PCR was also performed, and the PCR products were detected on 1% agarose gels.

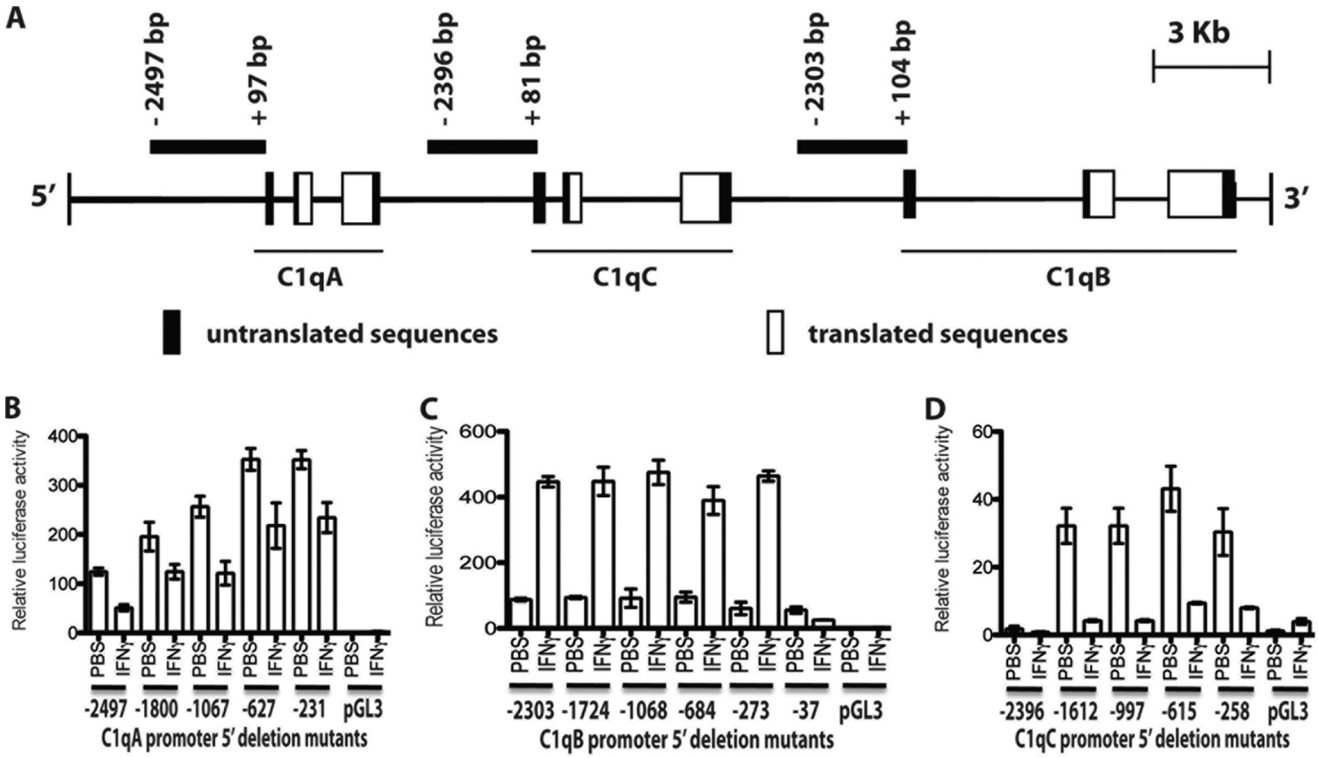

FIGURE 2. **Basal and IFNγ-stimulated activities of the C1qA, C1qB, and C1qC gene promoters.** The anticipated promoter regions for C1qA, C1qB, and C1qC were specified and cloned into the pGL3-basic vector (*A*). The cloned C1qA (*B*), C1qB (*C*), and C1qC (*D*) gene promoters were transfected into RAW264.7 cells to measure the basal activities, and cells were treated with mouse IFNγ (10 ng/ml) to measure IFNγ-stimulated activities. After 5′ deletions, the shortened promoters were examined similarly. In each experiment, a *Renilla* luciferase reporter plasmid under the β-actin promoter was co-transfected to normalize the firefly luciferase activities derived from C1q promoters. Data were presented as means ± S.D. of triplicate experiments.

IFNγ. These results are not in favor of the first hypothesis that synchronized transcription is achieved by having the relevant *cis*-acting element duplicated in the three independent C1q gene promoters.

Search for Discrete Regions for IFNγ Inhibition in Cloned C1qA and C1qC Promoters—A series of 5′ deletions were introduced in the C1qA and C1qC gene promoters in an attempt to determine whether specific regions exist that mediated IFNγ inhibition of these promoters. For the C1qA promoter (−2497 bp), deletions were introduced at −1800, −1067, −627, and −231 bp, whereas the C1qC promoter (−2396 bp) was truncated at −1612, −997, −615, and −258 bp. A progressive 5′ deletion of the C1qA promoter gradually increased its basal activity, *e.g.* the −627-bp promoter was 3-fold stronger than the −2497-bp promoter (Fig. 2*B*). Further deletion to −231 bp had no additional effect. However, all of these truncated C1qA pro-

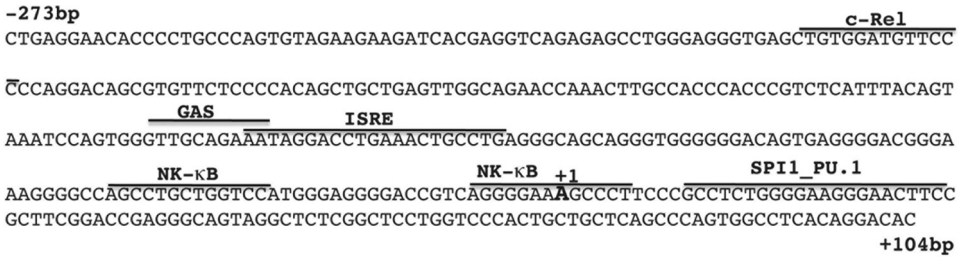

FIGURE 3. **Predicted *cis*-acting elements in the B273 promoter.** The sequence of the −273-bp C1qB promoter (B273) was analyzed using MatInspector software, and the predicted *cis*-acting elements were highlighted. Two noticeable elements identified are the GAS-ISRE chimeric site proximal to the TSS (+1) and an upstream c-Rel site.

moters remained inhibited by IFNγ, showing that the remaining promoter region of the C1qA gene, despite the increased basal activity, still lacks adequate *cis*-acting elements to confer IFNγ induction as observed in the endogenous C1qA gene.

Deletion of the C1qC promoter from −2396 to −1612 bp dramatically raised the otherwise trivial basal activity, but further deletion to −997, −615, and −258 bp had no additional effects (Fig. 2*D*). All of these truncated promoters also remained inhibited by IFNγ. It should be noted that even the raised basal activity of the truncated C1qC promoter remained much lower than that of the C1qB promoter. Furthermore, the truncated C1qA and C1qC promoters remained inhibited by IFNγ. It shows that the C1qA and C1qC genes are not effectively regulated by their 5′ regions upstream of the TSS, and additional endogenous mechanisms may exist, in the chromosomal context, for synchronized transcription of the three C1q genes.

Identification of Novel IFNγ-stimulated Region in C1qB Promoter—Similar 5′ deletions of the C1qB promoter (−2303 bp) at −1724, −1068, or −684 bp did not affect its strong basal and IFNγ-stimulated activities. Deletion to −273 bp only slightly reduced its basal activity, but the IFNγ-stimulated activity remained intact. Deletion further to −37 bp halved the basal activity, but it completely eliminated the IFNγ-stimulated activity (Fig. 2*C*). It suggests that the 236-bp region between −273 and −37 bp is responsible for its IFNγ-stimulated activity and may also partially contributes to its basal activity (Fig. 2*C*).

Inspection of this region using the MatInspector program (Genomatix), revealed a TSS-proximal chimeric GAS-ISRE sequence and a c-Rel site further upstream (Fig. 3). 5′ deletion to −141 bp removed the c-Rel site but still retained the GAS-ISRE sequences in the promoter. This slightly reduced the IFNγ-stimulated activity (Fig. 4*A*). However, deleting a further 8 bp to −133 bp began to impair the IFNγ-stimulated activity: a 50% reduction compared with the −273-bp promoter (Fig. 4*A*), suggesting a significant contribution of the 11-bp segment between −144 and −133 bp, which is 5′ outside of the GAS-ISRE region, to the ability of the promoter to respond to IFNγ. Deletion of another 8 to −125 bp still maintained the integrity of the GAS-ISRE region (Fig. 4*B*), but it completely abolished the IFNγ-stimulated activity (Fig. 4*A*). These results suggest that the putative GAS-ISRE region is inadequate for the IFNγ response of the C1qB gene if it plays any roles.

To further assess this GAS-ISRE region, 3′ deletions were made using the −133-bp promoter as a template. 3′ deletion to −28 bp, which removed the TSS, dramatically reduced its basal activity, but the promoter still retained some IFNγ-inducible activity (Fig. 4*B*). Progressive deletion to −45, −72, and −81 bp showed no impairment of this IFNγ-stimulated activity (Fig. 4*B*). However, deletion to −90 bp, which began to disrupt the ISRE region, completely abolished IFNγ-stimulated activity. These 5′ and 3′ deletion studies identified a basic 53-bp region in the C1qB promoter (−133 to −81 bp) that was sufficient and essential to confer most of IFNγ-stimulated activity to the promoter.

The involvement of different regions along this 53-bp fragment to IFNγ-stimulated activity was further evaluated by site-directed mutagenesis (Fig. 4*C*). Most of these mutants showed varying degrees of impairment in the IFNγ-stimulated activity. The activity appeared to be completely abolished by some single nucleotide mutations, *i.e.* A96G, A102G, A120G, A121G, T122C, T127C, and T128C (Fig. 4*D*). It shows that the integrity of this 53-bp element is essential for the C1qB gene promoter to respond to IFNγ. Interestingly, although the A96G mutation completely inactivated this element, mutations at the flanking nucleotides, *i.e.* C95T and G97A, substantially raised the basal activity of the promoter without compromising IFNγ-stimulated activity. This implies a potent regulatory role of this CAG trinucleotide segment in C1q gene transcription.

3′ B273 Promoter Can Correct 5′ C1qA and C1qC Promoters across Luciferase Reporter Gene—At this point, we evaluated the second hypothesis, *i.e.* that the expression of the three C1q genes is synchronized through a core *cis*-acting element within this gene cluster. We reckoned that the B273 promoter was such a candidate element. B273 was cloned at the 3′ end of the luciferase reporter gene in the pGL-C1qA-Luc and pGL-C1qC-Luc vectors, which had 5′ C1qA and C1qC promoters, respectively (Fig. 5*A*). This placed the luciferase gene under the influence of a 5′ promoter (C1qA or C1qC) and a 3′ B273 promoter (Fig. 5*A*). As a control, B273 was cloned at the 3′ end of the luciferase gene, which lacked a 5′ promoter (Fig. 5*A*). The B273 promoter clearly exhibited both basal and IFNγ-stimulated activities from the 3′ end of the luciferase gene in the absence of a 5′ promoter (Fig. 5*B*). When the C1qA promoter was present at the 5′ end, the 3′ B273 promoter had no obvious effect on its basal activity across the luciferase gene, but it reverted the 5′ C1qA promoter from a negative to a slightly positive response to IFNγ (Fig. 5*B*). Likewise, the 3′ B273 promoter also reverted the 5′ C1qC promoter from inhibition to induction by IFNγ (Fig. 5*B*). To a lesser extent, a shorter (−258 bp) C1qC promoter was similarly superseded by the 3′ B273 promoter. This experimental design mimicked the natural chromosomal ori-

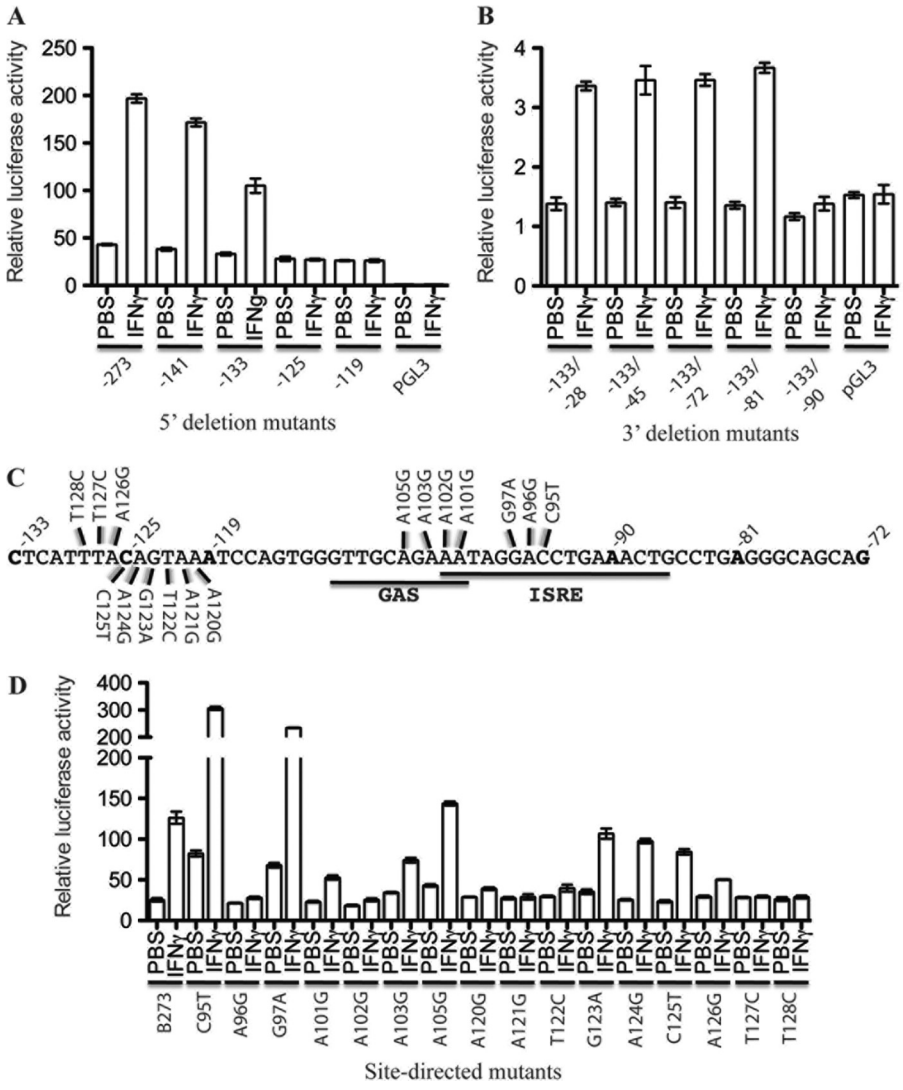

FIGURE 4. **Identification of a 53-bp IFNγ-stimulated *cis*-acting element in the C1qB promoter.** *A*, further 5′ deletions of the B273 promoter at −144, −133, −125, and −119 bp were made to identify the 5′ boundary of the IFNγ-stimulated region in the C1qB promoter. *B*, 3′ deletions were made at −28, −45, −72, −81, and −90 bp to identify the 3′ boundary of the IFNγ-stimulated site. *C*, illustration of site-directed mutagenesis of the B273 promoter to determine the involvement of specific nucleotides for B273 response to IFNγ. *D*, basal and IFNγ-stimulated activities of site-directed B273 mutants. Data were presented at mean ± S.D. of triplicate experiments.

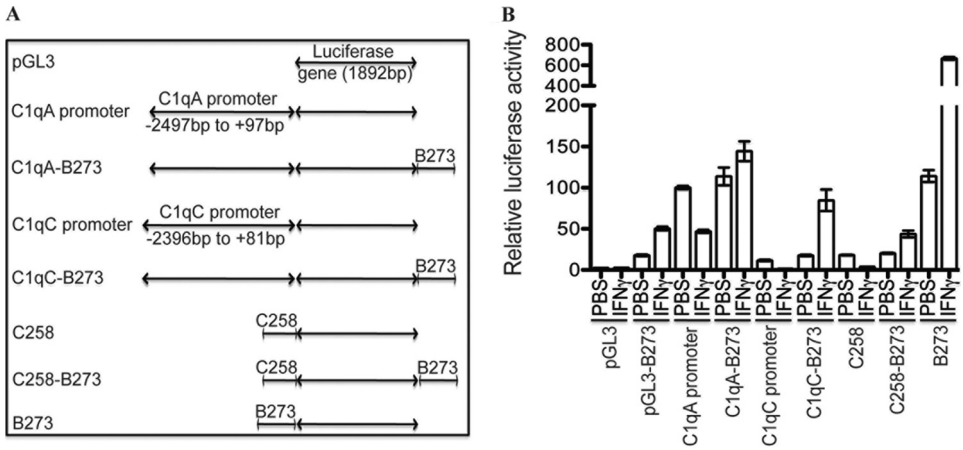

FIGURE 5. **Superseding regulation of 5′ C1qA and C1qC promoters by a 3′ B273 promoter.** *A*, schematic of expression constructs in which the B273 promoter was cloned at the 3′ end of the luciferase reporter gene that either lacked a 5′ promoter (pGL3-B273) or were flanked by a 5′ C1qA (C1qA-B273), C1qC (C1qC-B273), or shortened C1qC (C258-B273) promoters. Control plasmids were also illustrated. *B*, the basal and IFNγ-stimulated activities of these constructs were compared with the control constructs. Data were presented as mean ± S.D. of triplicate experiments.

entation of the B273 promoter relative to the C1qA and C1qC genes, and the results suggest that the B273 promoter, which may fall in the usual C1qB promoter region, may actually act as a core promoter element for all three C1q genes *in vivo*.

PU.1 and IRF8 Are Required for C1qB Promoter to Respond to IFNγ—ISRE and GAS, which form an essential region of the 53-bp element (Fig. 4*C*), are well defined IFNγ-stimulated elements (33–35), and, in some genes, they appear as either ISRE-GAS or GAS-ISRE chimera sequences to confer IFNγ responsiveness (36, 37). IRF1, IRF8, STAT1, and PU.1 interact with some forms of these elements (33–35), and these were evaluated for their involvement in C1qB promoter regulation firstly by shRNA knockdown. RAW264.7 cells were transfected with the B273 promoter and co-transfected with IRF1, IRF8, PU.1, or STAT1 shRNA plasmid, and the knockdown of each transcription factor in these transfected RAW264.7 cells was validated by Western blotting (Fig. 6). For PU.1 and IRF8, two different shRNA plasmids were used.

Knockdown of IRF1 in RAW264.7 cells showed no inhibition to the IFNγ -stimulated activity of the B273 promoter (Fig. 6*A*). On the contrary, it slightly but consistently increased B273 response to IFNγ. Knockdown of STAT1, IRF8, or PU.1 all markedly impaired B273 response to IFNγ (Fig. 6). Therefore, these three transcription factors are required for the C1qB promoter to respond to IFNγ.

PU.1 and IRF8 Bind to 53-bp IFNγ-stimulated Element—To determine whether PU.1, IRF8, and STAT1 directly interact with the C1qB promoter, we synthesized the 53-bp element as a double-stranded fragment to examine whether it was able to pull down these transcription factors from the nuclear extracts. It was synthesized with a 3′-biotin tag and then immobilized on streptavidin-Sepharose resins. From the nuclear extracts of untreated macrophages, none was co-precipitated (Fig. 7). However, when the macrophages were treated with IFNγ for 24 h, IRF8 and PU.1 were both co-precipitated with the 53-bp element (Fig. 7). IRF1 and STAT1 remained absent from these precipitates. As a control, the resins without the 53-bp element immobilized failed to pull down any of these transcription factors. These results show direct IRF8 and PU.1 interaction with the 53-bp element and also suggest that the role for STAT1 in C1qB promoter regulation is indirect.

STAT1 Regulates IRF8 Expression—A likely mechanism by which STAT1 may regulate the C1qB promoter, without direct association with the 53-bp element, is its ability to regulate transcription factors (*e.g.* PU.1 or IRF8) that directly interact with this element. PU.1 was found constitutively expressed in RAW264.7 cells (Fig. 6), but IRF8 showed little expression unless the cells were stimulated with IFNγ (Fig. 8). In fact, it is known that IRF8 expression is regulated by STAT1 (38).

Whether STAT1 indeed regulates IRF8 expression in RAW264.7 cells was assessed using shRNA. IRF8 mRNA was effectively induced in IFNγ-treated RAW264.7 cells, and this was effectively knocked down when IRF8 shRNA was co-transfected (Fig. 8). As a control, it was not affected by scramble shRNA. This IFNγ-induced IRF8 mRNA expression was also suppressed by STAT1 shRNA, showing that STAT1 is required for IRF8 induction by IFNγ in RAW264.7 cells. Therefore, PU.1 and IRF8 directly interact with the C1qB promoter upon IFNγ stimulation, but IRF8 expression is dependent on IFNγ-induced STAT1 signaling.

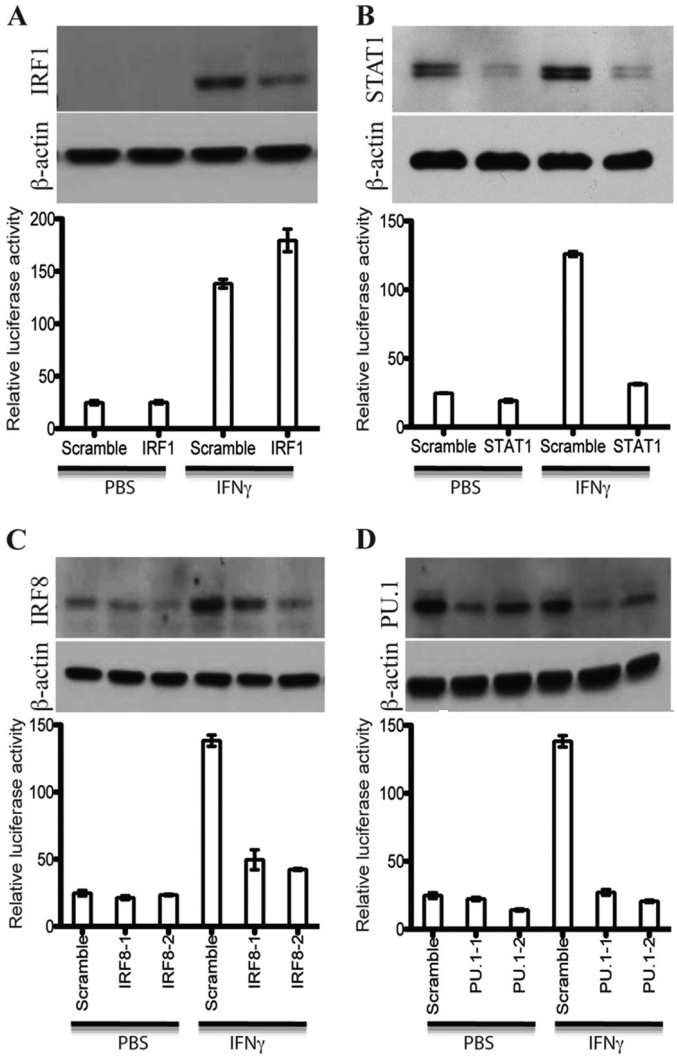

FIGURE 6. **Roles of PU.1, IRF8, STAT1, and IRF1 in IFNγ stimulation of the C1qB promoter.** RAW264.7 cells were transfected with the B273 promoter and also co-transfected with plasmids encoding shRNA for mouse IRF1 (*A*), STAT1 (*B*), IRF8 (IRF8–1, IRF8–2; *C*), PU.1 (PU.1-1, PU.1-2; *D*) or, as controls, with plasmids for scramble shRNA. Basal and IFNγ-stimulated activities were determined after the knockdown of these transcription factors. The knockdown of IRF1, STAT1, IRF8, and PU.1 expression was verified by Western blotting (*upper panels*). As a control, the expression of β-actin was monitored (lower panels). The luciferase data were presented as mean ± S.D. of triplicate experiments.

Association of Endogenous PU.1 and IRF8 with C1q Promoters—Whether PU.1 and IRF8 bind to the C1qB promoter *in vivo* was examined by ChIP using macrophages. After formaldehyde fixation, nuclei were isolated from macrophages, sheared, and immunoprecipitated using PU.1, IRF8, STAT1, or IRF1 antibodies. C1qB promoter sequences were sought in the precipitated chromatin fragments by PCR using primers spanning −167 and −31 bp, which contained the 53-bp element. C1qA and C1qC gene promoter sequences were also PCR-amplified from the precipitated chromatin fragments, *i.e.* C1qA (−122 to + 79 bp) and C1qC (−110 to +81 bp). Immunoprecipitation of IRF1 and STAT1 failed to co-precipitate any of the three C1q gene promoters regardless of IFNγ treatment (Fig. 9).

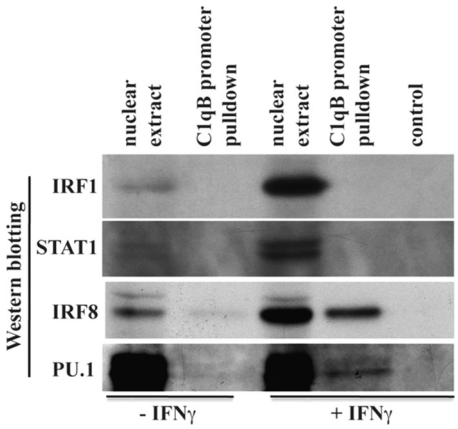

FIGURE 7. **Binding of PU.1 and IRF8 to a 53-bp fragment of the C1qB promoter (−133 to −81 bp).** This 53-bp DNA fragment was synthesized with 3′-biotin tag, which was then immobilized on streptavidin-Sepharose resins. Nuclear extract was prepared from untreated (PBS) and IFNγ-stimulated (IFNγ) RAW264.7 cells and then incubated with the immobilized 53-bp DNA fragment. After washing, the precipitated proteins were analyzed by Western blotting to detect IRF1, STAT1, IRF8, and PU.1 with specific antibodies. As a positive control, nuclear extract (10% of the input amount) was also included in the blots. Proteins precipitated with streptavidin-Sepharose without the 53-bp DNA fragment were used as negative controls.

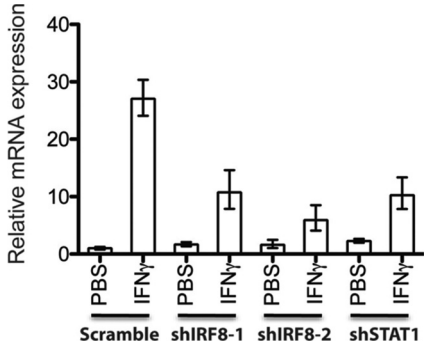

FIGURE 8. **STAT1 is required for IFNγ-stimulated IRF8 expression.** IRF8 mRNA was detected in RAW264.7 cells after transfection with IRF8 shRNA (shIRF8–1 and shIRF8–2) or STAT1 shRNA plasmids (shSTAT1). As a control, the cells were transfected with scramble shRNA plasmid. Cells were either untreated (PBS) or IFNγ-stimulated (IFNγ) for 24 h and IRF8 mRNA was measured by real-time PCR. Data were presented as mean ± S.D. of triplicate experiments.

With regard to IRF8, it failed to precipitate the C1qA and C1qC promoters. However, it co-precipitated with the C1qB promoter, but this only occurred after macrophages were treated with IFNγ (Fig. 9). It shows that, in live macrophages, IFNγ induces IRF8 expression enabling its binding to the C1qB promoter. PU.1 also co-precipitated with the C1qB promoter, and this was also dependent on IFNγ stimulation (Fig. 9). Regardless of IFNγ treatment, co-precipitation between PU.1 and the C1qA promoter was not detected. However, PU.1 co-precipitated with the C1qC promoter irrespective of IFNγ treatment.

Overall, the ChIP experiments validated the DNA pulldown results regarding IFNγ-dependent PU.1 and IRF8 binding to the C1qB promoter and were in line with PU.1 and IRF8 being shown to mediate IFNγ-stimulated C1qB promoter activity. The question remains how the C1qA promoter, which showed no interaction with PU.1 or IRF8, may respond to IFNγ. The implications of constitutive C1qC promoter association with PU.1 are not understood. However, our finding that the C1qB promoter potentially regulates all three C1q genes suggests contributions from multiple regions in this C1q gene cluster for the synchronization of basal and inducible expression of all three genes.

DISCUSSION

A number of reasons warrant investigation of the C1q gene promoters. First, genetic C1q deficiency, which impairs the production of functional C1q, has profound impact on the immune system as it almost certainly causes SLE. Although genetic C1q deficiency is rare, acquired C1q deficiency is more frequently found in SLE patients, which could contribute to SLE pathogenesis. For example, increased IFNα production by plasmacytoid DCs could result from reduced C1q levels (39). The reduced C1q levels could attribute partly to inflammatory consumption and anti-C1q autoantibodies (27, 28). However, C1q expression is also versatile, which is affected by diverse microbial stimuli, cytokines, hormones, and drugs (19). Second, C1q production is largely restricted to DCs and macrophages, but the transcription mechanisms are unknown. Thirdly, C1q is a precise assembly from 18 subunits, equally from three different subunits. Deficiency in a single subunit gene abolishes the production of the entire C1q molecules. This interdependence of the three subunits demands transcriptional synchronization.

We proposed two hypotheses regarding the transcriptional regulation of the three C1q genes: 1) the three C1q gene promoters contain replicated *cis*-acting elements so as to respond to common regulatory signals that are unique to DCs and macrophages, and 2) the three C1q genes lack strong independent promoters and synchronization is achieved through a superseding core promoter inside the C1q gene cluster. Our results support the second hypothesis and suggest that the C1qB promoter functions similar to such a core promoter for the cluster.

The IFNγ-stimulated C1qB promoter activity, which can regulate genes from the 3′ as well as 5′ end of coding genes, has been defined from both the *cis*- and *trans*-acting facets. First, a 53-bp region in the C1qB promoter was essential to its response to IFNγ induction. Second, this 53-bp element was found directly recognized by PU.1 and IRF8, which are two macrophage/DC-associated transcription factors and known to mediate IFNγ stimulation of target genes (33–35). The finding that STAT1, a key intracellular messenger in IFNγ receptor signaling (40), regulates the C1qB promoter through IRF8 induction appears to complete a signaling pathway that helps explain the IFNγ-induced C1qB gene expression (Fig. 10). Our data suggest that, through the C1qB promoter, the C1qA and C1qC genes are similarly and coordinately regulated. This "core promoter" concept reconciles with the highly conserved clustering of the three C1q genes (32).

Although these data help explain the synchronized induction of the three C1q genes by IFNγ, these data are insufficient to understand how the basal expression of the three C1q genes is coordinated. The ability of a 3′ C1qB promoter to regulate the activity of 5′ C1qA and C1qC promoters across the coding gene implies that C1qB promoter somehow also synchronizes the basal expression of the three C1q genes.

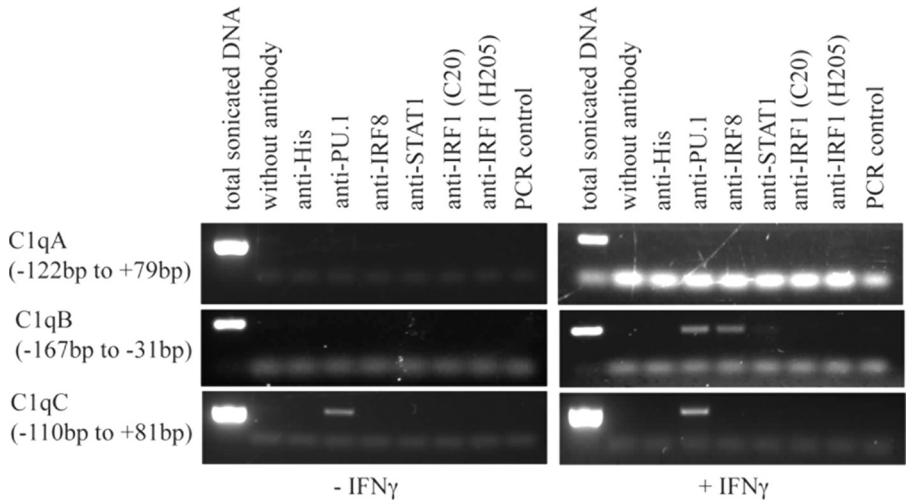

FIGURE 9. **Endogenous PU.1 and IRF8 association with the C1q gene promoters.** Macrophages were cultured from human blood monocytes and fixed with formaldehyde. Nuclear fraction was harvested and sonicated to generate nuclear lysate. The chromatin fragments were incubated with protein G-Sepharose immobilized with antibodies specific for IRF1, IRF8, STAT1, and PU.1. As negative controls, immunoprecipitation was performed with anti-His antibody or without antibody. Precipitated chromatin fragments were eluted, and DNA was extracted for PCR detection of specific C1qA, C1qB, and C1qC promoter regions. DNA isolated from sonicated nuclei before immunoprecipitation was used as a positive control. PCR products were examined on 1% agarose gels.

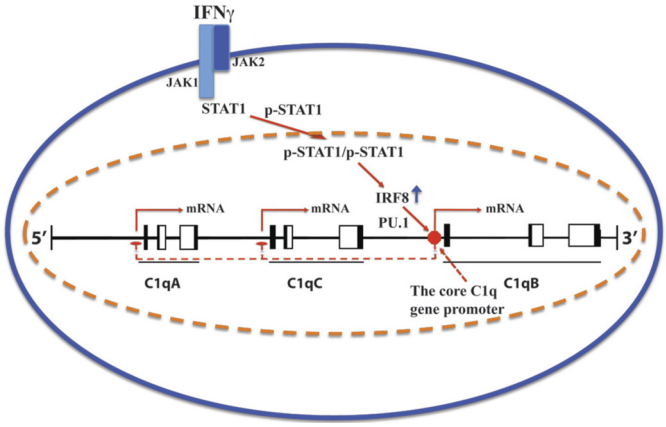

FIGURE 10. **A proposed core promoter function for the C1qB promoter.** The B273 promoter falls in the classic promoter region of its downstream C1qB gene. However, its upstream C1qA and C1qC genes lack the activities expected from results obtained with the endogenous genes. The fact that the B273 promoter is able to supersede these expected C1qA and C1qC gene promoters from the 3′ ends, across the luciferase reporter gene, suggests B273 regulation of C1qA and C1qC gene expression from the 3′ ends of these genes. This model explains the highly conserved chromosomal clustering and synchronized transcription of the three C1q genes.

C1q is preferentially, if not exclusively (41), produced by DCs and macrophages. The finding that PU.1 and IRF8 play key roles in the regulation of C1q gene expression help explain DC and macrophage production of C1q. First, PU.1 is a key transcription factor in DCs and macrophages, which is required for lineage commitments and is not significantly associated with other cell lineages (42, 43). Second, IRF8 is also strongly associated with macrophage and DC development and functions (44). In zebrafish, in which the three C1q genes are in even denser cluster (32), IRF8 is a definitive developmental switch in favor of macrophages over neutrophils (45).

In this context, it must be noted that whether PU.1 and IRF8 interact with wider regions of the three promoters has not been assessed. For example, PU.1 appears to associate with the C1qC promoter regardless of IFNγ stimulation (Fig. 9). More extensive ChIP studies may result in a comprehensive map of PU.1/IRF8-binding regions in the three promoters and help understand DC/macrophage-associated expression of C1q.

C1q is a prototype for non-collagen proteins that contain Gly-Xaa-Yaa collagen-like regions in their primary amino acid sequences. These collagen-like regions determine and facilitate the polymerization of multiple subunits into large molecular structures. However, C1q remains distinct from the rest of these other molecules for its assembly from three different subunits. Cbln1 and adiponectin are similar to C1q in primary sequences over both the collagen and non-collagen regions, but they are each assembled from a single type of subunit encoded by a single gene (46, 47). The sequence similarity between C1q and collectins and ficolins is limited to the collagen-like region and these C1q-like molecules are also each assembled from a single type of subunit (3, 48). Therefore, synchronized transcription remains a C1q-specific affair.

We hypothesize that, by having an independent promoter for each C1q subunit gene, the transcription of the three genes is difficult to synchronize. The three independent C1q genes are likely to drift apart during evolution resulting in three distinct, C1q-like molecular identities. The highly conserved 1:1:1 assembly of C1q from its three subunits must have offered key physiological advantages that are not compensated by C1q-like molecules assembled from a single type of C1q subunit. The fact that deficiency in a single gene is able to cause SLE (49) testifies the essentiality of this 1:1:1 assembly of C1q to the host immune system. C1q therefore offers an excellent model for dissecting SLE pathogenesis and a genomic template for understanding synchronized gene transcription.

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