INTERFERON REGULATORY FACTOR 7 (IRF-7) PLAYS AN IMPORTANT ROLE IN INNATE IMMUNITY, WHERE, TOGETHER WITH IRF-3, IT CONTROLS THE EXPRESSION OF INTERFERON A/B GENES AS WELL AS CHEMOKINE RANTES (REGULATED ON ACTIVATION NORMAL T CELL EXPRESSED AND SECRETED). PREVIOUSLY, WE CHARACTERIZED HUMAN IRF-7 PROMOTER AND SHOWED THAT AN INTERFERON-STIMULATED RESPONSE ELEMENT SITE IN THE FIRST INTRON BINDS INTERFERON-STIMULATED GENE FACTOR 3 (ISGF3) AND CONFFERS THE RESPONSE TO INTERFERON. HERE WE REPORT THE STIMULATION OF IRF-7 EXPRESSION BY 12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA) AND TUMOR NECROSIS FACTOR α (TNFα) IN HUMAN PERIPHERAL BLOOD MONOCYTES. USING PROMOTER ANALYSIS IN COMBINATION WITH ELECTROPHORETIC MOBILITY SHIFT ASSAYS, WE HAVE DEMONSTRATED THAT AN NFκB SITE LOCATED NEXT TO THE TATA BOX, BINDS p50 AND p65 HETERODIMER AND IS REQUIRED FOR THE INDUCTION OF THE IRF-7 GENE BY TPA AND TNFα. IN ADDITION, WE REPORT STIMULATION OF IRF-7 GENE EXPRESSION BY TOPOISOMERASE II (TOPII) INHIBITORS. WE SHOW BY CHROMATIN IMMUNOPRECIPITATION ASSAY THAT TREATMENT WITH THE TOPII INHIBITOR ETOPOSIDE INDUCES ASSOCIATION OF ACETYLATED HISTONE 3 WITH THE PROMOTER OF IRF-7 GENE, INDICATING THAT TOPII-MEDIATED CHANGES IN CHROMATIN STRUCTURE COULD BE RESPONSIBLE FOR THE INDUCTION. THIS SUGGESTS THAT THE IRF-7 GENE IS LOCALIZED IN THE CONDENSED AREA OF THE CHROMOSOME WHERE IT IS INACCESSIBLE TO TRANSCRIPTION FACTORS THAT WOULD PROMOTE ITS CONSTITUTIVE EXPRESSION.

Interferon regulatory factors (IRFs) belong to a family of transcription factor now consisting of nine members and several viral IRF homologs. IRFs bind, through their highly homologous N-terminal DNA binding domain, to an AANNNGAAA consensus sequence on the DNA to mediate diverse biological events, including antiviral defense, cell growth regulation, and development and maturation of the immune system. IRF-7 was originally cloned as a repressor associated with Epstein-Barr virus type III latency, where it binds the Qp promoter of the EBNA-1 gene. It has since been demonstrated, by us and other groups, that IRF-7 plays an important role in innate immunity where, together with IRF-3, it controls the expression of type I interferon (IFNα/β) as well as chemokines such as RANTES (regulated on activation normal T cell expressed and secreted) in virus-infected cells. The induction of IFNα/β by IRF-3 and IRF-7 is believed to consist of two phases (8, 9). In the early phase, viral infection triggers the phosphorylation and subsequent nuclear translocation of IRF-3. Together with c-Jun and ATF-2, the IRF factors assemble with p300/ CBP to form a transcription complex enhanceosome, on the promoter region of IFNβ gene. The newly synthesized IFNβ in turn stimulates expression of IRF-7 via an autocrine/paracrine pathway. In the late phase, the IRF-7 forms a heterodimer with IRF-3 and translocates into the nucleus, resulting in the induction of different IFNα subtype gene expressions. This biphasic induction model is supported by studies both in vitro and in vivo (10–12). 2FTGH cells are a human fibrosarcoma-derived cell line in which the IRF-7 locus is silenced due to promoter hypermethylation (13). We have demonstrated that, upon virus infection, 2FTGH cells cannot synthesize IFNα unless expression of IRF-7 is reconstituted, supporting a critical role of IRF-7 in IFNα expression (10). In addition, we have demonstrated a dramatic decrease in interferon production when IRF-3 expression is inhibited by a targeting ribozyme (11). Studies in vivo demonstrated a dramatic reduction in IFNα/β synthesis in infected IRF-3−/− mice, and IFNα/β induction was completely abolished in IRF-3−/− and IRF-9−/− double knock-out mice, which do not express IRF-7 (or express very low levels) as a result of the defective interferon signaling pathway (12).

IRF-7 is expressed predominantly in lymphoid tissues, and its expression can be further stimulated in multiple cell types upon virus infection and interferon treatment. Previously, we have cloned and characterized the human IRF-7 promoter and demonstrated that an interferon-stimulated response element (ISRE), located in the first intron of IRF-7 gene, binds the ISGF3 complex and is responsible for the stimulation of the IRF-7 gene by interferon (13). Recently, we reported the induction of IRF-7 gene expression by 12-O-tetradecanoylphorbol-13-acetate (TPA) in promonocytic U937 cells and further demonstrated the importance of IRF-7 induction in TPA-induced monocyte differentiation (14). In this paper, the mechanism by which TPA induces IRF-7 expression is further characterized. Here, we show that an NFκB site located next to the TATA box on the IRF-7 promoter is responsible for induction by TPA. In addition, we report for the first time that a proinflammatory cytokine TNFα also induces IRF-7 expression and that the induction requires the same NFκB site. Finally, we provide system (2).
evidence that the expression of endogenous IRF-7 gene is induced upon modulation of the chromatin structure by TOPII inhibitors, indicating that the IRF-7 gene (ID 3665), which is localized in a GC-rich area of chromosome 11p15.5 (15), is probably inaccessible to transcription factors that are already present in uninduced cells. Thus our study reveals multiple levels of regulation of IRF-7 gene expression.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Human peripheral blood mononuclear cells were isolated from the blood of healthy donors by a density gradient centrifugation method and cultured in RPMI 1640 medium containing 10% FBS. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. U937 and Namalwa cells were cultivated in RPMI 1640 medium plus 10% FBS. Reporter DNA Constructs—The generation of luciferase reporter vectors containing a 1.6-kb fragment of human IRF-7 promoter region wild type (WT) and the version with mutated ISRE site (ISREM) were described previously (13) (see Fig. 2). The luciferase containing wild type IRF-7 was used as a parent copy to generate a mutant vector reciprocally mutated at positions 525–539 (13) with a defined ISRE site, designated as ISREM, which was utilized as described (13) to mutate the NF-κB site on the IRF-7 promoter.

Northern Blot Analysis—Total cell RNA was isolated using the Trizol method (Invitrogen) and Northern blot analysis was carried out as described (13, 16). Briefly, 15 μg of total RNA was separated by electrophoresis on a 1% denaturing agarose gel. The gel was blotted onto a nylon membrane, and hybridization was carried out with 32P-labeled IRF-7 cDNA as a probe.

**Transient Transfection and Luciferase Assay**—Cells were transfected in 60-mm dishes using Superfect (Qiagen) according to the manufacturer’s recommendation. One microgram of the reporter plasmid DNA and 0.1 μg of β-galactosidase-expressing plasmid (internal control) were used for each transfection. In co-transfection experiments, 1:1 ratios of the reporter and expression plasmids (1 μg of each) were used. The final concentration of transfected DNA was kept constant in all co-transfection assays. Transfected cells were split 12 h after transfection into 35-mm-well plates and incubated for another 6 h, after which fresh medium was added and different treatments, as indicated in the legend, were carried out. Twelve hours later, cells were lysed, and luciferase activity was measured as described previously (13).

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared as described (13) from TPA- or TNFα-treated cells and from untreated control cells. After treatment, the cells were incubated with lysis buffer (50 mM KCl, 25 mM HEPES, pH 8.0, 100 μM dithiotheriol, 1% Nonidet P-40, and protease inhibitor mixture from Sigma) for 5 min on ice. The nuclei were pelleted by centrifugation and resuspended in extraction buffer (500 mM KCl, 5 mM HEPES, pH 8.0, 100 μM dithiotheriol, 10% glycerol, and protease inhibitor mixture) at 4 °C for 30 min with constant shaking. After centrifugation, the supernatants were stored at −70 °C until use. Aliquots of 5 μg of nuclear extracts were incubated in 25 μl of total reaction volume containing 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM MgCl2, 1 mM dithiotheriol, 10% glycerol, 40 μg/ml poly(dI-dC), and 50 mM KCl) with a 32P-labeled oligonucleotide probe for 20 min at room temperature. The reaction mixtures were analyzed by electrophoresis in a 4% native acrylamide gel. The sequence of the probe used for the EMSA was: 5’-GGCCCTTGCACGAAAATCGCTGG-3’.

**Chromatin Immunoprecipitation Assay**—Chromatin immunoprecipitation assay was performed as described previously (17, 18). Briefly, 1 × 106 Namalwa cells were treated with etoposide or trichostatin A for 12 h, and the proteins bound to DNA were subsequently cross-linked by addition of 11% formaldehyde to the culture medium to a final concentration of 1%. Cross-linking was allowed to proceed for 30 min at room temperature, and the reaction was stopped by the addition of glycine to a final concentration of 0.125 M. The fixed cells were washed and resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) on ice and lysed by sonication twice for 10 s each. Samples were diluted 10-fold with dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl). For each immunoprecipitation, 700 μl of diluted lysate was precleared by addition of 30 μl of blocked protein A beads (50% slurry protein A-Sepharose, 0.5% mg/ml fatty acid-free bovine serum albumin, and 0.1 mg/ml salmon sperm DNA). Samples were immunoprecipitated at 4 °C with 1 μg of activated histone 3 (H3) antibodies (Upstate Biotechnology) for 12 h. The immunocomplexes were extensively washed and treated with RNase, 0.5% SDS, and proteinase K. The cross-linked DNA and protein complexes were reverted by heating at 65 °C for 6 h. DNA was purified by phenol/chloroform extraction and immunoprecipitated with ethanol. PCR amplification was performed using specific primers for human IRF-7 promoter: sense, 5’-GGGCCCTTCCGGGAAAACTCCTGGGG-3’; antisense, 5’-GGGCGGTTCAGGCTCCGGGAAAAGCGG-3’. The condition for the PCR amplification is: 94 °C 1 min, 57 °C 1 min, 72 °C 1 min, for 30 cycles.

**RESULTS**

**Role of NFκB Site in Induction of IRF-7 Gene Expression by TPA and TNFα**—IRF-7 is predominantly expressed in lymphoid tissues, and its expression can be further up-regulated in multiple cell types upon virus infection and interferon treatment. Here, we report the induction of IRF-7 gene expression by two novel stimulants, TPA and TNFα. As shown in Fig. 1, both TPA and TNFα induced IRF-7 mRNA expression in a time-dependent fashion.

The mechanism by which TPA and TNFα stimulate IRF-7 gene expression was further examined. We previously have cloned the promoter region of the human IRF-7 gene, and a 1.6-kb promoter region was analyzed in detail (13). Our studies revealed that an ISRE site located in the first intron is responsible for induction of the IRF-7 gene by interferon. It is well documented that both TPA and TNFα activate the NFκB signaling pathway (19), and interestingly, there is a potential NFκB binding site located at 10 bp upstream of the TATA box. We mutated this NFκB site and inserted this mutant into a luciferase reporter vector. This new construct, designated as NFκB, was transiently transfected into HeLa cells, and its promoter activity was compared with the wild type promoter construct (WT) and ISRE site mutant (ISREM). First, we wanted to determine whether this potential NFκB site could confer the transcription activation mediated by NFκB family transcription factors. WT and NFκB reporter vectors were transfected into HeLa cells together with p50, p65, and both, and the activity of the promoter was measured. As shown in Fig. 2A,
co-transfection of WT with either p50 or p65 increased transcription activity of the IRF-7 promoter and co-transfection of both further stimulated the activity of the promoter. However, the response to p50 and p65 decreased dramatically when the NFκB site was mutated. This result suggests that the NFκB binding site present in the IRF-7 promoter is functional. Next, in independent experiments.

To assess the activation of the IRF-7 promoter by TPA and TNFα, we used a luciferase reporter plasmid, containing 1.6 kb of IRF-7 promoter region in front of the luciferase gene (13) together with p50- or p65-expressing plasmids (1 μg) and 0.1 μg of β-galactosidase encoding plasmid in 60-mm dishes. Twelve hours after transfection, cells were split into 35-mm dishes, and luciferase activity was measured 24 h later. Twelve hours after transfection, cells were split into 35-mm dishes, and the luciferase activity was measured 24 h later. B, activation of IRF-7 promoter by TPA and TNFα requires an NFκB site. Transient transfection was conducted as described above. Luciferase activity was measured 24 h after treatment with IFN, TPA, or TNFα. WT, wild type IRF-7 promoter construct; ISREM, IRF-7 promoter with mutated ISRE site (13); NFκBM, IRF-7 promoter with mutated NFκB site (mutation include change from CTCC to CCAA). Values were normalized to the constant levels of β-galactosidase and represent a summary of three independent experiments.

TF.2. Induction of IRF-7 expression by TPA and TNFα requires an NFκB site. A, HeLa cells were transfected with 1 μg of the reporter plasmid, containing 1.6 kb of IRF-7 promoter region in front of the luciferase gene (13) together with p50- or p65-expressing plasmids (1 μg) and 0.1 μg of β-galactosidase encoding plasmid in 60-mm dishes. Twelve hours after transfection, cells were split into 35-mm dishes, and the luciferase activity was measured 24 h later.

B, activation of IRF-7 promoter by TPA and TNFα requires an NFκB site. Transient transfection was conducted as described above. Luciferase activity was measured 24 h after treatment with IFN, TPA, or TNFα. WT, wild type IRF-7 promoter construct; ISREM, IRF-7 promoter with mutated ISRE site (13); NFκBM, IRF-7 promoter with mutated NFκB site (mutation include change from CTCC to CCAA). Values were normalized to the constant levels of β-galactosidase and represent a summary of three independent experiments.

FIG. 2. Induction of IRF-7 expression by TPA and TNFα requires an NFκB site. A, HeLa cells were transfected with 1 μg of the reporter plasmid, containing 1.6 kb of IRF-7 promoter region in front of the luciferase gene (13) together with p50- or p65-expressing plasmids (1 μg) and 0.1 μg of β-galactosidase encoding plasmid in 60-mm dishes. Twelve hours after transfection, cells were split into 35-mm dishes, and the luciferase activity was measured 24 h later. B, activation of IRF-7 promoter by TPA and TNFα requires an NFκB site. Transient transfection was conducted as described above. Luciferase activity was measured 24 h after treatment with IFN, TPA, or TNFα. WT, wild type IRF-7 promoter construct; ISREM, IRF-7 promoter with mutated ISRE site (13); NFκBM, IRF-7 promoter with mutated NFκB site (mutation include change from CTCC to CCAA). Values were normalized to the constant levels of β-galactosidase and represent a summary of three independent experiments.

co-transfection of WT with either p50 or p65 increased transcription activity of the IRF-7 promoter and co-transfection of both further stimulated the activity of the promoter. However, the response to p50 and p65 decreased dramatically when the NFκB site was mutated. This result suggests that the NFκB binding site present in the IRF-7 promoter is functional. Next, we sought out to determine whether this NFκB binding site is responsible for activation of the IRF-7 promoter by TPA and TNFα. As shown in Fig. 2B, both TPA and TNFα stimulated activity of the IRF-7 promoter. Activation by TPA was about 3-fold, and stimulation by TNFα was about 2.5-fold. Activation of the IRF-7 promoter by IFNα was used as a positive control. When the ISRE site was mutated, the IRF-7 promoter (ISREM) lost the response to IFN, but maintained the response to both TPA and TNFα, suggesting the involvement of different transcription factors in activation of the IRF-7 promoter by TPA and TNFα. Interestingly, the response to TPA and TNFα disappeared after the NFκB binding site on the IRF-7 promoter was mutated (NFκBM), even though the response to IFN remained. This result suggests that activation of IRF-7 promoter by IFN requires the ISRE site, whereas activation by TPA and TNFα requires the presence of the NFκB binding site.

Binding of NFκB Factors to the NFκB Site of IRF-7—The interaction of NFκB family members with the NFκB binding site was assessed by EMSA. Nuclear extracts from HeLa cells before or after treatment with TPA or TNFα were collected, and EMSA was carried out using a DNA probe containing the NFκB site. As shown in Fig. 3A, a distinct DNA-protein complex was detected using the nuclear extract from TPA- treated cells. This complex was not detected when the nuclear lysates from untreated HeLa cells were used. Formation of the complex was completely abolished in the presence of 100-fold molar excess of unlabeled cold probe, but it was not competed out by the oligodeoxynucleotide corresponding to the unlabeled NFκB mutant probe. This result indicates that the formation of this DNA-protein complex requires a functional NFκB site. The identity of the proteins bound to DNA in this complex was further characterized by supershift analysis with
the anti-p65, -p50, and -c-Rel antibodies. Fig. 3A shows complete or partial disappearance of the complex in the presence of p50 and p65 antibodies and simultaneous emergence of several supershifted bands. However, the c-Rel antibody had no effect on the formation of this complex, indicating that the DNA protein complex, formed after TPA treatment, requires the NFκB binding site and consists of p65 and p50 of NFκB family members. The same experiment was repeated with a nuclear extract of TNFα-treated cells. As seen with TPA treatment, the DNA binding complex detected after TNFα treatment also contained p65 and p50 (Fig. 3B). Taken together, our data indicate that treatment with TPA or TNFα stimulates binding of the p50 and p65 heterodimer to the NFκB site present on the IRF-7 promoter.

**Induction of IRF-7 Gene Expression by TOPII Inhibitors**—During the course of our study aimed at identifying the signaling pathways involved in the TPA-induced IRF-7 gene expression, we noticed that a tyrosine kinase inhibitor, genistein, induced IRF-7 expression (Fig. 4A). Genistein is commonly used as a nonspecific tyrosine kinase inhibitor, but it is also an estrogen receptor agonist and a TOPII inhibitor (20). Further study revealed that other TOPII inhibitors were able to induce IRF-7 expression (Fig. 4A and data not shown). The results in Fig. 4A show that treatment of HeLa cells with several TOPII inhibitors such as genistein, daidzein, doxorubicin, mitoxantrone, and etoposide induced IRF-7 gene expression; the maximal increase in the relative levels of IRF-7 mRNA (4-fold) was obtained with etoposide. However, a DNA synthesis inhibitor mitomycin C and a tyrosine kinase inhibitor herbimycin had no effect on IRF-7 gene expression. Furthermore, the induction of IRF-7 expression by etoposide was abolished in the presence of the RNA synthesis inhibitor actinomycin D, suggesting that the TOPII inhibitors induce transcription of IRF-7. In contrast, a significant increase in the relative levels of IRF-7 mRNA (12.9-fold) was observed when etoposide was used in the presence of the protein synthesis inhibitor cycloheximide. Whether the cycloheximide enhancement occurs at the level of transcription or is due to the increased stability of IRF-7 mRNA is unclear.

In an attempt to investigate the mechanism by which TOPII inhibitors stimulate IRF-7 gene expression, we studied the combined effect of TPA, etoposide, and histone deacetylase inhibitor trichostatin A on IRF-7 gene expression. Treatment with genistein was included as a positive control. As shown in Fig. 4B, etoposide, TPA, and trichostatin A were each able to induce expression of IRF-7 gene in Namalwa cells. Interestingly, when the cells were treated with the combination of either etoposide and trichostatin A or TPA and etoposide, an additive effect on IRF-7 expression was observed. However, no such effect was observed when the cells were treated with TPA and trichostatin A. Similar findings were observed in monocyte cell line U937 cells (Fig. 4C), suggesting that the observed enhancement by trichostatin A is not limited to Namalwa cells.

**Changes in Chromatin Structure Caused by TOPII Inhibitors**—To further examine the mechanism by which TOPII inhibitors induce IRF-7 gene expression we studied the effect of etoposide as well as TPA and trichostatin A on the activity of the IRF-7 promoter. In HeLa cells transiently transfected with the reporter plasmid containing the IRF-7 promoter in front of the luciferase gene, treatment with TPA and trichostatin A resulted in a 3- and 9-fold stimulation, respectively. However, treatment with etoposide did not stimulate the IRF-7 promoter. Furthermore, no additional effect was seen when the cells were treated with combinations of TPA and etoposide, TPA and trichostatin A, or trichostatin A and etoposide.

Since it is well known that in transiently transfected cells the plasmid DNA does not form a proper chromatin structure, we used a stable line in which the IRF-7 reporter construct was stably integrated into the chromosome (13). In this cell line, TPA and trichostatin A treatment stimulated the activity of the IRF-7 promoter to a level similar to that seen in a transient transfection. Surprisingly, however, in these cells activation of the IRF-7 promoter (about 2-fold increase) was consistently observed upon treatment with etoposide. Furthermore, treatment of these cells with the combination of TPA and etoposide showed an additive effect, while trichostatin A and etoposide gave a synergistic effect (Fig. 5B). Therefore, the response of integrated IRF-7 promoter was distinct from that of the transiently transfected reporter plasmid where the integrated IRF-7 reporter plasmid responded very much like the endoge-
dependent experiments. The results represent a summary of three in-

Table 1. Etoposide stimulation of IRF-7 expression.

| Treatment   | Relative luciferase activity (%) |
|-------------|----------------------------------|
| Control     | 50                                |
| TPA         | 70                                |
| ETO         | 90                                |
| TrA         | 110                               |

These results indicate that the induction of IRF-7 gene expression by TOPII inhibitors occurs only in the context of an intact chromatin structure.

**Etoposide Treatment Is Associated with Increased Histone Acetylation in the IRF-7 Gene**—The status of histone acetylation is often associated with active gene transcription (21). The current concept is that the core histones are acetylated around the actively transcribed genes, whereas around silenced genes the core histones are in a hypoacetylated state. We therefore examined the status of histone acetylation in the IRF-7 gene locus before and after etoposide treatment by using the chromatin immunoprecipitation assay. To this end, Namalwa cells were treated with etoposide or trichostatin A for 12 h, fixed with formaldehyde solution, and immunoprecipitated with anti-acetylated histone 3 antibodies as described under “Experimental Procedures.” The DNAAs present in the immunoprecipitates were then purified and amplified by using a specific primer set corresponding to the promoter region of the IRF-7 gene. As shown in Fig. 6, the fragment corresponding to the IRF-7 promoter was amplified from the nuclear DNA used for immunoprecipitation. The fragment corresponding to the IRF-7 promoter was also amplified from DNA immunoprecipitated with anti-acetylated H3 antibodies from etoposide and trichostatin A-treated cells that were used as a positive control. To show the specificity of the chromatin immunoprecipitation assay, anti-FLAG antibody was used for immunoprecipitation of the protein cross-linked to DNA. No amplification of the IRF-7 promoter was detected from anti-FLAG immunoprecipitates.

**DISCUSSION**

As described in our previous paper (13), we characterized the IRF-7 promoter and showed that an ISRE site located in the first intron is required for the response to interferon. Here, we report the identification of two novel stimulators of IRF-7 gene expression, namely TPA and TNFα. Further analysis of the IRF-7 promoter revealed that an NFκB site next to the TATA box was responsible for the induction by both TPA and TNFα and that the response to TPA or TNFα treatment was lost after the NFκB site was mutated. NFκB family transcription factors participate in both innate and adaptive immune responses (19, 22). NFκB is composed of a homodimer or heterodimer of Rel, family proteins including p50, p52, p65 (RelA), c-Rel, and RelB. Among them, only p65, c-Rel, and RelB contain transcription activation domains. p50 and p65 heterodimer is the most common form of dimerization (19, 22). In cells treated with TPA or TNFα, we detected binding of a specific DNA binding complex to the NFκB site that was composed of p50 and p65 heterodimer.

The identification of the NFκB site that is sufficient to induce the IRF-7 expression provides new information on the regulation of the IRF-7 gene. Previously, we had shown that an ISRE site located in the first intron binds ISGF3 and is responsible for IRF-7 induction by interferon. The present finding that the NFκB site is also sufficient to trigger IRF-7 expression indicates that IRF-7 expression could be regulated by stimuli that activate NFκB, such as proinflammatory cytokines. Recently, LMP-1 and LPS have been shown to induce IRF-7 expression (5, 23), and both LMP-1 and LPS are known activators of the NFκB pathway (24, 25). This finding has physiological implications. Several studies have indicated that the level of IRF-7 expression is one of the critical determinants of interferon production. The induction of IRF-7 expression by proinflammatory cytokines such as TNFα could increase the IRF-7 level in cells and thus prime them for an increase in the IFN response to incoming viral infection.

In this paper, we also report for the first time that TOPII inhibitors stimulated IRF-7 gene expression. Topoisomerase II enzyme catalyzes DNA topological reactions via a DNA breakage/reunion mechanism (26, 27). The DNA topological reactions allow the enzyme to segregate interlocked chromosomal DNA at mitosis and to remove excess DNA supercoils gener-
ated during processes such as DNA replication, RNA transcription, and chromosomal condensation. The breakage/reunion reaction of TOPII is ATP-dependent and can be interrupted by many TOPII inhibitors (26, 27). The inhibitors stabilize or trap the intermediate in this reaction by blocking/preventing the religation step and give rise to protein-associated double strand breaks, leading to DNA relaxation and chromatin decondensation. The induction of IRF-7 by TOPII inhibitors could be a direct transcription event, such as a direct activation of NFκB transcription factor(s) (19, 25) and consequent stimulation of IRF-7 gene transcription or an event secondary to the chromosomal structure changes inflicted on the DNA by the TOPII inhibitors, such as relaxation of the chromatin structure, exposure of transcription factor(s) binding sites, and activation of IRF-7 gene by the transcription factor(s) already present in cells. Several of our results seem to support the latter scenario.

First, the IRF-7 promoter construct has been very active in transient transfection in all cell types tested, even though expression of endogenous IRF-7 gene was very low in these cells (data not shown). One possible explanation is that the promoter of the endogenous IRF-7 gene is not accessible to the transcription factor(s) in unstimulated cells. Second, the activity of a transiently transfected IRF-7 promoter construct was not stimulated by treatment with TOPII inhibitor; however, this treatment enhanced the activity of the IRF-7 promoter construct stably incorporated into the chromosome. These data indicate that stimulation by TOPII inhibitors is the result of modulation of the chromatin structure in the vicinity and at the promoter site. Up-regulation of c-fos gene expression by the TOPII inhibitor adriamycin was associated with conformational changes downstream of the transcription initiation site (28).

The assembly of DNA into compacted arrays of nucleosomes limits the accessibility for binding of the transcription factors. One group of enzymes that may have evolved to contend with the inhibitory effects of nucleosome assembly is the family of ATP-dependent chromatin remodeling enzymes such as SWI/SNF, which utilize the energy of ATP hydrolysis to alter chromatin structure and to enhance the binding of transcription factors to DNA (29–31). Sequence-specific transcription activators are assumed to help recruit SWI/SNF complex to a specific promoter. A large body of evidence shows the requirement of both the ATP-dependent chromatin remodeling complex and histone acetyltransferases (HAT) for gene expression (21). However, since the chromatin remodeling status of each individual gene locus could be distinct, the different genes may have different requirements for ATP-dependent chromatin remodeling (21). For example, genes localized in a less condensed area of the chromosome can be expressed without much help from an ATP-dependent chromatin remodeling complex, while for expression of other genes, chromatin alteration mediated by the remodeling complex is critical (32, 33). Based on the studies of gene expression in mitotic condensed chromosomes, Krebs et al. (34) proposed a histone tail liberation model. That is, in condensed mitotic chromosomes the targets of the histone acetyltransferase enzyme, the histone tails, are obscured and only after the ATP-dependent remodeling are these tails visible to histone acetyltransferase (34). We suggest that the IRF-7 gene is localized in a condensed area of the chromosome, and therefore its promoter is inaccessible to transcription factors without prior chromosome remodeling. The mapping of the 11p15.5 region (15, 36) has shown that the homolog of Ha-Ras oncogene is localized about 180 kb telomeric and the large imprinting region, which starts with h19 (untranslated gene), is about 1320 kb centromeric from IRF-7 (35). Interestingly, in addition to IRF-7 this region contains other IFN-induced genes, such as IFN-induced transmembrane 1 and 2 genes (15).

The 15.5 region of chromosome 11 is 54.7% GC-rich and contains large numbers of CpG islands (35, 36). This region contains several genes involved in neoplasia and genetic disease. A number of the genes in the 15.5 region show functional imprinting by methylation in cancer cells and are subjected to loss of heterozygosity in several tumors, such as breast and ovarian carcinoma, rhabdosarcoma, and Wilms' tumors (37–39). Interestingly, the loss of IRF-7 expression in several tumor lines was also associated with hypermethylation of the IRF-7 promoter region (13). It will thus be of interest to gain further insight into the potential functional imprinting of IRF-7 in tumors. Whether inactivation of IRF-7 expression contributes to the tumorigenicity is yet to be determined. We have shown previously that IRF-3 is localized on chr.19q13.3 (40) and IRF-5 on chr.7q32 (41). These data indicate that although all these three IRF5s function as direct transducers of virus-induced signaling, these genes are not linked or clustered, and their roles in uninfected cells may be distinct.

Examination of the status of core histone acetylation in the IRF-7 locus also supports the notion that this gene is present in the transcriptionally inactive part of the chromatin. In recent years, acetylation of core histones has emerged as one of the key steps of transcriptional control in all eukaryotic cells. Acetylation levels are controlled by a variety of histone acetyltransferases and deacetylases, which are recruited to promoters by sequence-specific activators and repressors, respectively, and mediate their transcriptional activities (41–43). Association of TOPII with a histone deacetylase has been demonstrated (44). Also, the chromatin remodeling complex CHRAC was shown to contain both ATPases and TOPII (45). The IRF-7 locus is present in a hypoacetylated state in Namalwa cells, which is consistent with the low levels of IRF-7 expression. However, after etoposide treatment, the core histones in the IRF-7 locus were in a hyperacetylated state, and IRF-7 was expressed. We propose that creation of DNA double strand breaks by TOPII inhibitors results in relaxation of chromatin structure at the IRF-7 promoter and exposure of the binding sites for the transcription factors present in the uninduced cells. However, whether the modulation of IRF-7 expression by the TOPII inhibitors is entirely due to the perturbation of torsional strain (45) or whether additional mechanisms, such as blocking the interaction of TOPII with the transcription factors and co-factors or diminished recruitment of histone deacetylase to the IRF-7 promoter, are involved has to be yet examined.

The results presented in this study suggest that two different types of transcription factors are involved in the regulation of IRF-7 gene expression. One group, which includes activated transcription complexes such as ISGF3 and NFκB, may be able to bring chromatin-remodeling complex to the IRF-7 locus and consequently triggers IRF-7 gene expression. The second group...
of transcription factors, present constitutively in the cell, requires prior chromatin remodeling to be able to bind the promoter and activate expression of the IRF-7 gene.

Taken together, our studies have revealed some interesting features of IRF-7 gene regulation (Fig. 7). First, we have identified two enhancer-binding sites, namely ISRE and NFκB, each of which is sufficient to trigger IRF-7 expression. The presence of these two enhancer sites renders IRF-7 gene expression subject to the modulation by interferon as well as proinflammatory cytokines and another reagents, such as TPA and LPS, that can activate NFκB transcription factors. A second level of regulation of IRF-7 gene expression is associated with the promoter hypermethylation, which was reported in our previous paper (13). The promoter region of the IRF-7 gene contains CpG clusters that are methylated in some cancer cells resulting in the silencing of expression of the IRF-7 gene. Third, this study has revealed a new feature of IRF-7 gene regulation, namely regulation by gene accessibility. Localization of IRF-7 to chromosome 11p15.5 indicates that the IRF-7 gene is located in the GC-rich area of the chromosome, which may be condensed and which is inaccessible, without prior chromosome remodeling, even to the transcription factors constitutively present in the cells.

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