

indicate an elevated risk for HCC in patients with chronic HCV infection.

Hu et al. [36] found that an AFP level of 15.0 mg/mL could detect severe fibrosis with a sensitivity of 22.8% and specificity of 94.5%. Moreover, they reported, during observation for 6 months of patients with chronic hepatitis C, that AFP levels stayed within the normal range (<10 ng/mL) in 60%, were persistently elevated in 24%, and fluctuated in the remaining 15%. By multivariate analysis, they identified AST, INR, and fibrosis as risk factors for AFP levels of >10 ng/mL. In view of the correlation between AFP levels and fibrosis stages, the AFP level at the time of liver biopsy was taken into account in the analysis in the present study; ALT levels are reported to be persistently elevated in the majority (60%) of patients with chronic hepatitis C.

Liver biopsy is the gold standard for assessing hepatic fibrosis [8, 9]. However, the needle liver biopsy has a sampling error and is too invasive as a routine procedure [10, 11]. Therefore, AFP levels may be used as a noninvasive and predictive marker in place of the fibrosis stage. The platelet count is known to reflect the severity of chronic hepatitis C [12, 13], and is used to estimate the degree of fibrosis without resort to liver biopsy [12–14]. Previous reports have shown low platelet counts to represent a risk factor for HCC in cirrhotic patients [13, 15, 16]. Matsumura et al. [13] reported that age and serum platelet count were significant risk factors for the development of HCC, and as such, they were a major clinico-laboratory means of evaluating the fibrosis stage. In the present study, however, the platelet count was not an independent risk factor for HCC development. When Cox regression analysis was performed on variables other than the fibrosis stage, platelet count and serum albumin levels were identified as independent risk factors for the development of HCC (data not shown).

IFN has been used to treat patients with HCV infection. Failure to achieve an SVR to IFN-based therapies, and preexisting advanced hepatic fibrosis and/or cirrhosis, are major predictors of HCC [6, 23, 25, 39, 40]. In the present study, SVR emerged as an independent risk factor for the development of HCC, while non-SVR was not. However, the cumulative incidence rate of HCC in patients with non-SVR was lower than that in those without IFN therapy. These results suggest that the use of IFN in patients with HCV-related liver disease may be beneficial in preventing the development of HCC. Several Japanese cohort studies have demonstrated that IFN therapy reduces the incidence of HCC, not only in sustained virological responders but also in transient responders who have failed to eliminate HCV [6, 41–45]. In cirrhotic patients, Nishiguchi et al. [39] reported that the relative risk of patients with IFN- $\alpha$  treatment developing HCC was 0.067 in comparison with the control

group. In contrast, Valla et al. [46] could not prove any significant benefit for the prevention of HCC between patients with and without IFN treatment. Camma et al. [47] suggested a slight preventive effect of IFN on HCC development in patients with HCV-related cirrhosis. Shiffman et al. [48] have reported that continuous IFN therapy led to a decline in hepatic fibrosis despite the persistence of viremia. In addition, there are case reports that IFN therapy reduced AFP levels in virological nonresponders [49]. Murashima et al. [50] showed that IFN therapy, but not Strong Neo-Minophagen C (SNMC) (Glycyrrhizin, Tokyo, Japan), universally reduced basic AFP levels. In an *in vitro* study of the effects of IFN on an HCC cell line, IFN exhibited anti-tumor effects [51]. Taken together, these findings suggest that AFP levels may be useful for predicting the development of HCC during IFN-based treatments, including long-term low-dose IFN therapy.

There have been several reports on the relationship between chronological trends in platelet counts, AST or AFP levels, and the development of HCC [11, 26, 27, 52–54]. Tarao et al. [52, 53] showed that in patients with HCV-related cirrhosis, those with persistently high serum ALT levels had a high risk of developing HCC and multicentric carcinogenesis, whereas those with persistently low ALT levels faced a very low risk. Likewise, the dynamics of AFP levels in patients with chronic HCV infection may be useful to estimate the risk of developing HCC. Recently, Bruce et al. [32] found serial measurements of AFP helpful in identifying persons with advanced fibrosis. They used an AFP level of 8 ng/mL, the test manufacturer's upper limit of normal, as the evaluation of the risk of development of HCC. It is not certain whether or not AFP would be a risk factor of HCC development in patients with chronic liver disease of etiologies other than persistent HCV infection. Velazquez et al. [55] reported that an AFP level of >5 ng/mL at study entry was associated with the development of HCC in their univariate analysis but not in their multivariate analysis. They speculated that this could have been because the main causative factor of liver cirrhosis in their series was alcohol. Taken together, the findings of various studies suggest that the baseline AFP level may be more reliable as a predictive factor for the development of HCC in patients with HCV-related liver disease than in those with liver disease of other etiologies.

In conclusion, AFP is a noninvasive predictive marker for the development of HCC in patients infected with HCV. The present study indicates that not only high AFP levels ( $\geq 20$  ng/mL) but also slightly elevated AFP levels, between 6 and 20 ng/mL, could indicate substantial risks for the development of HCC, complementing the fibrosis stage. In contrast, AFP levels of <6 ng/mL indicate a low risk of HCC development, irrespective of the liver fibrosis stage. IFN therapy significantly reduces the risk of the

development of HCC, especially in patients with an SVR to the therapy.

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# Higher-Order Chromatin Regulation and Differential Gene Expression in the Human Tumor Necrosis Factor/Lymphotoxin Locus in Hepatocellular Carcinoma Cells

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**The three-dimensional context of endogenous chromosomal regions may contribute to the regulation of gene clusters by influencing interactions between transcriptional regulatory elements. In this study, we investigated the effects of tumor necrosis factor (TNF) signaling on spatiotemporal enhancer-promoter interactions in the human *tumor necrosis factor (TNF)/lymphotoxin (LT)* gene locus, mediated by CCCTC-binding factor (CTCF)-dependent chromatin insulators. The cytokine genes *LT $\alpha$* , *TNF*, and *LT $\beta$*  are differentially regulated by NF- $\kappa$ B signaling in inflammatory and oncogenic responses. We identified at least four CTCF-enriched sites with enhancer-blocking activities and a TNF-responsive TE2 enhancer in the *TNF/LT* locus. One of the CTCF-enriched sites is located between the early-inducible *LT $\alpha$ /TNF* promoters and the late-inducible *LT $\beta$*  promoter. Depletion of CTCF reduced *TNF* expression and accelerated *LT $\beta$*  induction. After TNF stimulation, via intrachromosomal dynamics, these insulators mediated interactions between the enhancer and the *LT $\alpha$ /TNF* promoters, followed by interaction with the *LT $\beta$*  promoter. These results suggest that insulators mediate the spatiotemporal control of enhancer-promoter associations in the *TNF/LT* gene cluster.**

Chromosomal regions harboring different tissue-specific or cellular-state-specific gene clusters may be influenced by long-range regulatory elements and higher-order chromatin organization (45, 53, 60). Recent studies suggest that transcriptional regulatory elements, such as enhancers, promoters, and chromatin insulators, contribute to gene activation and inactivation via genome accessibility and chromosomal interactions (8, 18). Among these, chromatin insulators are boundary elements that partition the genome into chromosomal subregions, probably through their ability to block interactions between enhancers and promoters when positioned between them (enhancer-blocking effect) (7, 17, 41). However, the precise mechanisms responsible for the enhancer-blocking effect and the relationship with long-range chromatin interactions remain unclear (47, 49). The CCCTC-binding factor CTCF is a highly conserved 11-zinc-finger protein that plays crucial roles at insulator sites (44). CTCF is also reported to function in transcriptional activation (62, 73) and repression (16, 36). In the *IGF2/H19* locus, CTCF binds to the differentially methylated region (DMR) of the *H19* gene to form a predicted chromatin loop structure (6, 22, 42). Genome-wide analyses identified the distribution of the putative CTCF-binding sites and their consensus sequences (4, 27, 28, 69). We and other groups recently determined that CTCF is enriched with cohesin in at least 14,000 sites on the human genome (46, 54, 65). CTCF and cohesin cooperatively form compact chromatin loops, leading to the colocalization of gene promoters and their common enhancer in the human *apolipoprotein* gene locus (40). CTCF has been reported to interact with nuclear substructures (71, 72), chromatin remodeling factors (26, 33), RNA polymerase II (10), and CTCF itself (34, 72), as well as undergoing several posttranslational modifications of the protein (12, 29, 37, 70).

Inflammation involves the activation of a highly coordinated gene expression program (43). The tumor necrosis factor (TNF) superfamily members, TNF (initially termed TNF- $\alpha$ ), lymphotoxin- $\alpha$  (LT $\alpha$ , also termed TNF- $\beta$ ), and lymphotoxin- $\beta$  (LT $\beta$ ), are major proinflammatory cytokines that mediate inflammatory responses in autocrine/paracrine manners (63). TNF and LT $\alpha$  form homotrimers and act as soluble ligands for the TNF receptor. In contrast, LT $\beta$  forms a heterotrimer with LT $\alpha$  and functions as a membrane-bound ligand for the LT $\beta$  receptor. In addition to their physiological roles, the aberrant or unbalanced expression of these cytokines is linked to pathological conditions, such as tissue damage/remodeling (38), metabolic diseases (14, 20), and cancer development (19, 23). Hepatic TNF expression is closely related to steatohepatitis (64), and LT $\beta$  expression is significantly involved in liver regeneration (3) and hepatocellular carcinomas (HCCs) (23, 67). The *TNF/LT* genes are clustered within the major histocompatibility complex (MHC) class III region on human chromosome 6p21.3, which is the most gene-dense region of the human genome (68). Interestingly, it is reported that NF- $\kappa$ B does not directly interact with the proximal human TNF promoter (9, 15, 59) and that NF- $\kappa$ B activation induced by TNF treatment in-

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fluences expression of the *TNF/LT* genes, resulting in the amplified inflammatory response (25). Several DNase-hypersensitive sites, generally suggestive of the presence of regulatory elements, have been found in the *TNF/LT* locus (5, 50, 56, 58). However, a transcriptional mechanism and higher-order chromatin regulation in the human *TNF/LT* locus are unknown.

Investigation of the *TNF/LT* locus identified at least four CTCF/cohesin-enriched insulators and a TNF-responsive TE2 enhancer in human hepatic cells. These CTCF-bound sequences possessed enhancer-blocking activities, and one of the insulators was located between the early-inducible *LT $\alpha$ /TNF* promoters and the late-inducible *LT $\beta$*  promoter. Chromosome conformation capture (3C) analyses determined that after TNF stimulation, these CTCF-bound insulators initially associated with the TE2 enhancer and the *LT $\alpha$* , *TNF*, and *LT $\beta$*  promoters, followed by a persistent interaction with the TC3 insulator, the TE2 enhancer, and the *LT $\beta$*  promoter. These late-phase interactions were consistent with the formation of a place in which the late-inducible *LT $\beta$*  gene was transcriptionally active. TNF stimulation thus induces dynamic changes in higher-order chromatin organization of the overall locus, together with differential expression of the *TNF/LT* genes. Based on our findings that insulators mediate the spatio-temporal control of enhancer-promoter interactions, we propose a dynamic chromatin conformation model and enhancer-blocking mechanism mediated by insulators in the *TNF/LT* locus.

## MATERIALS AND METHODS

**Cell culture.** Hep3B, HCT116, and HeLa cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F-12 nutrient medium (DMEM/F12; Sigma) supplemented with 10% (vol/vol) fetal bovine serum (FBS). NeHepLxHT cells were cultured in DMEM/F12 supplemented with 10% (vol/vol) FBS,  $10^{-7}$  M dexamethasone,  $10^{-7}$  M insulin, and 50  $\mu$ g/ml G418. For TNF stimulation, Hep3B and NeHepLxHT cells were treated with recombinant human TNF- $\alpha$  (210-TA; R&D Systems) at concentrations of 5 ng/ml and 0.5 ng/ml, respectively. For inhibition of NF- $\kappa$ B signaling, BAY11-7082 (10  $\mu$ M) was added to the medium for 1 h before treatment of the cells with TNF for 0.5 or 1 h.

**ChIP and quantitative PCR (qPCR) analysis.** Hep3B and NeHepLxHT cells were cross-linked with 1% formaldehyde at 37°C for 10 min. Crude cell lysates were sonicated to generate DNA fragments of 200 to 500 bp. Chromatin immunoprecipitation (ChIP) was performed with anti-CTCF (07-729; Millipore), anti-RAD21 (ab992; Abcam), anti-acetylated histone H3 (06-599; Millipore), anti-acetylated histone H4 (06-866; Millipore), anti-p65 (sc-372; Santa Cruz), anti-p300 (sc-585; Santa Cruz), or anti-RNA polymerase II (phosphor-S5) antibodies (ab5131; Abcam) or with control rabbit IgG (sc-2027; Santa Cruz) (26). Cells were cross-linked for an additional 10 min when anti-p65 and anti-p300 antibodies were used.

DNA enrichment in ChIP samples was determined using qPCR analysis with an ABI Prism 7300 system (Applied Biosystems) and SYBR green fluorescence. The threshold was set to cross a point where PCR amplification was linear, and the cycle number required to reach the threshold was recorded and analyzed using the Microsoft Excel software program. PCR was performed using precipitated DNA and the input DNA. Primer sequences are listed in Table S1 in the supplemental material. Other antibodies used were anti-lamin A/C (sc-7292; Santa Cruz).

**Electrophoretic mobility shift assay (EMSA).** The CTCF protein was synthesized using a coupled *in vitro* transcription/translation reaction with the TNT T7 Quick system (Promega), according to the manufacturer's protocol. For supershift assays, the reaction mixture was combined with 1  $\mu$ l anti-CTCF antibodies (612148; BD Biosciences) (40). The sequences of the probes were as follows: H19 DMR, 5'-TGG CAC GGA ATT

GGT TGT AGT TGT GGA ATC GGA AGT GGC CGC GCG GCG GCA GTG CAG GCT CAC ACA TCA CAG CCC GAG CCC GCC CCA ACT-3'; TC1, 5'-TCT CCA GCA CTT CTT GCT CAG GCA GCA CCC AAA GGG GCC GCC TGG GAG CAG CAG AGA CCA GGC CCA AAG CTG CGG GCT TAC AAC AGG TTA GCC ATC CCA-3'; TC2, 5'-AGA CCC TGG TGT CCT CTC TGG CCT TAT T TA CTC CTG GTC CTC TGC CAG CCC TGC CAC CAG ATG GCC TTC TAA CTC CTT GGT TGA AAG GCC CAT CTC ATT C-3'; TC3, 5'-CCC GGT ACA GAG AGC TGC GCA GCG TGA CCG AGC GG CCC TGG GGG TCC CCG CCG CCA GGG GGC GCC CGG CCC CGG TAG CCG ACG AGA CAG TAG AGG-3'; TC4, 5'-CTT CAC CCA GGT CTC TCC AGA GAG CCT CAG GCC GCT GCC TTT ACT TAG TTC TGT GTT CAA TGC CAG AAT GCT GCC TCC TAC AGG AAG TCC ACC TGT ATT GCC CAC ACC TCC T-3'; negative control, 5'-TGG CAA AAA GAA AGG ACA GGG CTG CAA GGA GAG TAC AGA CAT GTG CTG GTG AGT GCA CTG TCT GCA TAG TTA CAC CAG AGC ATC TTA TCA ATC AGA AAC TTA TC-3'.

**Luciferase reporter assay.** The reporter vector pIHLE consisted of the *luciferase* gene driven by the mouse *H19* promoter (-818 to +6 from the transcription start site), simian virus 40 (SV40) enhancer, and a 1.8-kb AatII-HindIII fragment containing the *H19* DMR insulator. The plasmid pIHLE was constructed by inserting the 1.8-kb *H19* DMR fragment between the *luciferase* gene and the enhancer. pIHLE plasmids were constructed by inserting fragments of about 200 bp, including TC1, TC2, TC3, and TC4, between the *luciferase* gene and the enhancer (pIHLE-1F/1R, -2F/2R, -3F/3R, and -4F/4R, respectively). For pIHLET, TC fragments were inserted downstream of the enhancer in pIHLE (pIHLET-1F/1R, -2F/2R, -3F/3R, and -4F/4R). To prepare pIHLE with mutations (pIHLE-1 M, -2 M, -3 M, and -4 M), base substitutions were introduced in CTCF consensus sequences at the TC1, TC2, TC3, and TC4 sites using a PCR-based mutagenesis method.

The reporter vector pPL consisted of the SV40 promoter and the *luciferase* gene and is identical to the pGL3-Promoter vector (Promega). pTPL, pAPL, and pBPL contained the *TNF* promoter (-1044 to +54 from the transcription start site), *LT $\alpha$*  promoter (-924 to +43 from the transcription start site), and *LT $\beta$*  promoter (-971 to +12 from the transcription start site), respectively, instead of the SV40 promoter of pPL. TE1 and TE2 sequences were PCR amplified and inserted upstream of pPL, pTPL, pAPL, and pBPL (pTE1-PL, pTE2-PL, pTE1-TPL, pTE2-TPL, pTE2-APL, and pTE2-BPL). The primer sequences used to prepare the TE1 and the TE2 sequences were as follows: TE1-S, CCT GTG GCT GGA TGA AAT CT; TE1-AS, CCT GGG CAA CAA AGT GAG AC; TE2-S, CCA GGG GAG TTG TGT CTG TAA; TE2-AS, GCA GTT CGG TTC CTT GTT CT.

Reporter vectors (0.05 pmol) were transfected into Hep3B cells ( $1.0 \times 10^5$  cells) in a 12-well plate, using FuGene6 reagent (Roche Applied Science), and analyzed using a luciferase reporter assay system (Promega) after 24 h. For dual luciferase activities (26), values are shown as means and standard deviations of the results from at least three independent experiments.

**qRT-PCR.** Total RNA was isolated from cultured cells with TRIzol (Invitrogen). The cDNA synthesis used 2  $\mu$ g of total RNAs that was reverse transcribed using a High Capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions. Quantitative PCR was performed using an ABI Prism 7300 system (Applied Biosystems) and SYBR green fluorescence. Each experiment was performed at least three times. The relative fold enrichment was quantified by normalization to  $\beta$ -actin or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression. Primer sequences are listed in Table S1 in the supplemental material.

**siRNA-mediated knockdown.** Small interfering RNAs (siRNAs) for GL3, CTCF, and siRad21 were used as previously reported (40). RELA silencer select validated siRNA (s11914; Ambion) was used for p65 knockdown. siRNAs were transfected using the Lipofectamine RNAiMAX reagent (Invitrogen) for 48 h.

**3C assay.** For the chromosome conformation capture (3C) assays (21, 52), formaldehyde-cross-linked chromatin from Hep3B and NeHepLxHT cells was digested with DpnII overnight, followed by ligation with T4 DNA ligase at 16°C for 4 h. To prepare control templates for standard curves, a bacterial artificial chromosome spanning the *TNF/LT* locus RPC111.C-47E16 was digested with Sau3AI, which is insensitive to Dam methylase, followed by random religation. After reversing the cross-links, genomic DNA was purified by phenol extraction and ethanol precipitation. The ligated products were assessed using qPCR with an ABI Prism 7300 system (Applied Biosystems) and Thunderbird SYBR qPCR Mix (Toyobo). The efficiency of DpnII digestion was evaluated after the entire 3C treatment using qPCR to amplify uncut fragments spanning the DpnII site. More than 80% of the individual restriction sites were digested in these experiments. The 3C-qPCR data were normalized to a loading control, using internal primers located in the *TNF/LT* gene locus. We gained similar results after normalization with internal primers located in *GAPDH* (data not shown). The relative frequencies of interactions between the reference and its physically close site in the control state were finally normalized to 1. Examples of the calculation for relative interacting frequencies are described in Results. Statistical analysis was performed using Student's *t* test for the results of more than three independent experiments. Primer sequences are listed in Table S1 in the supplemental material.

**Immunofluorescence analysis.** Cultured human cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Fixed cells were rinsed three times in PBS for 5 min and permeabilized with PBS containing 0.2% Triton X-100 and 0.5% normal goat serum (NGS) for 5 min on ice. Cells were rinsed three times in PBS containing 0.5% NGS for 5 min and then incubated with rabbit anti-p65 (sc-372; Santa Cruz) for 60 min followed by secondary donkey Cy3-conjugated or Alexa-488-conjugated antibodies for 60 min. Labeled cells were washed three times in PBS for 10 min each. Samples were analyzed using a fluorescence microscope system (Orca-ER1394; Olympus).

**Patients and histological assessment.** A total of 38 patients (male, 29; female, 9) with HCC, who had undergone tumor resection at the National Cancer Center Hospital, Tokyo, Japan, between May 2003 and December 2005, were enrolled in the present study. The median patient age and follow-up period were 63 years and 1,719 days, respectively. Among the 38 HCC patients, 12 were immunologically positive for hepatitis C virus (HCV) infection, and 16 for persistent hepatitis B virus (HBV) infection (hepatitis B surface antigen positive), and 10 were negative for both HCV and HBV infection. Histological examination of noncancerous liver tissue samples revealed findings compatible with chronic hepatitis in 22 and cirrhosis in 9 and no remarkable histological findings in 7. The 38 HCCs were histologically classified into 3 well-differentiated, 27 moderately differentiated, and 8 poorly differentiated tumors. All patients were followed for more than 100 days. Clinical and pathological profiles were obtained from the medical records of the patients. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan, and written informed consent was obtained from all patients.

**IHC.** Immunohistochemistry (IHC) for TNF and LT $\beta$  was performed using a polymer-based method Envision+ Dual Link system-horseradish peroxidase [HRP] (DK-2600 Glostrup; Dako). Sources and dilutions of primary antibodies were as follows: anti-TNF- $\alpha$  (ab9579, 1:100; Abcam) and anti-LT $\beta$  (ab64835, 1:50; Santa Cruz Biotechnology). Formalin-fixed, paraffin-embedded serial tissue sections (4  $\mu$ m) were placed on silane-coated slides for IHC. Sections cut through the maximum tumor diameter were selected for IHC evaluation. The sections were deparaffinized and rehydrated in xylene and grade-diluted ethanol (50 to 100%) and submerged for 20 min in 0.3% hydrogen peroxide with absolute methanol to block endogenous peroxidase activity. Antigen retrieval for TNF and LT $\beta$  was carried out by heating in target retrieval solution (Tris-EDTA buffer, pH 9; Dako Cytomation) at 121°C for 10 min by a pressure cooker. After protein blocking, the sections were incubated with each primary antibody at room temperature for 1 h, followed by incubation

with Envision+Dual Link reagent at room temperature for 30 min, and visualized using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. Finally, the sections were counterstained with hematoxylin. Sections were gently rinsed in PBS between incubation steps. The primary antibody was omitted from the reaction sequence as a negative control.

All sections were evaluated by two pathologists, Y. Kanai and H. Ojima, with no knowledge of any clinical or pathological information. Immunoreactivities of TNF and LT $\beta$  were defined as follows: negative, no cytoplasmic staining was observed or the intensity of cytoplasmic staining was lower than that for noncancerous hepatocytes within the same section in more than 50% of cancer cells; positive, the intensity of cytoplasmic staining was equivalent to or higher than that of noncancerous hepatocytes in more than 50% of cancer cells.

**Statistical analysis.** Differences between groups were analyzed using Student's *t* test. A *P* value of <0.05 was considered statistically significant.

## RESULTS

**Distribution of CTCF-enriched sites in the human *TNF/LT* gene cluster.** CTCF-enriched sites in the human *TNF/LT* gene region were investigated by checking several genome-wide CTCF-binding profiles available on websites and in our published data (40, 65). At least four CTCF-enriched sites (TC1, TC2, TC3, and TC4) were identified in this locus and were conserved among the cells tested (Fig. 1A; see also Fig. S1A in the supplemental material). There were no probe sets for the TC2 site in genome tiling arrays because of the presence of frequent repeat sequences (shown by asterisks in Fig. S1A in the supplemental material). Interestingly, TC3 was located between the *TNF* and *LT $\beta$*  gene promoters, forming the possible boundary between these adjacent chromosomal subregions.

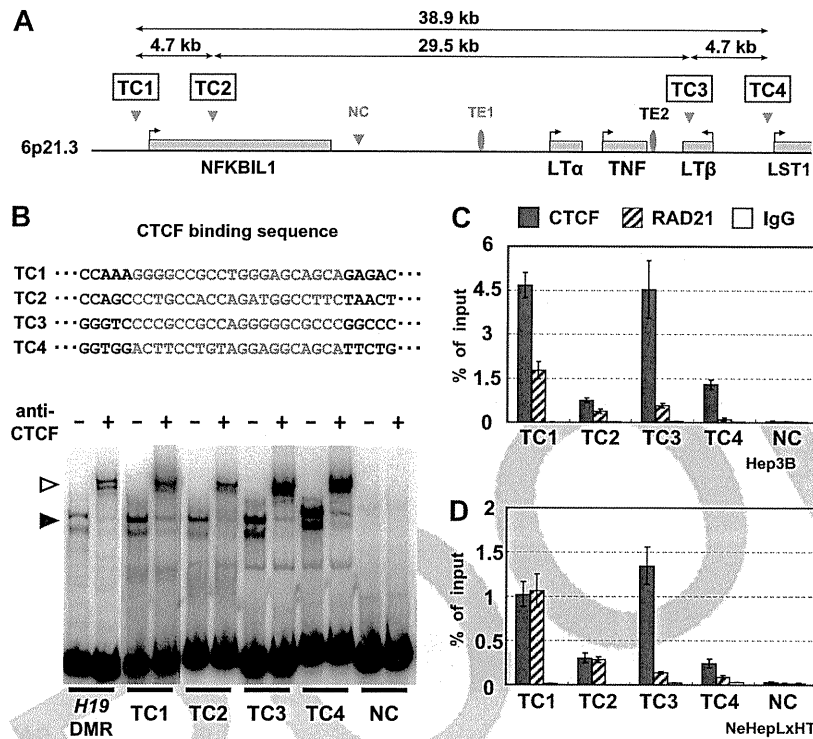
Based on previous reports (28, 69), each TC site contained a 20-bp consensus CTCF-binding motif (Fig. 1B). To determine if CTCF bound directly to these TC sequences, we performed electrophoretic mobility shift assays (EMSA) using radiolabeled duplex probes of approximately 100 bp for each TC site and the *in vitro* transcribed/translated CTCF protein. Similar to the DMR insulator of the *H19* gene used as a control (40), the TC probes formed complexes with CTCF and were further supershifted by anti-CTCF antibodies. In contrast, negative-control (NC) probes, which had sequences located downstream of the *NFKB1L1* gene, did not bind to CTCF. In addition, competition assays using mutated TC probes carrying base substitutions within the consensus motif showed that mutated probes did not bind to the CTCF protein (see Fig. S1B and C in the supplemental material), indicating that CTCF specifically bound to the TC sequences.

In order to clarify the localization of CTCF and the cofactor cohesin RAD21 in hepatic cells, we performed chromatin immunoprecipitation (ChIP) analyses using anti-CTCF and anti-RAD21 antibodies, followed by quantitative PCR (qPCR) (Fig. 1C and D). We used standard cell lines: Hep3B, which originates from human HCC, and NeHepLxHT, which is a telomerase-immortalized human neonatal hepatocyte line (51). Both CTCF and RAD21 bound to the TC sites but not to the NC site. RAD21 was relatively enriched with CTCF at TC1 in the *TNF/LT* locus. The CTCF enrichment at the TC sites in Hep3B cells may be remarkable due to the high expression of this gene (see Fig. S1D in the supplemental material) compared with that in NeHepLxHT cells.

**Differential regulation of *TNF/LT* genes under TNF stimulation.** To examine the transcriptional regulation of the *TNF/LT* genes, we performed quantitative reverse transcription (RT)-PCR



Watanabe et al.



**FIG 1** CTCF-enriched sites in the human *TNF/LT* gene cluster locus. (A) CTCF-enriched sites in the *TNF/LT* locus on human chromosome 6p21.3. In addition to the *NFKBIL1*, *LTα*, *TNF*, *LTβ*, and *LST1* genes, a newly identified TE2 enhancer is indicated by a red oval. Based on genome-wide CTCF-binding profiles available from websites and our published data (Fig. S1A in the supplemental material), four enriched sites were designated TC1, TC2, TC3, and TC4. NC is used as a negative control, and TE1 is a site with no enhancer activity. (B) Direct binding of CTCF to TC sequences. Predicted CTCF-binding sequences within TC1, TC2, TC3, and TC4 sites are indicated, together with the 20-bp consensus motif (red). For EMSAs, radiolabeled duplex probes of approximately 100 bp for each TC site were incubated with anti-CTCF antibodies and synthesized CTCF. Solid and open arrowheads indicate CTCF DNA and the supershifted complexes, respectively. The *H19* DMR insulator and an intergenic unrelated sequence (NC) were used as controls. (C and D) Existence of CTCF and the cofactor cohesin RAD21 at TC sites. Chromatin immunoprecipitation analyses were carried out with anti-CTCF (solid bars), and anti-RAD21 (hatched bars) antibodies and control IgG, followed by quantitative PCR with specific primers for each TC site in Hep3B cells (C) or NeHepLxHT cells (D).

(qRT-PCR) analyses with Hep3B and NeHepLxHT cells stimulated by TNF-induced NF- $\kappa$ B activation (Fig. 2A; see also Fig. S2A and B in the supplemental material). Expression of *LTα* and *TNF* mRNAs was markedly increased in Hep3B cells 1 h after stimulation, but *LTβ* mRNA was not simultaneously induced. Moreover, *TNF* expression seemed to be variable after the 1-h peak, while *LTα* and *LTβ* expression did not peak until 24 h after TNF treatment. Early induction of the *LTα* and *TNF* genes also occurred in NeHepLxHT cells, with subsequent expression of the *LTβ* gene. The patterns of *TNF/LT* expression differed between these cell lines, probably due to the constitutively low activation of the NF- $\kappa$ B pathway in Hep3B cells (see Fig. 4A) (11, 55).

Nuclear translocation of NF- $\kappa$ B is critical for its activation (24), and we therefore investigated its subcellular localization under TNF stimulation, using immunofluorescent staining of p65, a subunit of the NF- $\kappa$ B heterodimer (Fig. 2B; see also Fig. S2C in the supplemental material). Cytoplasmic p65 translocated to the nucleus at 30 min after stimulation, and this translocation was inhibited by the addition of BAY11-7082, a specific inhibitor of I $\kappa$ B $\alpha$  phosphorylation (48). The translocated p65 was found to decrease at 1 h after the stimulation (see Fig. S2D in the supplemental material). The expression status of the *TNF/LT* genes was analyzed in parallel using qRT-PCR analyses (Fig. 2C and D). TNF-induced expression of *TNF*, *LTα*, and *LTβ* was attenuated by NF- $\kappa$ B inhi-

bitation. Since the use of BAY11-7082 had cytotoxic effects at late time points after TNF stimulation, we carried out siRNA-mediated knockdown of p65 (see Fig. S2G and H in the supplemental material). The induction of the *TNF*, *LTα*, and *LTβ* genes was consistently inhibited by depletion of p65, indicating that the *TNF/LT* genes are regulated by NF- $\kappa$ B in the TNF-treated hepatic cells. Expression of the neighboring *NFKBIL1* gene was unaffected by the stimulation. TNF treatment caused no significant cell damage throughout the study (see Fig. S2E and F in the supplemental material). Thus, the *TNF/LT* genes are differentially induced by TNF-activated NF- $\kappa$ B signaling.

**CTCF-dependent enhancer-blocking activity in the *TNF/LT* gene locus.** Previous studies demonstrated that the *H19* DMR insulator contains multiple CTCF-binding sites, which are essential for enhancer-blocking activity (6, 22, 26). Luciferase reporter assays were performed with Hep3B cells to test the enhancer-blocking effects of TC1, TC2, TC3, and TC4 (Fig. 3). The presence of TC1, TC2, TC3, and TC4 between the enhancer and promoter reduced the luciferase activities to approximately 60% of those for the control pIHLE vector (pIHLE-1F, pIHLE-2F, pIHLE-3F, and pIHLE-4F). TC sequences in the opposite direction showed similar results (pIHLE-1R, pIHLE-2R, pIHLE-3R, and pIHLE-4R), indicating that the TC sites possess enhancer-blocking activities that are independent of the orientation of the

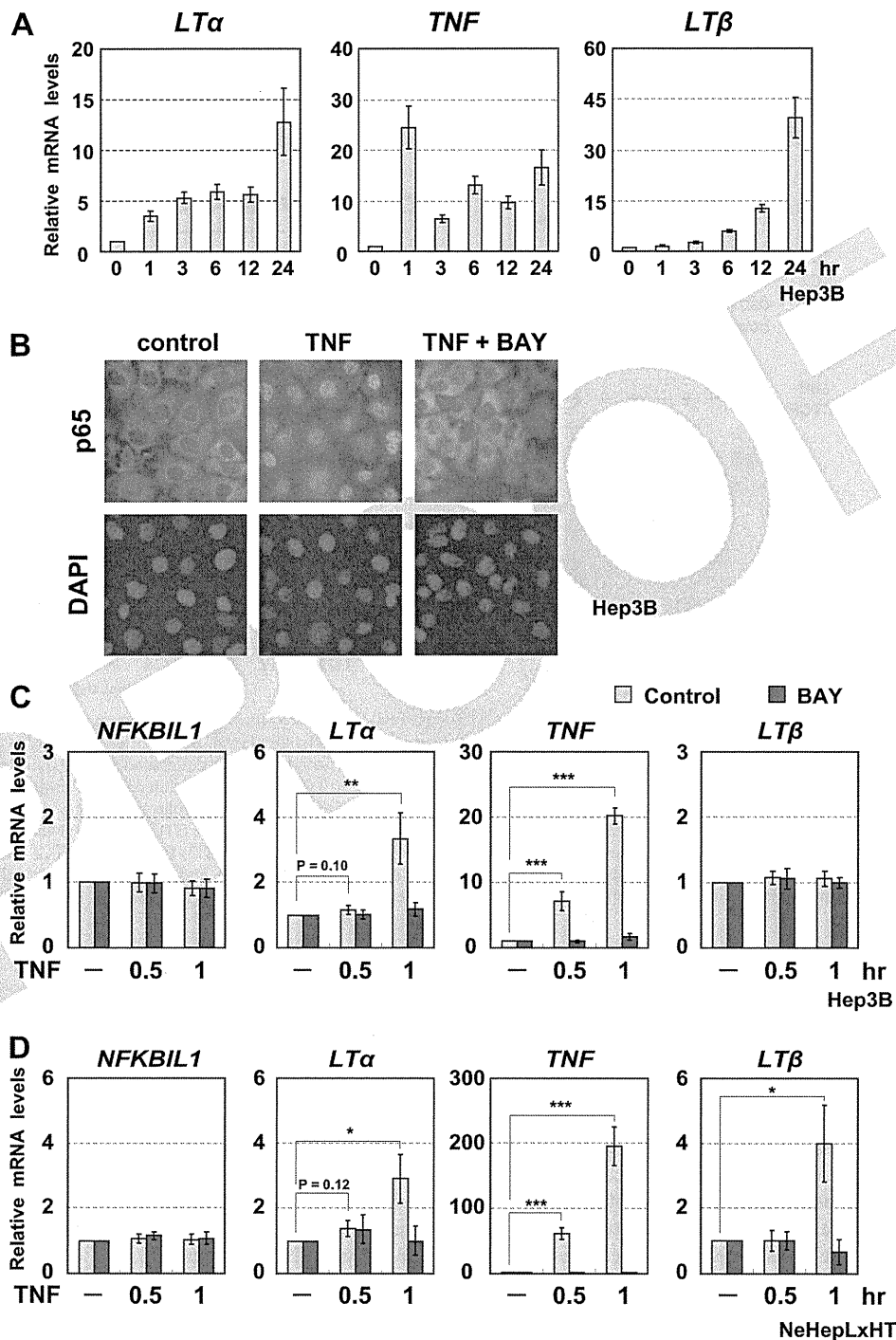


FIG 2 Differential regulation of *TNF/LT* genes under TNF stimulation. (A) Effect of TNF stimulation on *TNF/LT* expression in Hep3B cells. qRT-PCR analyses were performed with Hep3B cells under TNF treatment. (B) Nuclear translocation of NF- $\kappa$ B induced by TNF stimulation. The subcellular localization of the p65 subunit of the NF- $\kappa$ B heterodimer was analyzed by immunofluorescent staining of TNF-stimulated Hep3B cells, together with the use of BAY11-7082, an inhibitor of NF- $\kappa$ B activation. (C and D) NF- $\kappa$ B-dependent expression of the *TNF/LT* genes. TNF-induced expression of the *TNF/LT* genes was examined by qRT-PCR analyses in Hep3B (C) or NeHepLxHT (D) cells in combination with NF- $\kappa$ B inhibition. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ .

sequences. To exclude the possibility that the TC sites exhibit silencer-like activities, the TC sequences were placed downstream of the enhancer (pIHLET-1F, pIHLET-1R, pIHLET-2F, pIHLET-2R, pIHLET-3F, pIHLET-3R, pIHLET-4F, and pIHLET-4R). Lu-

ciferase activity was not reduced by TC sites in this position, suggesting that TC sites do not possess silencer-like functions. The use of mutant TC sites lacking CTCF-binding function, as described above (see Fig. S1B and 1C in the supplemental material),



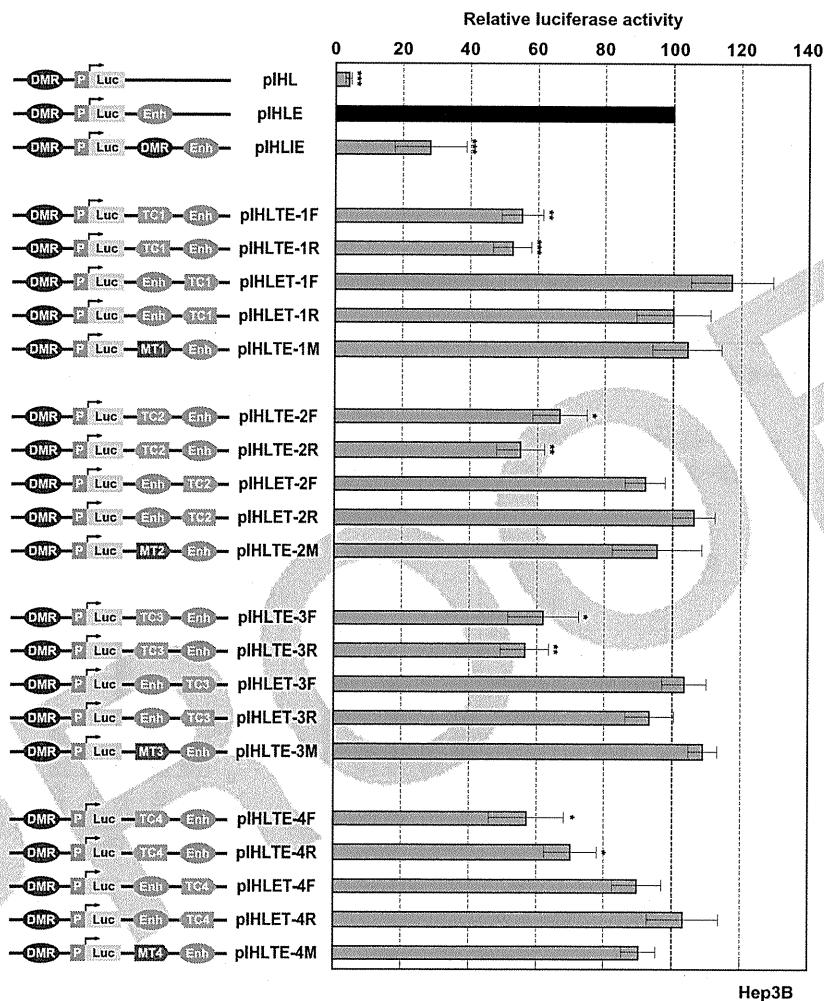


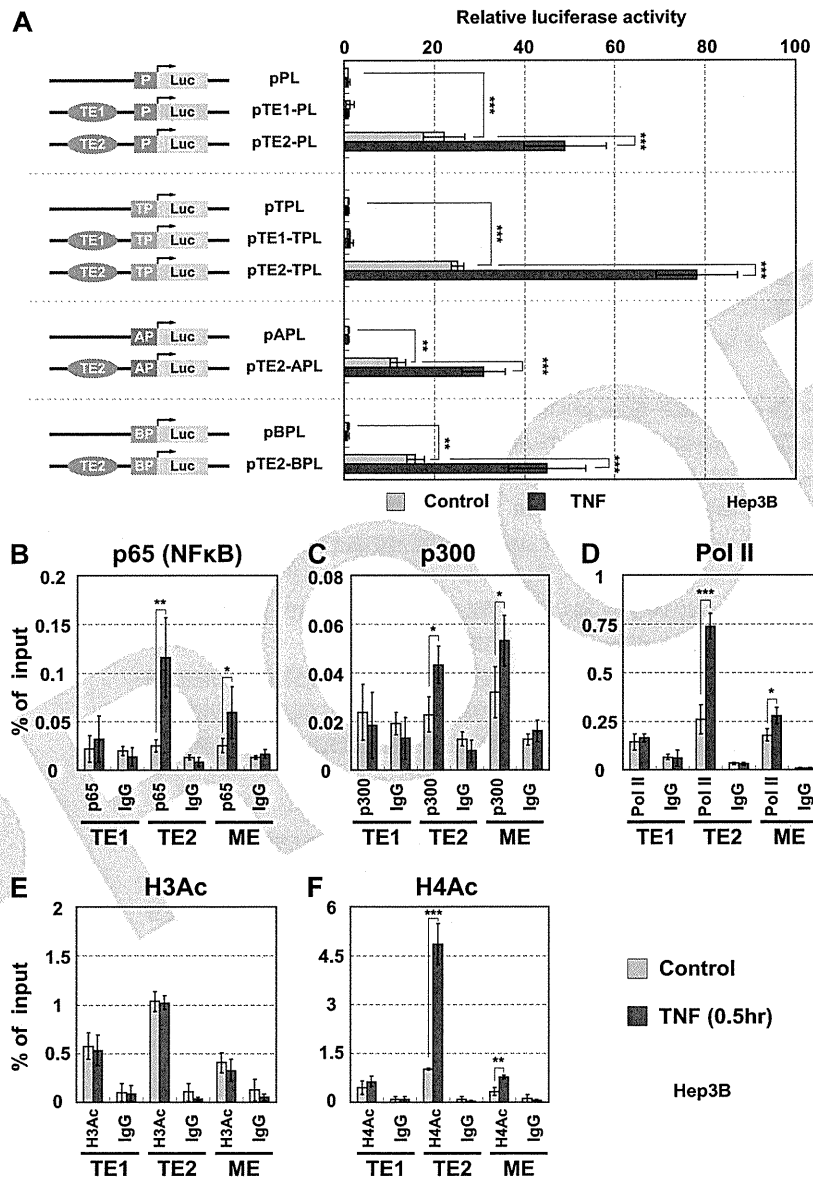
FIG 3 CTCF-dependent enhancer-blocking activity of TC sequences. pIHLE plasmids were constructed by inserting fragments of approximately 200 bp containing wild-type or mutant-type TC (lacking the CTCF binding function) between the promoter and the enhancer in pIHLE. The *H19* DMR insulator was used as a control. For pIHLET, TC fragments were inserted downstream of the enhancer in pIHLE. The luciferase activities from pIHLE were normalized to 100. The values are given as means and standard deviations of the results from more than three independent experiments. Luc, luciferase gene; P, *H19* promoter; Enh, SV40 enhancer; DMR, *H19* DMR insulator; TC1-TC4, CTCF-enriched sites; MT1 to MT4, the mutant TC sequences. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ .

demonstrated no enhancer-blocking effects (pIHLE-1 M, pIHLE-2 M, pIHLE-3 M, and pIHLE-4 M), further suggesting that the insulator activities of the TC sites depend on CTCF. These results suggest that TC1, TC2, TC3, and TC4 are functional insulators.

**Characterization of a TNF-responsive hepatic enhancer in the human *TNF/LT* locus.** In order to understand the overall regulatory mechanisms in the *TNF/LT* locus, we investigated the role of transcriptional enhancers in hepatic cells. Based on several DNase-hypersensitive sites in the locus (56), modified histones, p300 binding, previously reported enhancers (HSS-9 and HSS+3) in mouse T cells (58), and  $\kappa B$ -responsive elements conserved among humans, mice, and rats (30, 31), we chose two candidates, named TE1 and TE2, which were located about 3.5 kb upstream of the *LT $\alpha$*  gene and just downstream of the *TNF* gene, respectively (Fig. 1A; see also Fig. S1A in the supplemental material). Luciferase reporter assays were performed with Hep3B cells to determine if TE1 and TE2 act as enhancers (Fig. 4A). Compared to the con-

trol (pPL) and TE1 (pTE1-PL), TE2 significantly increased transcription from the *SV40*, *TNF*, *LT $\alpha$* , and *LT $\beta$*  promoters (pTE2-PL, pTE2-TPL, pTE2-APL, and pTE2-BPL), probably because of the constitutively low activation of NF- $\kappa B$  in Hep3B cells. Under TNF stimulation, these promoter activities were further elevated. These results indicate that TE2 has a TNF-responsive enhancing effect on the *TNF/LT* gene promoters. In addition, the effect of TE2 on the *LT $\alpha$*  promoter seemed to be weaker than that on the *TNF* promoter. The TNF-inducible enhancer activities of TE2 were also detected in other cell lines (see Fig. S3A in the supplemental material).

NF- $\kappa B$  p65 cooperates with histone acetyltransferase p300 (74), which functions as a transcriptional coactivator that accumulates in active enhancer elements (61). To validate the role of TE2 as an active enhancer, we investigated recruitment of p65 and p300 to TE2 by TNF stimulation in Hep3B cells, using ChIP-qPCR assays (Fig. 4B and 4C). A previously demonstrated enhancer of the *MCP-1* gene (ME) was used as a control (57). Recruitment of

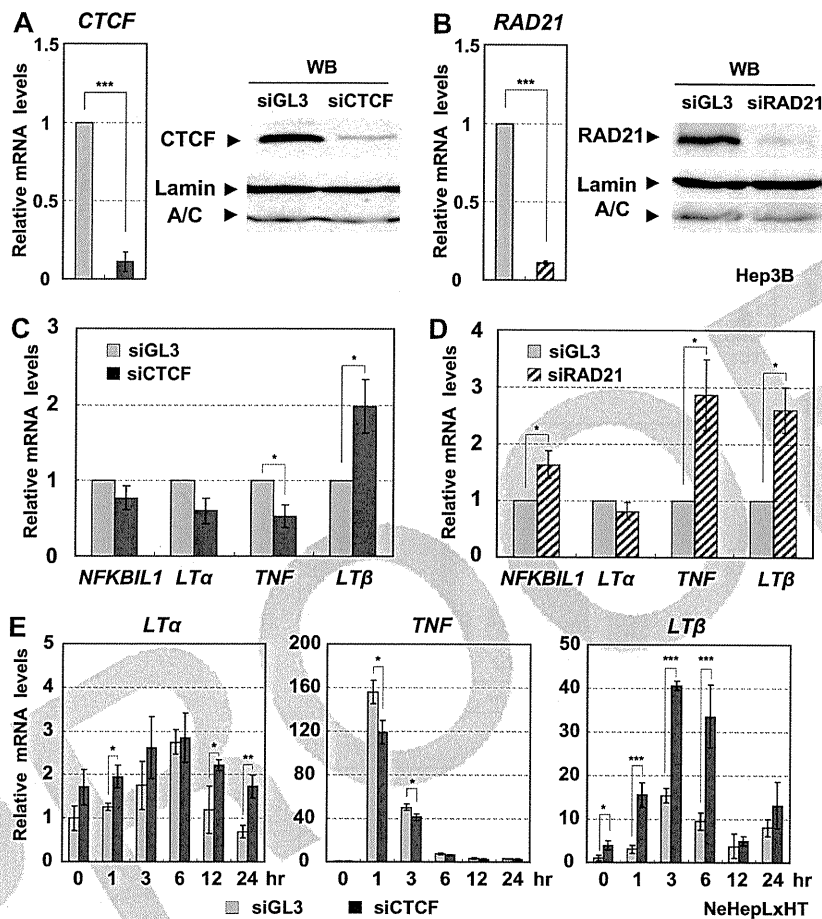


**FIG 4** Characterization of TNF-responsive enhancer in the human *TNF/LT* locus. (A) Enhancer activity of TE2. The *Luciferase* reporter vectors pPL, pTPL, pAPL, and pBPL contained the *SV40* promoter, *TNF* promoter, *LTα* promoter, and *LTβ* promoter, respectively. The candidate enhancers TE1 and TE2 were inserted in these vectors upstream of the promoter. Hep3B cells were transfected with the reporter vectors and treated with TNF for 3 h (solid bars). Luciferase activities were normalized to basal pPL, pTPL, pAPL, and pBPL. The values are given as means and standard deviations of the results from more than three independent experiments. P, *SV40* promoter; TP, *TNF* promoter; AP, *LTα* promoter; BP, *LTβ* promoter. (B to E) The chromatin state of the TE2 enhancer in TNF-stimulated Hep3B cells. ChIP assays were performed with antibodies against p65/NF-κB (B), p300 (C), RNA polymerase II (D), or acetylated histone H3 (E) or H4 (F). The *MCP1* enhancer (ME) was used as a positive control. The values are given as means and standard deviations of the results from more than three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ .

p65 and p300 to TE2 occurred at 0.5 h after TNF stimulation. Interestingly, RNA polymerase II (Pol II) and acetylated histone H4 were also significantly enriched at TE2 (Fig. 4D to F). In contrast, histone H3 acetylation showed no remarkable changes (Fig. 4E). It was previously reported that various stimuli, such as serum, interleukin 1β (IL-1β), gamma interferon (IFN-γ), and TNF induced the acetylation of histone H4 but not histone H3 (2, 13, 32). Similar data were obtained in NeHepLxHT cells (see Fig. S3B in the supplemental material). These results indicate that TE2 is an

active enhancer, which has four putative κB-binding motifs (see Fig. S3C in the supplemental material), under TNF-stimulated conditions in hepatic cells.

**CTCF and the cofactor cohesin are involved in transcriptional regulation in the *TNF/LT* gene cluster.** RNA interference-mediated knockdown in Hep3B cells was used to determine if CTCF and cohesin, which are enriched at the TC insulators, were involved in transcriptional regulation in the *TNF/LT* locus. Western blot and qRT-PCR analyses showed that CTCF and RAD21



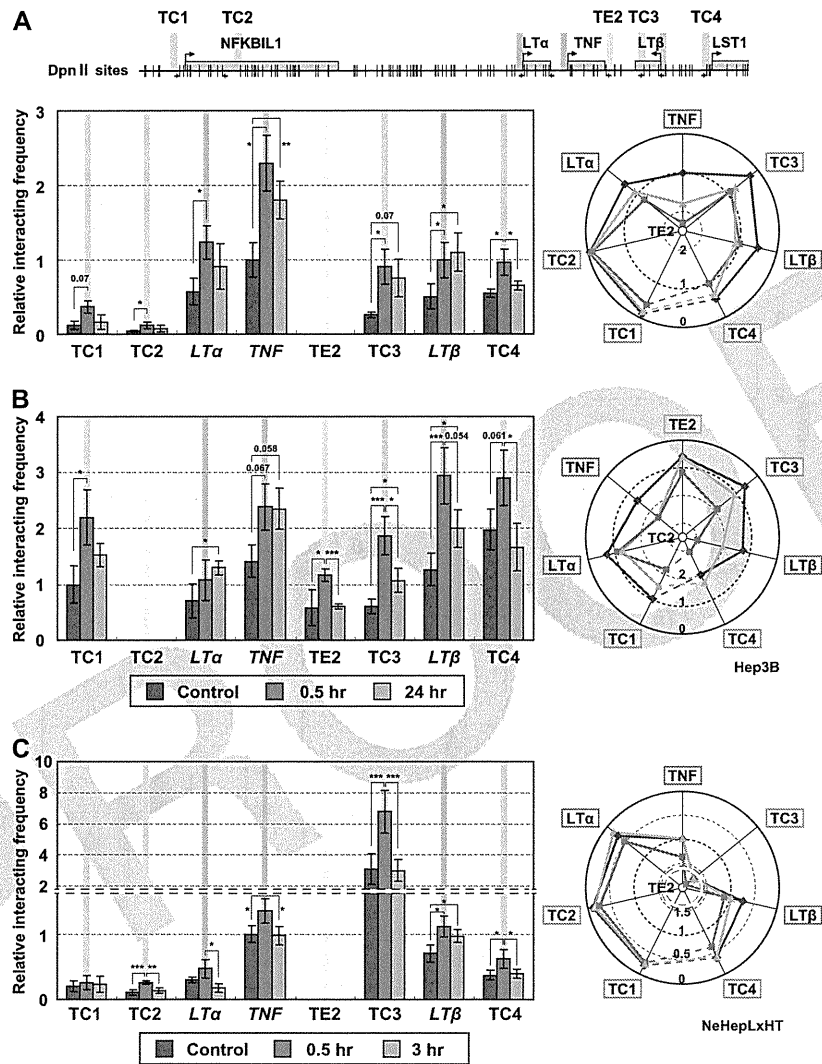
**FIG 5** CTCF-mediated insulators are involved in transcriptional regulation in the *TNF/IL6* gene cluster. (A and B) RNA interference-mediated knockdown of CTCF (A) and the cofactor cohesin RAD21 (B). Western blot and qRT-PCR analyses were carried out with Hep3B cells. As previously demonstrated (40), more than two distinct siRNAs against CTCF or RAD21 and control siRNAs were used in the experiments. (C and D) Effects of CTCF and RAD21 knockdown on the transcriptional status of the *TNF/IL6* genes. Using qRT-PCR analyses, the transcriptional levels of these genes were analyzed relative to that of  $\beta$ -actin and were normalized with the control GL3. (E) Effect of CTCF knockdown on *TNF/IL6* expression in TNF-stimulated NeHepLxHT cells. CTCF siRNAs were introduced into NeHepLxHT cells for 48 h, followed by TNF treatment for the indicated time period. Values are given as means and standard deviations of the results from more than three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ .

were depleted at both the protein and RNA levels (Fig. 5A and B). ChIP-qPCR confirmed that the amounts of CTCF and RAD21 were significantly reduced at each TC site in the knockdown cells (see Fig. S4A and B in the supplemental material). The effect of the knockdown on the constitutively low activation of the *TNF/IL6* genes in Hep3B cells was tested by qRT-PCR analyses (Fig. 5C and D). The loss of CTCF reduced *TNF* expression and increased *LTβ* expression, while RAD21 depletion increased *NFKBIL1*, *TNF*, and *LTβ* expression, suggesting that CTCF and cohesin have overlapping but certain distinct roles. Indeed, cohesin was reported to be able to behave as a transcriptional regulator, independent of CTCF (46, 54, 65).

We also analyzed the effects of CTCF knockdown on *TNF/IL6* genes in TNF-treated NeHepLxHT cells in which the *TNF/IL6* genes are normally silenced (Fig. 5E). The loss of CTCF reduced *TNF* expression and accelerated *LTβ* induction in the stimulated cells (Fig. 5E; see also Fig. S4C to 4E in the supplemental material). These results suggest that CTCF/cohesin-mediated insulators are involved in the transcriptional regulation of the *TNF/IL6* gene cluster. It is notable, however, that TNF stimulation itself did not

affect the degrees of CTCF and RAD21 enrichment at each TC site (see Fig. S4F and G in the supplemental material), suggesting that higher-order chromatin regulation may be involved in the expression of the *TNF/IL6* genes upon TNF stimulation. We assessed the knockdown effects with no significant cell damage throughout the study (see Fig. S4H and I in the supplemental material).

**Dynamics of higher-order chromatin conformation in the *TNF/IL6* locus.** 3C assays were performed with Hep3B and NeHepLxHT cells to investigate higher-order chromatin regulation in the *TNF/IL6* locus, where TE2 enhancer, gene promoters and TC insulators were identified as functional elements (Fig. 6; see also Fig. S5 in the supplemental material). Use of the 4-bp-recognizing restriction enzyme DpnII allowed us to examine these elements separately. Based on qPCR analyses of the intramolecular ligation products, the relative interacting frequencies of the reference site (yellow bar) with other 7 DpnII fragments containing each element in the *TNF/IL6* locus were measured, as further described in Fig. S6 in the supplemental material. TE2 and TC2 were mainly chosen as the reference sites because of their effectiveness in the experiments. The efficiency of DpnII digestion of



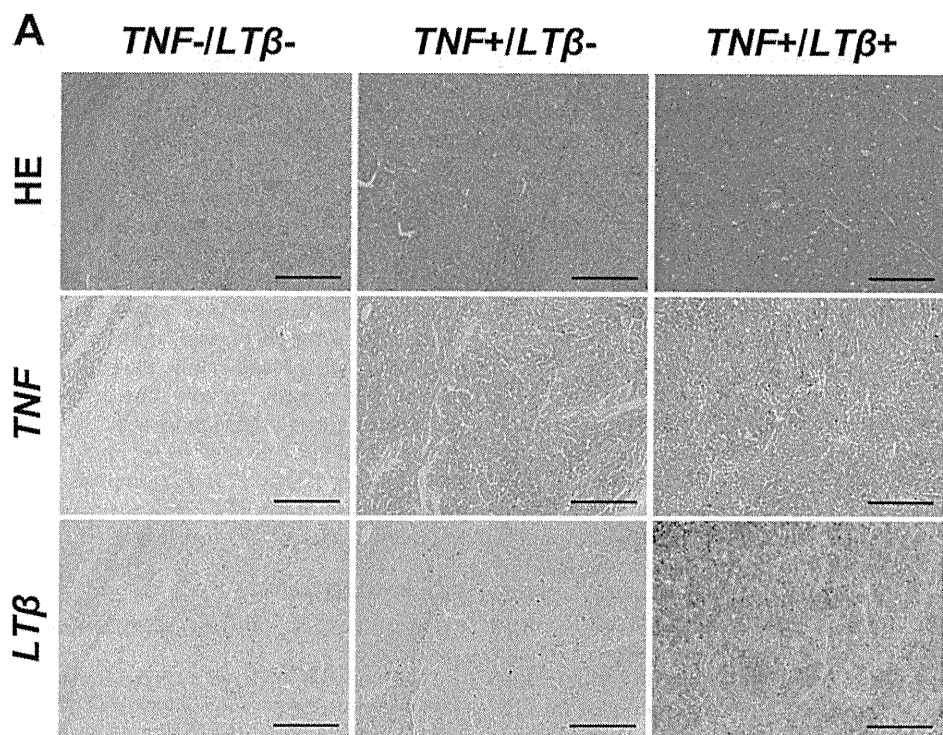
**FIG 6** Dynamic changes in higher-order chromatin conformation of the *TNF/LT* locus under TNF stimulation. DpnII digestion was used to design 3C analyses to allow the examination of individual fragments containing each TC site, TNF/LT gene promoter, and TE2 enhancer. (A) The relative interacting frequencies between the reference TE2 fragment (yellow bar) and other DpnII fragments were determined by qPCR analyses of at least three distinct samples from Hep3B cells under TNF treatment. The relative frequencies of interactions between the reference TC2 (yellow bar) and other DpnII fragments in Hep3B cells (B) or between the reference TE2 (yellow bar) and other DpnII fragments in NeHepLxHT cells (C) are shown. In the right panel, the radar chart shows the average relative frequencies of interactions between the reference (central yellow circle) and each functional element. PCR amplification using internal primers located in the *TNF/LT* locus was used for a loading control to normalize the amount of DNA fragments. Efficiencies of DpnII digestion and subsequent ligation were confirmed at each restriction site used. The relative frequencies of interactions between the reference and its physically close site in the control state were normalized to 1 (TE2-TNF [A and C] or TC2-TC1 [B]). Control basal state, blue; TNF-expressing state, magenta; TNF/LT $\beta$ -expressing state, green. TC sites, TNF/LT gene promoters, and TE2 enhancer are indicated by the same color bars in the locus (upper panel) and the 3C data. The values are given as means and standard deviations of the results from more than three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ ;  $P$  values are added.

individual sites was  $> 80\%$ , and samples without ligation gave no PCR-amplified products. We determined if CTCF knockdown affected the chromatin conformation of the *TNF/LT* locus in Hep3B cells (see Fig. S5A and B in the supplemental material). Compared with the basal control state, the frequencies of interactions of the referenced TE2 or TC2 with other fragments were mostly reduced to  $< 50\%$  in the CTCF-depleted cells, suggesting that CTCF is involved in the basal conformation of the locus.

To clarify the spatiotemporal chromatin dynamics of the *TNF/LT* locus, we then examined the frequencies of interaction

between these regulatory elements under TNF stimulation (Fig. 6). 3C assays were carried out in the cells under the basal control state, TNF-expressing state (0.5 h after stimulation), and TNF/LT $\beta$ -expressing state (24 or 3 h after stimulation). Compared with results for the basal control state, the frequencies of TE2 interaction with other sites tested in the locus were significantly augmented in TNF-expressing Hep3B cells (Fig. 6A), suggesting that intrachromosomal interaction occurred in the locus. Interestingly, TE2 maintained an interaction with the LT $\beta$  promoter and TC3 in the TNF/LT $\beta$ -expressing state while remaining separate

Watanabe et al.



**B**

<i>TNF</i> \ <i>LTβ</i>	+	-
+	12/38 (31.6 %)	10/38 (26.3 %)
-	0/38 (0 %)	16/38 (42.1 %)

**FIG 7** Expression of *TNF* and *LTβ* in human hepatocellular carcinoma tissues. (A) Representative immunohistochemical staining of human HCC. When the intensity of cytoplasmic staining was equivalent to or higher than that for noncancerous hepatocytes in >50% of cancer cells, the case was defined as positively stained. Three representative cases of the 38 cancer tissue samples tested are shown. Hematoxylin-and-eosin staining (upper) and immunostaining for *TNF* (middle) and *LTβ* (lower) are shown. Scale bar, 500 μm. (B) Percentages of *TNF*- and *LTβ*-stained cancer tissues. Cases with neither *TNF* nor *LTβ* expression (*TNF*- *LTβ*-), expression of both (*TNF*+ *LTβ*+), and *TNF* expression alone (*TNF*+/*LTβ*-) were found in 42.1%, 31.6%, and 26.3% of the cancer tissues, respectively. No cases expressed *LTβ* alone. The data for each tissue are summarized in Table S2 in the supplemental material.

from other elements. We also examined the frequencies of TC2 ligation with other fragments and found that TC2 enhanced the interaction with other fragments in the *TNF*-expressing state (Fig. 6B). However, TC2 maintained its close localization with the *TNF* and *LTα* promoters, but not with other fragments, in the *TNF*/*LTβ*-expressing state. Using the TE2 fragment as a reference, similar data were obtained in *TNF*-stimulated NeHepLxHT cells (Fig. 6C), except for some interactions of TE2 with the TC3, TC1, and *LTα* promoter. Using the TC2 fragment as a reference, we did not detect the interactions with other fragments in NeHepLxHT cells. Collectively, these data suggest that the enhancer-promoter interactions are selectively controlled by intrachromosomal association and subsequent dissociation of the *TNF*/*LT* locus upon activation of *TNF* signaling. To further demonstrate interactions between TC insulators in chromatin reorganization, we assessed their relative frequencies of interaction in these cells using TC4 as

a reference (see Fig. S5C and D in the supplemental material). These TC sites consistently showed association in the *TNF*-expressing state and subsequent dissociation in the *TNF*/*LTβ*-expressing state (modeled in Fig. S7 in the supplemental material).

**Expression of *TNF* and *LTβ* in human HCC tissues.** To examine whether the expression of *TNF* and *LTβ* is differentially regulated *in vivo*, we carried out immunohistochemical (IHC) analyses of HCC tissues (Fig. 7). Immunoreactivities of *TNF* and *LTβ* were assessed by comparison with the intensity of cytoplasmic staining of noncancerous hepatocytes within the same section. Representative images are shown in Fig. 7A, and the data for each tissue are summarized in Table S2 in the supplemental material. As summarized in Fig. 7B, neither *TNF* nor *LTβ* expression was detected in 16 out of 38 HCCs studied (42.1%), while both were densely stained in 31.6% of the cancer tissues. Interestingly, *TNF* alone was highly expressed in 10 of the 38 cancer tissues

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(26.3%), while *LTβ* alone was not detected in any cases. There may be at least two transcribed states *in vivo*, a TNF-expressing state and a TNF/*LTβ*-expressing state. We analyzed the correlation between the IHC data and clinical features and found no significant correlations between TNF and/or *LTβ* expression status and viral status, histological findings (differentiation grade of cancer, presence of chronic hepatitis or cirrhosis), or overall survival of the patients (data not shown). Although it is currently unknown whether the data for HCC tissues are related to higher-order chromatin states of the *TNF/LT* locus (shown in Fig. 6), these results suggest that differential expression of TNF and *LTβ* occurs *in vivo*.

## DISCUSSION

The present study demonstrates the significance of the spatiotemporal regulation of gene activities and higher-order chromatin dynamics in the human *TNF/LT* locus. We identified four CTCF-dependent insulators (TC1, TC2, TC3, and TC4) and an enhancer (TE2) in hepatic cells. The well-known *H19* DMR insulator contains four CTCF binding sites, while each TC site has single CTCF binding sequence with moderate enhancer blocking activities (Fig. 3). The *LTα/TNF* promoters and TE2 were located between TC2 and TC3, while the *LTβ* promoter was between TC3 and TC4, which may play a role in differential regulation of these three genes. The *LTα/TNF* genes were immediately induced by TNF stimulation in a fashion sensitive to inhibition of NF- $\kappa$ B signaling, while the *LTβ* gene was expressed later, as seen in other cell types (1, 39). Our previous report on the human *apolipoprotein* gene locus suggested that CTCF insulators play an essential role in clustered gene control (40). Furthermore, the current study shows that insulator interactions are likely to mediate intrachromosomal association and subsequent dissociation following TNF signaling. The dynamic enhancer-promoter associations and differential expression in the *TNF/LT* locus may be directed by the NF- $\kappa$ B-related regulatory molecules.

From the viewpoint of enhancer-promoter-insulator associations, we propose a spatiotemporal dynamics model in the human *TNF/LT* locus (see Fig. S7 in the supplemental material). In the basal state, CTCF-bound TC sites, the TE2 enhancer, and the *TNF/LT* promoters are located some distance apart in the chromatin structure. After TNF signaling activation, in the TNF-expressing state, the TC insulators, TE2, and *TNF/LT* promoters become colocalized and form a compact chromatin structure, resulting in interactions between TE2 and the *TNF* and *LTα* promoters. Because the *LTβ* gene is not fully induced at this stage, the *LTβ* promoter is likely to be sequestered by forming a possible chromatin loop between TC3 and TC4 (see Fig. S5C and D in the supplemental material). In addition, TC sites may be involved in stabilizing the interaction between TE2 and the *TNF* promoter because of the decrease of *TNF* expression in CTCF-depleted cells (Fig. 5C and E). In the *TNF/LTβ*-expressing state, TE2 significantly maintained its interaction with the *LTβ* promoter despite a reduced association with other elements. Thus, sequential chromatin conformation changes may contribute to switching of the enhancer-promoter interaction. Posttranslational modifications of CTCF and changes in the interacting molecules may be involved in the mechanism of intrachromosomal dynamics in the *TNF/LT* locus (47).

Our study revealed that TNF signaling can induce spatiotemporal remodeling of the clustered gene region and that CTCF in-

surators are likely to mediate higher-order control of transient enhancer-promoter interactions in the *TNF/LT* locus. Previous studies of the *TNF/LT* locus in hematopoietic cells suggested the presence of certain regulatory elements in intron 3 of the *TNF* gene and in the final exon of the *LTβ* gene (5, 66). The sequences, including the TC3 site, showed silencer activity in human T cells, though our study indicated that TC3 had a CTCF-dependent enhancer-blocking function, suggesting that the regulatory elements may differ among cell types. We showed that CTCF-mediated higher-order chromatin is involved in *TNF/LT* gene regulation. Persistent NF- $\kappa$ B activation in chronic inflammation may result in the chromatin conformation of the *TNF/LT* locus being deregulated and maintained in the *TNF/LTβ*-expressing state as an epigenetic memory. Indeed, constitutive NF- $\kappa$ B activation was recently noted to cause *LTβ* expression in inflamed hepatocytes and HCC cells *in vivo* (35), and *LTβ* was demonstrated to be an inducer of HCC (23). The proposed higher-order chromatin conformation of the *TNF/LT* locus may be involved in these *in vivo* situations.

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PROOF

# Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules

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## Abstract

To eliminate hepatitis C virus (HCV) from infected hepatocytes, we generated two therapeutic molecules specifically activated in cells infected with HCV. A dominant active mutant of interferon (IFN) regulatory factor 7 (IRF7) and a negative regulator of HCV replication, VAP-C (Vesicle-associated membrane protein-associated protein subtype C), were fused with the C-terminal region of IPS-1 (IFN $\beta$  promoter stimulator-1), which includes an HCV protease cleavage site that was modified to be localized on the ER membrane, and designated cIRF7 and cVAP-C, respectively. In cells expressing the HCV protease, cIRF7 was cleaved and the processed fragment was migrated into the nucleus, where it activated various IFN promoters, including promoters of IFN $\alpha$ 6, IFN $\beta$ , and IFN stimulated response element. Activation of the IFN promoters and suppression of viral RNA replication were observed in the HCV replicon cells and in cells infected with the JFH1 strain of HCV (HCVcc) by expression of cIRF7. Suppression of viral RNA replication was observed even in the IFN-resistant replicon cells by the expression of cIRF7. Expression of the cVAP-C also resulted in suppression of HCV replication in both the replicon and HCVcc infected cells. These results suggest that delivery of the therapeutic molecules into the liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

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## Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma [1,2]. Although the proportion of patients achieving a sustained virological response (SVR) has been increased by the recent used of combination therapy with pegylated-interferon- $\alpha$  (PEG-IFN $\alpha$ ) and ribavirin (RBV), half of patients still exhibit no response to this therapy, suggesting that the IFN signaling pathway is modulated by HCV infection. In addition, various side effects have been reported in more than 20% of patients treated with this combination therapy [3].

HCV belongs to the family *Flaviviridae* and possesses a single positive-stranded RNA genome that encodes a single polyprotein composed of about 3,000 amino acids. The HCV polyprotein is processed into 10 viral proteins by host and viral proteases. Viral structural proteins, including the capsid protein and two envelope proteins, are located in the N-terminal one third of the polyprotein, followed by nonstructural proteins. The NS2 protease cleaves its own carboxyl terminus and NS3 cleaves the downstream positions to produce NS4A, NS4B, NS5A and NS5B. Although laboratory strains of HCV propagating in cell culture (HCVcc) have been established based on the full-length genome of the

genotype 2a JFH1 strain [4], establishment of a robust cell culture system capable of propagating serum-derived HCV from hepatitis C patients has not yet been achieved.

Type I IFN exhibits potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs) which encode proteins involved in the establishment of antiviral state in cells [5]. IFNs induce transcription of ISGs through activation of the Jak-STAT pathway [6]. Binding of type I IFN to the IFN receptor induces phosphorylation of the receptor-associated tyrosine kinases, Jak1 and Tyk2, and then these kinases activate STAT1 and STAT2. The phosphorylated STATs migrate into the nucleus and activate ISG promoters through binding to the specific responsible elements. HCV infection has been suggested to impair the IFN production through multiple pathways. The IFN-induced Jak-STAT signaling is inhibited in cells and transgenic mice expressing HCV proteins and in the liver biopsy samples of chronic hepatitis C patients [7–9].

Induction of type I IFN upon infection with pathogens is crucial for innate immunity, and it is mediated by the activation of pattern-recognition receptors, including Toll-like receptors (TLRs) and cytosolic receptors, such as RIG-I and MDA5 [10–12]. The induction of type I IFN is primarily controlled at the gene transcriptional level, wherein a family of transcription factors known as IFN regulatory factors (IRFs) play a pivotal role. IRF3

and IRF7 are known to be essential for the RIG-I-, MDA5-, and TLR-mediated type I IFN production pathways. IRF3 is induced primarily by a response to initiate IFN $\beta$  production, whereas IRF7 is induced by IFN $\beta$  and participates in the late phase for IFN $\beta$  induction [13]. All TLRs, except for TLR3, activate the MyD88-dependent pathway, whereas TLR3 and TLR4 activate the TRIF-dependent pathway. HCV NS3/4A protease has been shown to impair the production of IFN $\beta$  as well as the subsequent IFN-inducible genes through the inactivation of the adaptor molecules involved in the TLR-dependent and -independent signaling pathways [14–18]. On the other hand, Vilasco *et al.* suggested that impairment of IKKi - which, along with TBK1, is one of the important factors participating in IRF3 phosphorylation and activation - in the HCV replicon cells plays at least a partial role in the restoration of type I IFN signaling pathways [19]. In addition, IRF7 was shown to participate in the positive feedback of type I IFN signaling through the IFN receptor [13]. Therefore, we tried to examine the effect of exogenous expression of IRF7 under the assumption that IRF7 is a potent type I IFN inducer and capable of modulating the viral propagation in hepatocytes infected with HCV.

In this study, we generated two therapeutic molecules consisting of a dominant active mutant of IRF7 or VAP-C, a negative regulator of HCV replication [20], followed by the C-terminal region of IFN promoter stimulator 1 (IPS-1), including the cleavage site of the HCV NS3/4A protease, which was modified so that the cleavage site localized on the ER membrane [21]. The expression of the plasmids encoding these molecules in the HCV replicon and HCVcc-infected cells resulted in a substantial suppression of HCV propagation, suggesting the possibility that these or other similar molecules could be used therapeutically to eliminate HCV from hepatocytes infected with HCV.

## Results

### IRF7m, a dominant active mutant of IRF7, activates the IFN promoters in cells replicating HCV

Previous studies have shown that an IRF7 mutant, IRF7m, lacking the amino acid residues from 284 to 454, a region that includes the auto-inhibitory domain (from amino acid residue 305 to 467), and an IRF3 mutant, IRF3m, carrying the substitution of Ser<sup>396</sup> to Asp in the carboxyl terminal region (Fig. 1A), induced a potent activation of type I IFN promoter in non-hepatic cell lines irrespective of viral infection [22–25]. We first examined the effect of the expression of the IRF dominant active mutants on the inhibition of HCV RNA replication through the production of type I IFN. HCV replicon cells and Huh7OK1 cells infected with HCVcc were transfected with the plasmids encoding either wild-type or dominant active mutant of IRF3 or IRF7 together with the reporter plasmids encoding a luciferase gene under the control of the promoters of IFN $\alpha$ 6, IFN $\beta$  and ISRE, respectively. Among these examined constructs, we observed significant activation of the promoters of IFN $\alpha$ 6 and ISRE in the replicon and HCVcc-infected cells compared with naïve and mock-infected cells upon expression of IRF7m, while we observed no activation of the IFN $\alpha$ 6 promoter in cells expressing IRF3m (Figs. 1B and 1C). Potent stimulation of the IFN $\beta$  promoter was observed in the replicon cells expressing IRF7m but not in cells infected with HCVcc. Next we examined the antiviral activity of the IRF constructs in both replicon (Fig. 1D) and HCVcc-infected cells (Fig. 1E). The expression of the plasmid encoding IRF7m resulted in potent suppression of viral protein and viral RNA syntheses in both cell types. Although expression of IRF3m induced a slight suppression of viral propagation in cells infected with HCVcc,

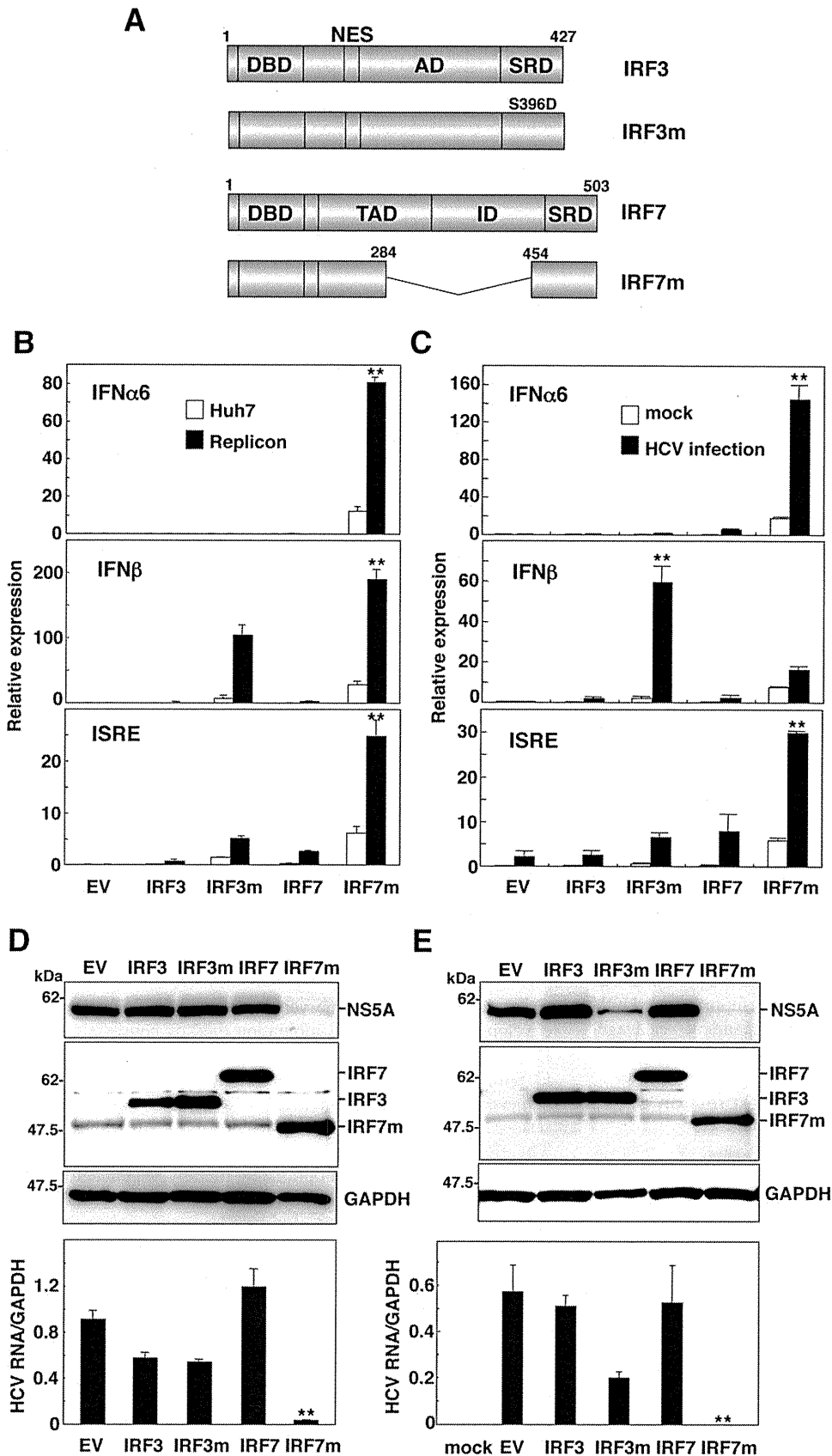
expression of the IRF constructs except for IRF7m did not induce the significant suppression of viral replication and propagation. These results suggest the possibility of elimination of HCV through a specific induction of type I IFN by the expression of IRF7m in HCV-infected cells.

### cIRF7, a chimeric construct of IRF7m, specifically activates the IFN promoters in cells replicating HCV

To induce IFNs in cells infected with HCV but not in uninfected cells through a selective activation of IRF7m, we constructed a chimeric IRF7 (cIRF7) consisting of the IRF7m fused with FLAG-tag and the C-terminal amino acid residues from 503 to 540 of IPS-1 modified to be localized on ER (Fig. 2A upper) [21]. HCV NS3/4A protease cleaves the carboxyl site of Cys<sup>508</sup> in the C-terminal domain of IPS-1. Although cIRF7 is anchored in the ER and exhibits no activation in uninfected cells, cIRF7 would be cleaved by the NS3/4A protease in cells infected with HCV and the released N-terminal fragment would migrate into the nucleus and activate various IFN promoters (Fig. 3). Immunoblot analyses revealed that cIRF7 was cleaved in 293T cells expressing HCV NS3/4A protease of a wild type but not in those expressing the mutant protease NS3/4A(S139A), and a mutant cIRF7(C508A) which has a substitution of Cys<sup>508</sup> to Ala, exhibited resistance to the cleavage by the HCV protease (Fig. 2A bottom). To assess a specific activation of the IFN promoters after cleavage of the cIRF7 by HCV NS3/4A, 293T cells expressing FLAG-tagged HCV proteases were transfected with the plasmids encoding the luciferase gene under the control of the promoter of IFN $\alpha$ 6, IFN $\beta$  or ISRE together with the plasmid encoding either cIRF7 or cIRF7(C508A). Expression of cIRF7 but not of cIRF7(C508A) induced the activation of the IFN $\alpha$ 6, IFN $\beta$  and ISRE promoters in cells expressing HCV NS3/4A protease but not in those expressing the mutant protease NS3/4A(S139A) (Fig. 2B). Next we examined the activation of the IFN promoters associated with the expression of the plasmid encoding cIRF7 in the replicon and HCVcc-infected cells. Expression of cIRF7 but not of cIRF7(C508A) induced the activation of the IFN promoters in both cell types (Figs. 2C and 2D). On the other hand, these promoters were not activated by the expression of cIRF7 in the replicon cells harboring subgenomic RNA of Japanese encephalitis virus (JEV) and Huh7 cells infected with JEV (Fig. 2E). These results suggest that the cIRF7 expression is a feasible method for specifically activating the IFN promoters in cells infected with HCV.

### Specificity of activation of the IFN promoters by the expression of cIRF7

To further examine the specificity of the activation of the IFN promoters by the expression of cIRF7 in cells replicating HCV, a plasmid encoding either cIRF7 or IRF7m was co-transfected with that encoding the luciferase gene under the ISRE promoter into the HCV replicon or HCVcc-infected cells and cultured in the presence or absence of inhibitors for HCV replication. Treatment with an HCV protease inhibitor (BILN2061) or cyclosporine A (CsA) inhibited the activation of the ISRE promoter by the expression of cIRF7 in the HCV replicon and HCVcc-infected cells in a dose-dependent manner, in contrast to the resistance to the treatments in cells expressing the IRF7m (Fig. 4A and Fig. 4B). Recently, it was shown that an NS3/4A protease of GB virus B (GBV-B), which is the virus genetically related most closely to HCV, also impairs the dsRNA-induced IFN production through a cleavage of IPS-1 [26]. Therefore, to assess the possibility of activation of cIRF7 by other flaviviral proteases, cleavage of cIRF7



**Figure 1. Dominant active mutant of IRF7 activates IFN promoters in cells replicating HCV.** (A) Structures of IRF3, IRF7 and the dominant active mutants, IRF3m and IRF7m. The DNA-binding domain, nuclear export sequence, transactivation domain, association domain, inhibitory domain, and signal response domain are indicated as DBD, NES, TAD, AD, ID, and SRD, respectively. Huh7 cells and HCV replicon cells ( $1 \times 10^5$  cells/

well) (B), and Huh7OK1 cells ( $7.5 \times 10^4$  cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h (C) were transfected with 100 ng of plasmid encoding the luciferase gene under the control of the IFN $\alpha$ 6, IFN $\beta$ , or ISRE promoter together with an empty vector (EV) or a plasmid encoding each of the IRF constructs. The relative luciferase activity of cell lysates was determined at 24 h post-transfection. HCV replicon cells ( $3 \times 10^5$  cells/well) (D) and Huh7OK1 cells ( $1.5 \times 10^5$  cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h (E) were transfected with EV or a plasmid encoding each of the IRF constructs and the expressions of NS5A, IRFs, and GAPDH (upper panel) and synthesis of viral RNA (lower panel) at 72 h post-transfection were determined by immunoblotting and real-time PCR after standardization with GAPDH, respectively. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (\*\* $P < 0.01$ ) versus the control cells or mock-infected cells.  
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and activation of the IFN promoters were evaluated in 293T cells expressing the viral proteases of HCV, GBV and JEV. Immunoblot analyses revealed that cIRF7 was processed by the viral proteases of HCV and GBV but not by that of JEV and the activation of the IFN promoters was well correlated with the cleavability of the cIRF7 (Fig. 4C). Although the GBV protease exhibited an efficient activation of cIRF7 comparable to HCV protease, processing of cIRF7 and activation of the IFN promoters by the GBV protease was not inhibited by the pretreatment with the HCV protease inhibitor (Figs. 4D and 4E). These results indicate that cIRF7 is capable of activating the IFN promoters through a specific cleavage by the protease in cells infected with HCV.

#### Nuclear localization of cIRF7 in cells expressing HCV protease

From these results, it was suggested that cIRF7 is cleaved by the HCV protease and the processed fragment migrates into the nucleus and activates IFN promoters (Fig. 3). To confirm the nuclear localization of the cleaved cIRF7, we constructed an EGFP-cIRF7 and determined its subcellular localization in cells expressing the HCV protease and in the HCV replicon cells by confocal microscopy. Nuclear accumulation of the cIRF7 was observed in cells expressing EGFP-cIRF7 together with NS3/4A, but not in those with NS3/4A(S139A) or NS5A and also not in cells co-expressing EGFP-cIRF7(C508A) and NS3/4A (Fig. 5A). Furthermore, expression of EGFP-cIRF7 but not of EGFP-cIRF7(C508A) induced a nuclear accumulation of cIRF7 in the HCV replicon cells, and nuclear localization of the cIRF7 abrogated the expression of viral antigen (NS3), in contrast to the co-localization of EGFP-cIRF7(C508A) and the ER marker PDI, which had no discernible antiviral effect (Fig. 5B). These results suggest that cIRF7 is capable of suppressing HCV replication through an HCV protease-dependent cleavage, migration into the nucleus and activation of the IFN promoters.

#### Suppression of HCV replication by the expression of cIRF7

To examine the inhibitory effect of the expression of cIRF7 on HCV replication, a plasmid encoding either cIRF7 or cIRF7(C508A) was transfected into the HCV replicon and HCVcc-infected cells, and HCV replication was evaluated by immunoblotting and real-time PCR. The expression of cIRF7 but not of cIRF7(C508A) resulted in cleavage by the HCV protease, and a clear reduction of viral protein and RNA syntheses in both replicon and HCVcc-infected cells (Figs. 6A and 6B). In addition, we examined the effect of cIRF7 on the replication of HCV in the 4 $\beta$ R replicon cells [27,28], which have been shown to exhibit more resistant to the IFN $\alpha$  treatment than Con1 replicon cells (Fig. 6C upper left). Expression of the cIRF7 in the 4 $\beta$ R replicon cells but not in those cured HCV RNA (4 $\beta$ Rc cells) induced an activation of the ISRE promoter (Fig. 6C lower left). Expression of cIRF7 but not of cIRF7(C508A) also resulted in processing by the HCV protease and suppression of viral protein and RNA syntheses in the 4 $\beta$ R replicon cells (Fig. 6C right panels).

#### Suppression of HCV replication by the expression of cVAP-C

Human vesicle-associated membrane protein-associated protein subtype A (VAP-A) and B (VAP-B) are known to be involved in the regulation of membrane trafficking, lipid transport and metabolism, and the unfolded protein response [29]. VAP-A and VAP-B have been shown to be involved in the replication of HCV, and we have shown recently that human VAP-C, a splicing variant of VAP-B, negatively regulates HCV replication by interfering with the interaction of VAP-A and VAP-B with HCV NS5B [20]. We next examined the possibility of using a selective activation of VAP-C to suppress HCV replication in cells infected with HCVcc. We generated expression plasmids encoding a chimeric VAP-C fused with the IPS-1 sequence (cVAP-C), a cVAP-C(C508A) which is made resistant to the HCV protease by a substitution in the cleavage site similar to the substitutions made in cIRF7(C508A), or VAP-C (Fig. 7A). The cVAP-C was cleaved in cells infected with HCVcc, and expression of cVAP-C and VAP-C suppressed expression of NS5A, in contrast to the weak reduction of NS5A in the infected cells expressing cVAP-C(C508A), probably due to a slight cleavage of cVAP-C(C508A) (Fig. 7B, top). Furthermore, the production of viral RNA and infectious particles in the culture supernatants of cells infected with HCVcc was also impaired by the expression of cVAP-C and VAP-C, but not of cVAP-C(C508A) in a dose-dependent manner (Fig. 7B, middle and bottom). Collectively, these results suggest that delivery of the therapeutic molecules into liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

#### Discussion

An effective prophylactic vaccine against HCV has not been developed yet. Although combination therapy consisting of PEG-IFN $\alpha$  and RBV has been introduced for the treatment of hepatitis C patients, and 50% of individuals infected with genotype 1 achieved a SVR, this treatment is sometimes associated with serious side effects, including depression and anemia [3]. Therefore, new anti-HCV drugs targeted to HCV protease and polymerase and capable of optimizing therapy are currently in the early stages of the development [30,31]. However, it is difficult to achieve a complete removal of viruses by antiviral drugs targeted to the viral enzymes from patients persistently infected with RNA viruses that exhibit a quasispecies nature, such as human immunodeficiency virus (HIV) and HCV. Viral quasispecies are not a simple collection of diverse mutants but a group of interactive variants capable of adapting to new environments [32]. Furthermore, introduction of antiviral drugs may induce an emergence of drug-resistant breakthrough viruses as seen in the case of HIV infection. Therefore, a novel therapeutic approach for hepatitis C patients in addition to the current chemotherapies is required to overcome serious adverse effects and improve the ratio of patients achieving SVR.

In this study, we have generated two therapeutic molecules, cIRF7 and cVAP-C, which are selectively activated in cells