Regulation of the Promoter Activity of Interferon Regulatory Factor-7 Gene

ACTIVATION BY INTERFERON AND SILENCING BY HYPERMETHYLATION

The molecular mechanism by which virus induces expression of the early inflammatory genes has not yet been completely elucidated. Previous studies indicated that the virus-mediated transcription of type I interferon (IFN) genes required activation of two members of IFN regulatory factor (IRF) family, IRF-3 and IRF-7, where the expression of IRF-7 was found to be indispensable for the induction of IFNA genes. To determine the factors that regulate expression of IRF-7 gene, as well as its inducibility by type I IFNs, we have isolated and characterized the promoter and first intron of the human IRF-7 gene. This region shows a presence of two potential interferon-sensitive response elements (ISRE/IRF-E). However, only the ISRE present in the first intron was functional and conferred interferon inducibility in a transient transfection assay. Using a pull-down assay with an oligodeoxynucleotide corresponding to this ISRE immobilized to magnetic beads, we have demonstrated that this ISRE binds ISGF3 complex and IRF-1 from the extract of IFN-treated cells but not from the untreated cells. We have further shown that the previously observed lack of expression of IRF-7 in 2fTGH fibrosarcoma cell line, correlated with hypermethylation of the CpG island in the human IRF-7 promoter. The repression of the promoter activity was relieved by treatment with DNA methyltransferase inhibitor 5-aza-deoxycytidine. In vitro methylation of IRF-7 promoter silenced IRF-7 directed expression of luciferase gene in HeLa cells that express endogenous IRF-7 gene. Whether silencing of IRF-7 by methylation is instrumental for the process of tumorigenesis remains to be determined.

Expression of eukaryotic genes is regulated at multiple levels including the accessibility of promoter DNA for binding of basic transcriptional machinery or the specific transcription factors and chromatin structure around the potential promoters. The molecular mechanism by which virus activates expression of the early inflammatory genes has not yet been completely elucidated. It was shown, however, that activation of the NFkB family of transcription factors in infected cells plays a critical role in the transcriptional activation of many cytokine and chemokine genes, since large number of these genes contains NFkB-binding sites in their promoters. Recently the importance of another family of transcription factors as the mediators of virus induced signaling has emerged. These factors designated interferon (IFN) regulatory factors (IRF) were shown to play an important role in the induction of the early inflammatory genes in infected cells as well as in development of cells of lymphoid lineage (1, 2).

The IRF-3 and IRF-7 have been identified as direct transducers of virus mediated signaling and were shown to play a critical role in the induction of Type I IFN genes (3–7). In infected cells, these factors are phosphorylated on C-terminal serines and majority of the phosphorylated IRFs was localized in the nucleus (8–11). There are, however, differences in their expression. IRF-3 is expressed constitutively in most of the cells examined and its expression is enhanced neither by viral infection nor IFN treatment (3, 12). In contrast, human IRF-7 is expressed efficiently only in lymphoid tissues, peripheral blood mononuclear cells and some cell lines of lymphoid origin and its transcription can be further stimulated by treatment with IFNa. However, none or very little expression of IRF-7 could be detected in established cell lines of fibroblast or epithelial origin (4). Recent studies demonstrated the critical role of IRF-7 in the virus-mediated induction of IFNA genes both in human and mouse cells and closely correlated the virus mediated, cell type-specific expression of IFN genes with the presence of IRF-7 in the cells (13–15). Thus, the cells that were not able to express IFNA genes upon viral infection were able to do so upon reconstitution of IRF-7 expression (13, 14). The role of IRF-7 was also implied in virus-mediated induction of IFNB and RANTES genes (7, 16). Furthermore, the levels of IRF-7 were found to be an important determinant in the regulated expression of the EBV-encoded EBNA-1 gene, where the silencing of Qp promoter, that regulates expression of EBNA-1 gene in EBV-associated tumors, was correlated with the expression of IRF-7 (17, 18). These results indicated that the levels of IRF-7 in the cells may not only modulate the virus-mediated inflammatory responses but may also affect the EBV-associated malignancies.

The hereditary silencing of gene expression can occur by mutational and epigenetic pathways. The silencing of gene expression by methylation of the CpG dinucleotides, especially those located in the CpG clusters, has been shown to occur relatively frequently in immortalized and transformed cells (19). The aberrant methylation has been associated with the
inactivation of tumor suppressor genes in human cancers and may also play a role in the control of cell type-specific gene expression. Recent studies have shown that the methyl-CpG-binding protein can bind transcriptional co-repressor Sin3A and recruits histone deacetylase to methylated DNA (20). This results in deacetylation of the chromatin and formation of a transcriptionally repressive chromatin around the methylated DNA. What determine the methylation status of CpG islands is still not clear. Several DNA methyltransferases (DNMT) were identified. The DNMT-1 was shown to preferentially methylate hemimethylated DNA and is therefore believed to function as maintenance methyltransferase (21). Elevated levels and activity of DNMT-1 were observed in cancer cells in vitro (22). However, the knock-out studies (23) suggested that two other methyltransferase, DNMT-3a and DNMT-3b, are responsible for de novo DNA methylation and mutations in DNMT-3b gene are associated with the ICF immunodeficiency syndrome (23, 24).

The aim of the present study was to determine the critical factors that regulate type I IFN-stimulated expression of IRF-7 gene as well as the mechanism by which expression of IRF-7 gene is silenced in some cell lines. To this effect we have isolated the promoter of the human IRF-7 gene and have shown by deletion and mutation analyses that it contained two interfering-stimulated response element (ISRE)/IRF-E like elements. However, only one of them was functionally active and able to confer the IRF-α-mediated activation of IRF-7 promoter. Furthermore, we have shown that hypermethylation of the CpG island in the IRF-7 promoter is responsible for the silencing of the IRF-7 gene in 2fTGH fibrosarcoma line. Expression of IRF-7 gene and stimulation by IFN could be rescued in non-expressing cells by treatment with 5-aza-deoxycytidine (5-AzaC).

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**Human cells HeLa, 2fTGH, and U2A were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Jurkat T cells were cultivated in RPMI plus 10% FBS. Rabbit polyclonal antibodies against IRF-1 (C-20), IRF-2 (C-19), STAT 1 (E-23), STAT 2 (N-17), and ISGF-3γ (C-20) were purchased from Santa Cruz Biotechnologies.

**Isolation of IRF-7 Genomic DNA**—A DASH II genomic library containing the BamHI genomic DNA fragment of human lung carcinoma NCI-H157 cell line, was screened by hybridization with full-length IRF-7 cDNA probe. The phage harboring the IRF-7 genomic DNA. Two plaques (from 2.50 × 10^5 plated) showing positive hybridization with IRF-7 DNA probe were purified by three rounds of purification. The corresponding phage DNA was purified (Qiagen λ phage DNA isolation kit) and digested with various restriction enzymes to construct a restriction map. The result indicated that the insert containing genomic DNA was about 12 kb (data not shown). The EcoRI-digested genomic DNA fragments were then cloned into pBluescript vector (Stratagene). The clone containing EcoRI insert with IRF-7 sequences was identified by colony hybridization, and PCR amplification. The insert DNA was sequenced and the sequences were compared with the IRF-7 cDNA sequence.

**Transient Transfection and Luciferase Assay—**Cells were transfected in 60-mm dishes by Superfect transfection method (Qiagen). One microgram of the reporter plasmid DNA and 0.1 μg of β-galactosidase expressing plasmid (internal control) were used for each transfection. In a co-transfection experiment, 1:1 ratio of the reporter and expression plasmid (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 6-well plates, and incubated at 37°C for another 6 h after which the medium was replaced with DMEM containing 2% FBS and recombinant IFN-α2 (500 units/ml). After an additional 12 h, the cells were harvested, lysed, and luciferase activity was measured as described previously (25). Experiments were repeated at least three times.

**Reporter Constructs Containing IRF-7 Promoter and Its Mutants—**A 1.6-kb fragment of IRF-7 promoter containing the TATA box and the first intron was amplified by PCR, using primer set RL1S, RL1AS (see Table I), and EcoRI fragment of IRF-7 genomic DNA as template. The fidelity of PCR transcription was confirmed by sequencing and the amplified fragment was inserted into XhoI and HindIII sites of pGL3-basic vector (Invitrogen). This plasmid (designated RL1) was used as the parental plasmid to generate (by PCR) a serial of mutation and deletion constructs (Fig. 2B). A PCR-based site-directed mutagenesis method (26) was used to mutate ISRE/IRF-E sites in IRF-7 promoter. The primers IRF-EMS and IRF-EMAS (Table I) were used for mutation of the IRF-E site (RL2 plasmid). The primers ISRE-MS and ISRE-MAS were used to mutate the ISRE site (RL3 plasmid). The deletion construct RL5 was generated using RL5S and RL1AS primers and RL1S and RL6AS primers were used to construct RL6. The plasmids containing IRF-E or ISRE sites and their flanking sequences (RL7, RL7M, RL8, and RL8M plasmids) were generated by inserting the respective double stranded oligodeoxynucleotide fragments into XhoI and HindIII sites of pGL3-promoter vector (Invitrogen). The oligonucleotides used for generation of RL7 plasmid were IRF-ES and IRF-EAS; for RL7M plasmid, IRF-EMS and IRF-E1MAS; for RL8 plasmid, ISRE-S and ISRE-AS; for RL7M plasmid, ISRE-MAS and RL7M plasmid ISRE-MS and ISRE-MAS (Table I).

**5-AzaC Treatment and Northern Blot Analysis—**HeLa cells were cultivated in the presence of 5 μM 5-AzaC for 4 days in DMEM and 10% FBS with fresh drug added every 24 h. Control cells were grown under identical conditions in medium without 5-AzaC. At day 5, cells were either treated with 500 units/ml interferon-α for 6 h or only incubated for additional 6 h. Total RNA from control and stimulated cells was analyzed by Northern blot.

Northern blot analysis was done as described before (4) with 10 μg of total RNA isolated with TRIZOL (Life Technologies, Inc.). Random-primed 32P-labeled human IRF-7 cDNA encoding the entire open reading frame was used as a probe.

**Oligodeoxynucleotide Pull-down and Immunoblot Assays—**Biotinylated antisense oligonucleotides (IRF-EAS and ISRE-A5) containing IRF-E/ISRE sites were synthesized (Life Technologies, Inc.).
annealed with the corresponding sense oligodeoxynucleotides. Biotinylated DNA (4 μg) was then mixed with 100 μg of DynaBeads M-280 streptavidin (Dynal) in 200 μl of binding buffer I containing 20 mM Tris, pH 8, 1 mM EDTA, and 0.1 mM NaCl. After 1 h incubation at room temperature, the beads were washed three times with binding buffer I and then resuspended in 230 μl of binding buffer II (10% glycerol, 12 mM HEPES, 5 mM MgCl₂, 60 mM KCl, 0.1 mM dithiothreitol, and 0.1 mM phenethylisulfonyl fluoride). Nuclear extracts were prepared as described before (13) from control cells and cells stimulated with 500 units/ml interferon-α for 2 h. Then 150 μg of nuclear extracts were incubated with 40 μl of DNA-conjugated beads, containing either IRF-α or ISRE oligodeoxynucleotides in binding buffer II, for 2 h at 4 °C. The beads were washed twice with binding buffer II three times before the bound proteins were eluted from the beads by boiling in sample buffer. The proteins were resolved on 10% SDS-polyacrylamide gel electrophoresis, and transferred to membranes. The membranes were blocked by 10% skim milk in phosphate-buffered saline containing 0.1% of Tween 20. The individual proteins were identified by immunoblotting with antibodies against IRF-1, IRF-2, PAS/IRF-9, STAT1, and STAT 2 used at a dilution of 1:1000. The signals on the blots were visualized by the enhanced chemiluminescence detection system. (ECL, Amersham Pharmacia Biotech) Sodium Bisulfite Modification—Genomic DNA was prepared as described (27). One microgram of DNA was denatured in 50 μl of 0.2 M NaOH for 10 min at 37 °C. For the chemical modification of DNA, 520 μl of 3 M sodium bisulfite (Sigma) and 30 μl of 10 M hydroquinone (Sigma) were added to the DNA solution, and the samples were incubated for 16 h at 50 °C. Modified DNA was purified on Wizard purification resin (Promega) and eluted in water (50 μl). As a final step, NaOH was added to a final concentration of 0.3 M and the samples were incubated for 5 min at room temperature. DNA was precipitated by ethanol and resuspended in water.

Methylation-specific PCR (MSP) and Genomic DNA Sequencing—DNA methylation status in the CpG islands of IRF-7 promoter was analyzed by treatment with sodium bisulfite and subsequent PCR amplification (MSP). The rationale of using MSP method to detect methylation status of the CpG-rich region is based on the conversion of cytosine to uracil after bisulfite treatment of DNA and has been previously validated for number of methylated genes (27). The sodium bisulfite treated DNA and MSP were performed as described previously (27). The primer sequences for the amplification of methylated and unmethylated DNA fragments are listed in Table I. The size of amplified DNA fragment using these primers is 226 bp. PCR conditions for the amplification of methylated and unmethylated DNA were as follows: reaction was started at 94 °C for 5 min before the addition of Taq polymerase (Promega). Amplification was 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The final extension was done at 72 °C for 7 min. Controls without DNA were performed for each set of amplifications. Amplified products were resolved on a 2% agarose gel, and visualized after staining with ethidium bromide. The amplified DNA was purified using a GENECLEAN kit (BIO-101) and sequenced.

In Vitro DNA Methylation and Stable Transfection—Plasmid DNA isolated from RL1 plasmid, containing a CpG island (as determined by GeneTool program) was methylated in vitro using SssI methylase (1 unit/μg; New England Biolabs), which methylates every CpG site. Complete methylation was verified by digestion with the methylation sensitive restriction enzyme HpaII. Only plasmids that showed a complete protection from HpaII digestion were used in the transfection experiments. To establish stably transfect cell lines, HeLa cells plated in 100-mm dishes were transfected with 10 μg of either the methylated or unmethylated RL1 plasmid together with 1 μg of p-galactosidase expressing plasmid, used as an internal control, and pBv neo vector which confers resistance to G418 (1 μg). The transfectants were selected in medium containing 800 μg/ml G418, and surviving colonies were pooled after 4 weeks. The pooled cells were treated with recombinant IFN α/β (500 units/ml) for 12 h and luciferase activity was measured in cell lysates prepared from treated and untreated cells.

RESULTS

Isolation of IRF-7 Genomic DNA Containing the 5'-Untranslated Region—Using the full-length IRF-7 cDNA as probe we have screened the human genomic library (see “Experimental Procedures”), and isolated a 3-kb fragment of genomic IRF-7 DNA containing 5'-flanking DNA and the first intron of IRF-7 gene. The sequence of the 1.3-kb fragment containing the promoter region and the first intron of the IRF-7 gene are shown in Fig. 1 (GenBank accession number AF277159). Using the PCR conditions for the amplification of methylated and unmethylated DNA were as follows: reaction was started at 94 °C for 5 min before the addition of Taq polymerase (Promega). Amplification was 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The final extension was done at 72 °C for 7 min. Controls without DNA were performed for each set of amplifications. Amplified products were resolved on a 2% agarose gel, and visualized after staining with ethidium bromide. The amplified DNA was purified using a GENECLEAN kit (BIO-101) and sequenced.

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### Transcriptional Activity of IRF-7 Promoter

IrF-7 genomic DNA was isolated from λ DASH II genomic library derived from normal human lung carcinoma NCI H157 cell line, using full-length IRF-7 cDNA as hybridization probe. The fragment containing 5'-flanking region and the first intron of the IRF-7 gene was isolated as described under “Experimental Procedures.” The IRF-7 promoter sequence was analyzed using TRANSFAC data base and the sequence of a 1.3-kb fragment of the IRF-7 promoter region including the first intron (boxed) is shown. The TATA box and some potential transcriptional factor-binding sites are shown. The transcriptional initiation site, and the transcriptional start sites of two IRF-7 isoforms: IRF-7H, IRF-7A, as well as the beginning of the second intron are marked with arrows.

**Fig. 1. The human IRF-7 promoter.** IRF-7 genomic DNA was isolated from normal human lung carcinoma NCI H157 cell line, using full-length IRF-7 cDNA as hybridization probe. The fragment containing 5'-flanking region and the first intron of the IRF-7 gene was isolated as described under “Experimental Procedures.” The IRF-7 promoter sequence was analyzed using TRANSFAC data base and the sequence of a 1.3-kb fragment of the IRF-7 promoter region including the first intron (boxed) is shown. The TATA box and some potential transcriptional factor-binding sites are shown. The transcriptional initiation site, and the transcriptional start sites of two IRF-7 isoforms: IRF-7H, IRF-7A, as well as the beginning of the second intron are marked with arrows.

**Identification of Functional ISRE That Confers IFN Inducibility to IRF-7 Gene—Sequence analysis of the 1.6-kb fragment containing the IRF-7 promoter and the first intron shows a presence of IRF-α/β ISRE binding sites (Table II). One is in the 5'-flanking region of the IRF-7 gene and its sequence closely resembles the IRF-E site, the second one that is closely related to ISRE is in the first intron of the IRF-7 gene (Fig. 1). In order to determine the role of the IRF-E and ISRE sites in the IFN-α mediated stimulation of IRF-7 gene, we have constructed reporter plasmids containing either the entire 1.6-kb region (RL1) or its deletion (RL5 and 6) and mutation fragments (RL2, -3, and -4) inserted 5' of the luciferase gene in
the pGL-3 vector (Fig. 2B). The transcriptional activities of these promoters were analyzed in a transient transfection assay in HeLa cells before and after induction with IFN-α. We have chosen HeLa cells for these studies because these cells express IRF-7 gene constitutively (IRF-7 mRNA can be detected in these cells by RT-PCR, data not shown) and its levels can be further enhanced by treatment with IFN-α (Fig. 2A). Furthermore, the transfection efficiency of these cells is consistently high. As can be seen in Fig. 2C, transfection of RL1 plasmid, that contains an entire 1.6-kb fragment of the 5' end flanking region and the first intron, resulted in a significant level of luciferase activity that was about 6 times higher than when the promoterless pGL-3 vector was transfected. These results indicated that in HeLa cells this promoter is activated constitutively which correlated with the observed constitutive expression of the endogenous IRF-7 gene. Treatment of the transfected cells with IFN-α for 12 h resulted in about 3.5-fold increase in luciferase activity. The mutation in the IRF-E site that is localized in the promoter region (RL2 plasmid) resulted only in a slight decrease in both constitutive and inducible promoter activity (81 and 72% of RL1 activity, respectively). These results suggested that this IRF-E alone cannot confer IFN inducibility. In contrast, mutation of the ISRE site that is present in the first intron (RL3 plasmid) essentially abolished the response to IFN-α while it had little effect on the constitutive expression of this promoter. These results suggested that ISRE is the functional element that confers the response to IFN-α. The RL4 plasmid in which both IRF-E and ISRE were mutated had the same activity as RL3, supporting the observation that ISRE is the functional element that confers the IFN-α-mediated stimulation.

To further confirm our findings with the mutated plasmids we constructed several deletion mutants and analyzed their expression. In the RL-5 plasmid we have deleted the 5'-upstream region of RL1 including the IRF-E and inserted the remaining 600-bp fragment that contains the ISRE site into pGL3 plasmid. When transfected into HeLa cells the overall transcription activity of this plasmid was lower than that of RL1 but the ability to respond to IFN treatment was preserved and the luciferase activity was increased by about 3 fold upon IFN-α stimulation. The RL6 plasmid contains only the 5'-flanking region of IRF-7 promoter and has the first intron including the ISRE site deleted. This construct had only low constitutive activity and did not respond to IFN-α. Thus, the deletion analysis supports the finding that only the ISRE site is able to mediate the response to IFN.

Finally, we wanted to further confirm our findings and to examine whether either IRF-E or ISRE can alone, confer the stimulation by IFN-α. We therefore inserted IRF-E or ISRE element together with the respective flanking sequences (about 35 bp in length) into pGL3-promoter vector, 5' of a basic SV40 promoter and luciferase gene. As a control, we inserted into this vector the same fragments, which contained mutations in the respective IRF-E/ISRE sites. These plasmids were transfected into HeLa cells and their expression was analyzed before and after IFN stimulation. The results showed that RL7 plasmid as well as its mutated analogue did not respond to IFN stimulation. In contrast, expression of RL8 plasmid was significantly increased (8-fold) in IFN-treated cells. Mutation in the ISRE site (RL8M) completely abolished the IFN-mediated stimulation. Thus, altogether, these data indicate that ISRE is the functional element that mediates the response of IRF-7 gene to IFN-α. These data also indicate that this element alone is able to confer IFN inducibility indicating that it functions as an enhancer in IFN-treated cells.

Activation of IRF-7 Promoter Is Mediated by ISGF3 Complex—We have demonstrated the importance of the ISRE domain in response of IRF-7 promoter to IFN-α stimulation. It was shown that the stimulation of interferon-stimulated gene

### Table II

| Gene          | Sequence                      | Ref. |
|---------------|-------------------------------|------|
| IRF-7 (IRF-E) | CA AAA GCGAAACT              |      |
| IRF-7 (ISRE)  | GG AAA GCGAAACC              |      |
| ISRE consensus| (A/G/NG AAA NNGAAACT)        | 29   |
| IRF-E consensus| G(A/AAA/G(C)/T/C)GA(A/G/C)/T/C | 28   |

### Fig. 2

A. Identification of functional ISRE site that confers IFN-α inducibility to IRF-7 gene. A, time course induction of the IRF-7 gene in HeLa cells by IFN-α. HeLa cells were cultivated in DMEM plus 10% FBS and stimulated with 500 units/ml IFN-α for the indicated times. Total RNA was isolated from IFN-treated cells and controls and analyzed on Northern blots as described before. Ethidium bromide (E.B.) staining of total RNA was used as an internal loading control. B, IRF-7 promoter constructs. Schematic representation of the 1.6-kb IRF-7 promoter region (top) and the mutation (RL2, RL3, and RL4) and deletion constructs (RL5 and RL6) that were inserted into pGL3 basic vector upstream of a luciferase reporter gene (Luc). The mutation sites are marked by a *. Wild type and mutated ISRE (RL8 and RL8M) as well as IRF-E (RL7 and RL7M) oligodeoxynucleotides were inserted into pGL-Promoter vector. C, activation of IRF-7 promoter constructs in HeLa cells by IFN-α. The IRF-7 promoter constructs were co-transfected with plasmid encoding β-galactosidase into HeLa cells as described under “Experimental Procedures.” In all transfection assays the levels of luciferase activity were normalized to a constant level of β-galactosidase that served as an internal control. The result are presented as percentage of a luciferase activity of the RL1 construct in IFN-α-treated cells that is considered 100%. The error bars represent the S.D. of three independent experiments.
In addition, a significant binding of IRF-1 to ISRE oligodeoxynucleotide was also detected in IFN-treated cells, while in untreated cells very little IRF-1 bound. Binding of IRF-1 from the IFN-treated cells to IRF-E was also detected, but the binding was much weaker and the signal was detectable only after a long exposure. The increased binding of IRF-1 in IFN-treated cells corresponded to the increase levels of IRF-1 in the lysates of IFN-treated cells (data not shown). Expression of IRF-1 was shown to be up-regulated in IFN-treated cells and to stimulate expression of several ISGs (33). Thus the observed binding of IRF-1 to ISRE may implicate a potential role of IRF-1 in virus-mediated induction of IRF-7 promoter. In addition to IRF-1 we have also observed binding of IRF-2 to both ISRE and IRF-E elements, but the binding was much stronger in the extracts from uninduced cells than from induced cells. Since IRF-2 is known to act as a repressor, which antagonizes the action of IRF-1 (34), the decreased binding of IRF-2 to ISRE site in IFN-treated cells, together with the binding of IRF-1 and ISGF3 suggested a possible competition between binding of IRF-2 and the other IRF factors.

In order to be able to distinguish between the contribution of IRF-1 and ISGF3 complex in the IFN mediated activation of IRF-7 promoter, we have examined the activity of ISRE containing plasmid RL8 in U2A cells that are lacking the functional p48/IRF-9. As shown in Fig. 3B in U2A cells expression of RL8 was not stimulated by IFN treatment. Overexpression of IRF-1 or IRF-7 in these cells did not restore IFN stimulation. However, when RL8 was transfected together with p48 expressing plasmid the IFN-mediated stimulation of ISRE was restored. These data indicated that ISGF3 is a critical determinant in IFN-mediated stimulation of IRF-7 promoter and confirmed the previous observation of the importance of p48/IRF-9 in the expression of IRF-7 gene in mice (14). Although IRF-1 was not able to restore IFN inducibility of IRF-7 promoter in U2A cells, the result of co-transfection assay of IRF-1 and RL1 expression plasmids indicated that IRF-1 could further stimulate activity of IRF-7 promoter in IFN-treated HeLa and 2TGH cells (data not shown). These data suggested that IRF-1 may contribute to the activation of IRF-7 promoter by IFN.

5-AzaC Treatment Restores IRF-7 Expression in 2fTGH Cells—We have shown previously that 2TGH cells do not express IRF-7 constitutively nor can IFN-α treatment stimulate expression of IRF-7 in these cells (4, 13). Surprisingly, however, transfected RL1 plasmid that contains 1.6 kb of IRF-7 promoter was constitutively expressed in these cells and its expression was further stimulated by treatment with IFN-α (data not shown). These data indicated that 2TGH cells do not have a defect in the basal transcription machinery that is required for the expression of the IRF-7 gene. Since no obvious genetic defect in the IRF-7 gene could be identified in 2TGH cells (data not shown) we explored the possibility that the expression of this gene is modified epigenetically by methylation. Silencing of a number of genes in immortalized and transformed cell lines as well as in primary tumors has been shown to be mediated by promoter hypermethylation (19). To examine the possibility that methylation is involved in the silencing of the IRF-7 gene in 2TGH cells, we treated these cells with 5-AzaC, a cytosine analogue which is a suicidal inhibitor of methyltransferase and can be used to reverse the inhibitory effect of methylation on gene expression. IRF-7 expression was analyzed 4 days after the initial addition of the drug. As shown in Fig. 4, in the absence of 5-AzaC, 2TGH cells failed to express IRF-7 mRNA even after IFN-α stimulation. However, after 5-AzaC treatment, IRF-7 mRNA could be detected by RT-PCR (data not shown) suggesting that 5-AzaC treatment alone was sufficient to elicit basic levels of IRF-7 expression and further enhancement could be seen in IFN-α-treated cells (Fig. 4).
Furthermore, upon virus infection, 5-AzaC-treated cells were able to synthesize biologically active IFN-α, while no IFN-α production could be detected in infected, untreated 2fTGH cells (data not shown).

The IRF-7 Promoter Contains a Putative CpG Island, Which Is Methylated in 2fTGH Cells—The restoration of IRF-7 gene expression by 5-AzaC treatment could be due to a direct demethylation of the IRF-7 gene or another gene, which expression is required for IRF-7 activation. Genes silenced by methylation generally contain CpG islands in their promoters, which are targeted by methylation. In general, CpG islands are rare in mammalian DNA with a typical expected:observed ratio of 0.3 or lower. In promoters with CpG islands, this ratio is 0.6 or higher. The 1.6-kb fragment of the IRF-7 promoter showed a presence of one CpG island (Fig. 5A) located around the TATA box spanning from −271 to +382 bp which has CpG frequency higher than 1.0. The methylation status of this CpG island was analyzed by MSP analysis (27). This method uses primers that are able to distinguish methylated from unmethylated DNA in bisulfite-modified DNA. Bisulfite treatment of DNA converts the unmethylated cytosine residues to uracil, while the methylated cytosine in CpG dinucleotides remain unchanged. The primer sequences chosen provided maximal discrimination between methylated and unmethylated CpG island of the IRF-7 promoter region (Table I). We first used these primers to amplify sequences from bisulfite-treated genomic DNA isolated from IRF-7 nonexpressing cell 2fTGH and IRF-7 expressing HeLa cells. As seen in Fig. 5B, methylation specific primers (MS1, MAS1) amplified a strong band from DNA of 2fTGH cells while the unmethylated primers (UMS1, UMAS1) did not amplify any band. In contrast, the unmethylated primers amplified a strong band from HeLa cells. These data show that in nonexpressing cells, 2fTGH, the IRF-7 promoter is hypermethylated while it is hypomethylated in expressing cells. Interestingly, methylated primers also amplified a weak band from HeLa cells suggesting a low occurrence of hypermethylated alleles in the population of HeLa cells used. To further confirm the findings from MSP analysis we cloned and sequenced the amplified fragments. The sequences of the first 110 bp of the amplified 226-bp fragments from bisulfite-treated DNA of 2fTGH and HeLa cells are shown in Fig. 5C. There are 13 CpG islands present in this region. The fragment amplified by the unmethylated primers from HeLa cells DNA shows that all

**Fig. 4.** 5-AzaC treatment restores IRF-7 expression in 2fTGH cells. 2fTGH cells were cultivated in DMEM with 10% FBS in the presence of 5 μM 5-AzaC for 4 days, with fresh drug added every 24 h. IFN-α was added at day 5 into 5-AzaC-treated and non-treated 2fTGH cells and total RNA was isolated 6 h later. Northern blot analysis was performed using the full-length IRF-7 cDNA as a probe. Epstein-Barr (E.B.) staining of total RNA was used as internal loading control. The experiment was repeated at least three times.

**Fig. 5.** The promoter of IRF-7 is methylated in 2fTGH cells. A, quantitation of CpG ratio (observed/expected) in the IRF-7 promoter region. CpG frequency was analyzed over a 1.5-kb region of IRF-7 promoter using GeneTool program (BioTools Inc.), where CpG island is defined as a region with a CpG ratio of 0.6 or higher. B, methylation-specific PCR. Genomic DNA was isolated from HeLa and 2fTGH cells and modified by sodium bisulfite treatment. PCR amplification of methylated and unmethylated IRF-7 fragments were conducted as described under “Experimental Procedures.” The amplified products were resolved on 2% agarose gels. C, sequence of bisulfite-modified DNA from 2fTGH and HeLa cells. The sequence of the first 110 bp of the PCR-amplified 226-bp fragments from bisulfite-treated and untreated genomic DNA from 2fTGH (2fTGH B.S.) and HeLa (HeLa B.S.) is shown. The methylated cytosines are marked by an asterisk, while the unmethylated cytosines are boxed. D, sequence of ISRE site along with the flanking region of bisulfite modified DNA from 2fTGH cell. The ISRE site is underlined.
CpG cytosines were converted to uracil, confirming the hypomethylated state of this CpG island in HeLa cells. In contrast when DNA from 2TGH cells was analyzed, every cytosine in CpG dinucleotide remained unchanged by bisulfite treatment, indicating that these cytosines are methylated. Thus, these results directly identified the methylated CpG islands in the promoter region of IRF-7.

Since there is a CpG dinucleotide present in the ISRE site, we further examined the region flanking ISRE site using methylation-specific primers (MS2, MAS2) from 2TGH cells and sequenced the amplified product. The partial sequence of amplified product, that contains the ISRE site (underlined), is shown in Fig. 5D. As expected, every cytosine in CpG dinucleotide remained unchanged in bisulfite-treated DNA, indicating that they are methylated.

**In Vitro Methylation of IRF-7 Promoter Blocks Its Expression in HeLa Cells**—To determine whether the methylation of IRF-7 promoter is sufficient to silence its expression in the cells that can express the endogenous IRF-7 gene, the RL1 plasmid that contains the entire CpG island was methylated in vitro by SssI, an enzyme that methylates every CpG dinucleotide. The methylated and unmethylated RL1 plasmids were transfected into HeLa cells together with a plasmid conferring resistance to neomycin and stably transfected clones were selected by growth in G418 and pooled. Luciferase activity in cell lysates of the transfected cells was determined before and after IFN-α treatment. As shown in Fig. 6, cells transfected with RL1 plasmid showed a constitutive luciferase activity that was further increased by treatment with IFN-α. In contrast, the expression level of methylated RL1 was 100-fold lower than that of their unmethylated counterpart. Furthermore, after IFN-α treatment no further stimulation of the promoter activity could be detected. However, treatment with 5-AzaC (5 μM) for 4 days could restore the activity of IRF-7 promoter and further stimulation (2-fold) was observed after IFN treatment (data not shown). β-Galactosidase activity was detected in all transfected cells and was used as internal control to normalize the luciferase activity. These results showed that methylation of CpG dinucleotides in IRF-7 promoter blocks its expression even in the cells that can express endogenous IRF-7 gene.

**DISCUSSION**

We have cloned, in this study, the promoter region of the human IRF-7 gene and analyzed the molecular basis of IFN-α-mediated transcriptional activation. In contrast to functionally related IRF-3, which is expressed constitutively, IRF-7 is expressed predominantly in cells of lymphoid origin and its expression is stimulated by IFN (4, 15). Consistent with this pattern of expression, IRF-7 promoter shows several common characteristics with the promoters of other inducible genes. It has the conserved canonic TATA box that is absent in the promoter of human IRF-1, IRF-2, IRF-3, and p48/IRF-9 genes. Upstream of the TATA box there is a NFkB site which overlaps with the STAT 6-binding site and Sp-1 site. Since the IRF-7 gene is transcriptionally activated by type I IFNs the presence of the ISRE site in the promoter was expected. The sequence analysis revealed the presence of GAAAGCGGAACTC domain similar to the IRF-E consensus sequence (28) localized about 260 bp upstream of the TATA box. However, the deletion analyses as well as mutation analyses of this IRF-E site showed that this IRF-E did not confer the IFN stimulated activation. Instead, the GGTTCGCTTTC sequence present in the first intron of the IRF-7 gene, that closely resembles the ISRE site (29) of ISG genes responded to IFN mediated signal and its deletion or mutation abolished the stimulation of the IRF-7 promoter in IFN-treated cells. The fact that a single copy of this element was able to mediate response to IFN-α indicated that this ISRE functions as an enhancer in IFN-α-treated cells. Accordingly binding of both IRF-1 and ISGF3 complex to this ISRE was detected in IFN-α-treated cells. However, no activation of IRF-7 promoter was observed in a cell line that was defective in p48/IRF-9 (U2A cells). Since reconstitution of p48/IRF-9 expression, but not overexpression of IRF-1, restored the response to IFN-α, we concluded that the stimulation is mediated by ISGF3 complex. These data are consistent with previous observations that the expression of IRF-7 gene is stimulated by Type I IFNs but not by IFN-γ, since the ISGF3 complex is assembled only in IFN-α-treated cells (29). The importance of p48/IRF-9 in the expression of IRF-7 gene was suggested by the results in p48/IRF-9−/− mice, which shown that the fibroblasts derived from these mice were not able to express IFNA after viral infection and the defect was related to the absence of IRF-7 (14). Only after the reconstitution of IRF-7 expression could IFNA expression be rescued. However, the results of transient transfection assay indicated that the human IRF-7 promoter has a low constitutive activity. Furthermore, we have detected low levels of constitutive expression of IRF-7 gene in lymphoid tissues (4) as well as in primary peripheral blood mononuclear cells, macrophages, and dendritic cells. Thus, it is likely that the transcription activities of the mouse and human IRF-7 promoter are not identical.

The role of NFκB site in IRF-7 promoter remains to be clarified. Recently it was shown that IRF-7 is expressed at high levels in some EBV-transformed B cell lines and it was suggested that the EBV-encoded integral membrane protein, LMP-1, which activates NFκB, stimulates the expression of the IRF-7 gene (17). However, co-transfection of the reporter RL1 plasmid with LMP-1 expression plasmid to HeLa cells did not result in significant activation of IRF-7 promoter (data not shown). These results suggested that the LMP-1mediated activation of IRF-7 may be cell type-specific and that NFκB activation may require cooperation with other transcription factors. Thus, further studies are needed to determine the role of the NFκB site in the transcriptional activity of IRF-7 in infected cells.

Previous finding from several laboratories, including ours, indicated that there is a close correlation between the induction of IFNA genes expression in infected cells and the ability of this cell to synthesize IRF-7 (13–15). The inability of the virus to induce expression of IFNA genes in fibrosarcoma cell line

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**FIG. 6. In vitro methylated IRF-7 promoter is silenced in HeLa cells.** RL1 plasmid which contains IRF-7 promoter with the entire CpG island, was methylated in vitro using SssI methylase. Permanently transfected HeLa cells containing either methylated or unmethylated RL1 plasmid were generated as described under "Experimental Procedures." The transfected cells were pooled and treated with IFN-α (500 units/ml) overnight or left untreated. Luciferase activity was determined in lysates of both IFN-α-treated and untreated cells. The error bars represent the S.D. of three independent experiments.
suppressor function either by hypermethylation or mutation in the X chromosome in females and there is growing evidence that tumor suppressor gene IRF-7 is involved in the maintenance of genomic stability. Thus, clearly irradiation can activate IRF-7 (44) suggests that IRF-7 may play a role in the maintenance of genome stability. Therefore, further studies are needed to establish whether IRF-7 is instrumental for tumorogenesis or its progression.

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