Divergent Regulation of the Growth-promoting Gene \textit{IEX-1} by the p53 Tumor Suppressor and Sp1*

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Hee-Jeong Im‡, Mark R. Pittelkow§, and Rajiv Kumar¶¶

From the Departments of \textit{†}Internal Medicine, Biochemistry, and Molecular Biology and \textit{§}Dermatology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

\textit{IEX-1}, a recently discovered early response gene, regulates cell growth and apoptosis. \textit{IEX-1} gene expression is regulated by a variety of factors such as x-irradiation, ultraviolet radiation, steroids, growth factors, and inflammatory stimuli. By systematic examination of the \textit{IEX-1} promoter, we show that \textit{IEX-1} gene expression is controlled by multiple conserved gene regulatory elements and that \textit{IEX-1} is a downstream target of the p53 tumor suppressor and Sp1. In addition, p300, Sox, nuclear factor-xB, and AP4 appear to be modulators of \textit{IEX-1} gene expression to a lesser degree. We found that there is at least one Sp1 element that functions as an activator and contributes to high basal transcriptional levels of the \textit{IEX-1} gene. We demonstrate the presence of a p53 response element that represses \textit{IEX-1} promoter activity in HaCaT keratinocytes, indicating that Sp1 and p53 have opposite effects on \textit{IEX-1} gene expression. We conclude that \textit{IEX-1} expression in cells is regulated by the p53 tumor suppressor and Sp1, thus providing a direct mechanism for control of cell proliferation.

Expression of immediate-early genes is rapidly and transiently induced in response to growth factors and other extracellular signals. Genes unregulated during growth factor stimulation include nuclear proteins (e.g. c-Fos, c-Jun, c-Myc, zinc finger proteins, and nuclear hormone receptors), secretory molecules (e.g. cytokine-related factors), and components of the cytoskeleton and extracellular matrix. Nuclear factors encoded by immediate-early genes regulate expression of other genes that are required for cell cycle progression toward the G1/S phase transition (1). \textit{IEX-1} (immediate-early response factor X) represents a recently characterized member of the immediate-early gene family that may be critical for control of cell proliferation in several cell types.

\textit{IEX-1} (also known as \textit{Dif}-1 and \textit{PRG}1), the human ortholog of murine \textit{gly96}, was first identified in human squamous carcinoma cells as a radiation-inducible immediate-early gene (2–5). The \textit{IEX-1} gene encodes a 17-kDa, 156-amino acid protein that undergoes post-translational modification by glycosylation to yield a product of 27–29 kDa (5). Apart from the induction of \textit{IEX-1} in x-irradiated human tumor cells (5), this gene is known to be regulated at the mRNA level by ultraviolet B radiation (6), growth factors such as epidermal growth factor (6, 9), steroid hormones such as \(\alpha_1\),25-dihydroxyvitamin D\(_3\) (7), and inflammatory stimuli such as lipopolysaccharide and ceramide (8). Studies from our laboratory (6, 7) and others (9) suggest that \textit{IEX-1} plays a critical role in the control of keratinocyte cell growth and apoptosis. Consistent with this concept, \textit{IEX-1} is a nuclear protein whose cellular location is altered by steroid hormones such as \(\alpha_1\),25-dihydroxyvitamin D\(_3\) (7) that influence cell growth and differentiation. Recent reports have shown that disruption of \textit{IEX-1} expression by hammerhead ribozymes specifically reduces growth rate and influences cell cycle progression in 293 cells (10). In addition, 293 cells stably transfected with hammerhead concatameric ribozyme expression constructs are much less sensitive to Fas/CDE95-mediated apoptosis or the anticancer drugs etoposide and doxorubicin. Hence, \textit{IEX-1} may promote cell proliferation when growth factor conditions are favorable and facilitate apoptosis through death receptor activation under unfavorable conditions (10). Comparable results have been obtained in our laboratory using a keratinocyte cell system (11). Forced expression of \textit{IEX-1} significantly increases the growth rate of keratinocytes under basal conditions and increases the rate of apoptosis when cells are subjected to stress (11).

Because \textit{IEX-1} plays an important role in cell growth and apoptosis, the molecular mechanisms by which the \textit{IEX-1} gene is regulated require further investigation. Several regulatory factors involved in the transcriptional control of the \textit{IEX-1} gene such as p53 (12, 13) and NF-\(\kappa B\) (12, 14) have been identified. The tumor suppressor p53 is a crucial regulator of cell cycle progression, apoptosis in DNA-damaged cells, and maintenance of genomic stability (15, 16). The development of a wide range of malignant tumors is mediated by the mutational inactivation of p53 (17). Similar to the \(p21^{\text{Waf1}}\) gene (18), which is a well characterized p53 target gene that is directly involved in p53-dependent G1 cell cycle arrest (19), there is a p53-binding site in the human \textit{IEX-1} promoter. Schafer et al. (13) demonstrated that the p53-binding site in the \(p22\) (\textit{IEX-1}/\textit{Dif}-2) promoter mediates transcriptional activation of \(p22\) in a fashion similar to that observed for the \(p21^{\text{Waf1}}\) gene in HeLa and Hep3B cells. In this study, we have systematically examined the transcriptional elements that regulate \textit{IEX-1} gene expression in HaCaT keratinocytes. We demonstrate that the p53-binding site modulates the promoter activity of the \textit{IEX-1} gene in keratinocytes, but our evidence indicates that p53 functions as a transcriptional repressor rather than an activator of \textit{IEX-1}. We also found that transcription factor Sp1, but not Sp3, is a transcriptional activator of the \textit{IEX-1} gene. We propose that p53 suppresses Sp1-dependent activation of \textit{IEX-1} gene expression in keratinocytes.

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† To whom correspondence should be addressed: Mayo Clinic, 911A Guggenheim, 200 First Street S.W., Rochester, MN 55905. Tel.: 507-284-0020; Fax: 507-266-4710; E-mail: rkumar@mayo.edu.

¶¶ The abbreviations used are: NF-\(\kappa B\), nuclear factor-\(\kappa B\); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
The Renilla report gene was kindly provided by Dr. Mira O. Jung (Georgetown/H11002). The primer sequences are from 5’ to 3’. The core response elements replaced by SaI restriction enzyme sites are indicated in boldface, italic, underlined letters.

The primer sequences from 5’ to 3’. The core response elements replaced by SaI restriction enzyme sites are indicated in boldface, italic, underlined letters.

| Primers | Sequences |
|---------|-----------|
| −1IEX-rev | TCA GTT GCT AGG “Nhe1” |
| −575IEX-f | CTA CTA GAA GAA GGA CAGG GAGG |
| −279IEX-f | TAA AGT GAG CCC CTC TCC AGG TGC CAC |
| −200IEX-f | CAC ACA CTC ACA AGC TGC AGT TGG GCG |
| −150IEX-f | GCC AGT TGG TTA ATC GTC |
| −110IEX-f | CAA CCG CTC CCC AGC TGC GGG AGG AGG AGT |
| −70IEX-f | GAA GGA CCC GCC CAA TTT TCA GGA GCA CA |
| mp53/SaI-f | CCT CTC CAG GTG CCA GTC GAC TCG TAC ACA TGG GCC TGG TAC |
| mp53/SaI-r | GCT GCA GGC ACA TGT CCA GTC GAC TCG CAC GTG CAG GAG |
| mE-box/SaI-f | GAC TAC CAG ACT CAC AAC GTG GTC GAC GCC GCC TAG TAT TGC GTC TGT G |
| mE-box/SaI-r | CAC ATG CAC ATT CAG GTG CCC GAG GTG AGT TGG CAC |
| mNF-kB/SaI-f | CCT TTG TTT AAT CTT GGA GTC GAC GAC CCC GCT GCT GCC AAC |
| mNF-kB/SaI-r | GGC GCC AGG AGG AGG AGG AGT AAA CAA AGG |
| mp300/SaI-f | GTG CAT GTG AAC TGT CCA GTC GAC GCG GCC GTG CAC ATT AAA CAA AGG |
| mp300/SaI-r | GTC GAC GTC GAC GTC GAC CAC AGT |
| mSox/SaI-f | CAA GTA TCC ACC ACC ACC TCT CTT CCA GTC GAC TTA ATC GTC GAA TTG GCA GCA GAG |
| mSox/SaI-r | GCT GAA AAT TCC GAC GAT TAA GTC GAC GGA GGT GTA GGG TGA TTG |
| mAP4/SaI-f | GCC TGC TGC CAA CCG CTC CCG TCG ACC GCC GGG AGG AGG AGT TAG AAG GAC |
| mAP4/SaI-r | GTC CTT CTG ACT CCT CCC CCC CCC GTG CAG GAG CCG CAG TGG TCG GCA GCA GCC |
| mSp1/SaI-f | CCG GAG GAG GTA GAA GGA GAT CCA CCA CGA CAG |
| mSp1/SaI-r | GTC CTT CTG AAA ATT GTC GAC GTG CTC TGG AAA TTT TCA GGA GCA C |

**TABLE II**
Double-stranded synthetic oligonucleotides used as probes in electrophoretic mobility shift assays
The oligonucleotide sequences are from 5’ to 3’. The mutated base pairs (replaced by SaI sites) are indicated by boldface lower-case letters.

| Primers | Sequences |
|---------|-----------|
| SpI/IEX-1 | TAG AAG GAC CCG CCC CCC AAA TTT TCG |
| mSp1/IEX-1 | TAG AAG GAC gt gcac AAT TTT CAG |
| p53/IEX-1 | CAG GTC CCA CAT GCC TCA TGA TGC GCC |
| mp53/IEX-1 | CAG GTC CCA g tcgc TCA TGC GCC |
| Sp1 consensus | ATT CCA GTC GGG GGG GGG CAG |
| p53 consensus | TAC AGA ACA TGT CTA AGC ATG CTC GGG |

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Transient Transfection—**Human primary keratinocytes were isolated from neonatal foreskin specimens, and cell cultures were maintained in an undifferentiated replicative state by washing cells propagated in complete medium with standard medium and refeeding subconfluent cell cultures with standard medium.

**HαCaT cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum as described (21).** For transfection, cells were plated 24 h prior to transfection at a density of 1 × 10⁶ cells/plate in six-well plates and transiently transfected with 1 μg of IEX-1 promoter/firefly luciferase reporter gene constructs using 6 μl of FuGene 6 transfection reagent (Roche Molecular Biochemicals). The Renilla luciferase construct pRL-TK (100 ng; Promega) was included as an internal control for transfection efficiency. Cells were harvested 24 h post-transfection, and luciferase activity was assayed by the Dual-Luciferase reporter assay system using a luminometer (Turner Designs, Sunnyvale, CA) following the accompanying instructions. All transfection experiments were repeated four or more times in duplicate, utilizing plasmids that were independently prepared at least twice.

**Deletion and Site-directed Mutagenesis—**The IEX-1 promoter construct −1419pIEX-1 fused into the chloramphenicol acetyltransferase report gene was kindly provided by Dr. Mira O. Jung (Georgetown University Medical Center, Washington, D. C.). The −1419pIEX-1 DNA fragment was fused into the firefly luciferase reporter gene plasmid (pGL3-Basic) at the KpnI and Nhe1 restriction enzyme sites. For deletion mutagenesis, −575IEX-1, −279IEX-1, −200IEX-1, −150IEX-1, −110pIEX-1, and −70pIEX-1 PCR products were generated with sense and antisense primers containing appropriate restriction enzyme sites and template −1419pIEX-1 DNA. These PCR products were cloned into the pCR2.1-TOPO cloning vector using the TOPO TA cloning kit (Invitrogen), followed by subcloning into the promoterless pGL3-Basic plasmid at the KpnI and Nhe1 restriction enzyme sites. Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene). In brief, sense- and antisense-oriented primers complementary to each other and bearing SaI enzyme restriction sites were mixed with the template plasmid for generation of PCR products. This was followed by DpnI restriction enzyme digestion (37 °C for 3 h) to remove the template plasmid. The unmethylated PCR product was transformed into Escherichia coli XL1-Blue competent cells (Stratagene) and selected on ampicillin (100 μg/ml)-agar plates. The DNA sequences of all constructs were verified by KpnI/Nhe1 (IEX-1 promoter deletion constructs) or SaI (site-directed mutagenesis) restriction enzyme digestion, followed by dyeoxy DNA sequencing using an automated sequencer and the dyeoxy sequencing method of Sanger et al. (22). The PCR primers used for the promoter deletion constructs and site-directed mutagenesis are listed in Table I.

**Construction of Protein Expression Vectors—**Reverse transcription-PCR for the construction of Sox18 [Sry-like HMG box-containing transcription factor] cDNA was performed using the ThermoScript reverse transcription-PCR system (Invitrogen) as described by the manufacturer. The Sox18 cDNA obtained by reverse transcription-PCR was gel-purified and cloned into the pCR2.1-TOPO cloning vector using the TOPO TA cloning kit following the instructions provided by the manufacturer. The coding sequence in the pCR2.1-TOPO cloning vector was separated from the vector DNA by EcoRI restriction enzyme digestion, followed by agarose gel purification of the cDNA fragment. The purified cDNA was subcloned into the EcoRI restriction enzyme site of the pcDNA3 expression vector (Invitrogen). The resulting mammalian expression plasmid was verified by restriction enzyme digestion, and the orientation of the insert was confirmed by DNA sequencing. The primer set used for Sox18 reverse transcription-PCR is as follows: Sox18f, 5′-CAT CAG ACC TTC GAA GTC TTT GCA GAG-3′; and Sox18-r, 5′-TCA GCT CTT CCA CCA ATC GCC GGA GAG-3′. Other Sox cDNAs (Sox5, Sox6, and Sox9) were provided by Dr. Veronique Lefebvre (University of Texas, Houston, TX). pcCMV-Sp1 and pcCMV-Sp3 expression vectors were provided by Dr. Andre J. van Wijnen (University of Massachusetts Medical School, Worcester, MA), and pcCMV-p300 expression vector was provided by Dr. Ralf Janknecht (Mayo Clinic, Chicago).
Regulation of IEX-1 by p53 and Sp1

Fig. 1. Structural features of 5′-flanking sequences (nucleotides −1419 to +1) of the human IEX-1 gene. Nucalitide +1 corresponds to the translation start site (ATG). The multiple putative cis-acting elements in the upstream sequences of the human IEX-1 promoter are illustrated. Putative Sp1 and p53 response elements are shown as gray and black boxes, respectively. The deletion sites for the IEX-1 promoter deletion constructs (−1419, −575, −279, −200, −150, −110, and −70) used in this study to show the presence or absence of the putative cis-acting elements in the particular construct are indicated at the top. A TATA box is located 25 bp upstream from the transcription initiation site (TIS). SRE, serum response element; RE, response element; HRE, hormone response element; USF, upstream stimulatory factor.

Results

Delineation of Regulatory Elements in the Promoter of the Human IEX-1 Gene—To understand the cell growth regulatory mechanisms that control expression of the IEX-1 gene, we initiated studies to define the basal promoter, cis-acting elements, and cognate factors mediating IEX-1 gene transcription. A genomic DNA segment spanning −1.4 kb of the 5′-upstream region of the human IEX-1 promoter has previously been cloned (5). The 5′-region of the human IEX-1 gene contains multiple putative recognition motifs for distinct classes of transcription factors and a TATA box sequence located 25 bp upstream from the transcription initiation site (Fig. 1). The multiplicity of consensus elements in the IEX-1 promoter may provide many combinatorial control to regulate IEX-1 gene transcription.

To address the contribution of distinct promoter segments to IEX-1 gene transcription, we transfected a series of IEX-1 promoter deletion constructs fused to the firefly luciferase reporter gene into HaCaT and primary keratinocyte cells. Our results show that deletion of the promoter to bp −279 did not significantly affect transcriptional levels (Fig. 2). Subsequent deletion of the segment between bp −200 and −150 decreased promoter activity by 2–3-fold in HaCaT cells, and this level of promoter activity was 50−200-fold higher than that of a promoterless reporter gene construct, pGL3-Basic. A relatively high basal level of transcription was retained with the −150 promoter deletion construct. Deletion of promoter sequences between bp −150 and −110 strongly reduced transcription by 3-fold. Further deletion of the promoter from bp −110 to −70 further reduced transcriptional activity, which remained several fold higher than the background promoter activity observed with the promoterless luciferase construct (Fig. 2). Therefore, we conclude that the 5′-region of the IEX-1 gene contains a potent basal promoter region (bp −279 to +1) that encompasses a repressor element between bp −279 and −200. In addition, at least one strong activator region is noted between bp −150 and −110, and some modest transcriptional activity is conferred by a proximal region (bp −110 to +1). Similar results were obtained when the IEX-1 promoter deletion constructs were transiently transfected into human primary keratinocyte cells.

Divergent Control of IEX-1 Gene Transcription Involves Consensus Recognition Elements for p53 and Sp1 Factors—To assess whether consensus elements for distinct classes of gene regulatory factors are capable of modulating IEX-1 gene expression, we used site-directed mutagenesis to incorporate a systematic series of point mutations into reporter gene constructs driven by the minimal functional IEX-1 promoter (bp −279 to +1). Site-directed mutagenesis of regulatory response elements of the IEX-1 promoter was performed by replacing the core response element sequences with SalI restriction enzyme sites (see “Experimental Procedures”). We performed transient transfection analyses with this panel of mutant promoter/reporter gene constructs in HaCaT cells. One of the most striking results is that mutation of a putative recognition motif for the p53 tumor suppressor protein located in the −256 to −237 region increased transcription by 3-fold (Fig. 3). This result suggests that the putative p53 motif represents a bona fide cis-acting element that apparently plays an important role as a transcriptional repressor. On the other hand, mutation of the Sp1/GC box consensus motif located in the −63 to −55 region decreased basal promoter activity by at least 4-fold as shown in mutant constructs mSp1/−575, mSp1/−279, and mSp1/−150 (Fig. 3). Slight modifications of transcriptional effects were observed by mutating putative elements for p300 (mp300/−200; i.e. −40% reduction), Sox (mSox/−200; i.e. 30% reduction), NF-κB (mNF-κB/−200 and mNF-κB/−150; i.e. 30% reduction), and AP1 (mAP1/−200; i.e. 10–15% reduction) (Fig. 3). However, forced expression of p300, various Sox proteins (Sox5, Sox6, Sox9, and Sox18), and the NF-κB dominant-negative inhibitor IκBα showed no significant increase in promoter activity (data not shown). These data indicate that there are at least two distinct negative and positive cis-acting elements in the human IEX-1 promoter, which coincide with consensus recognition motifs for p53 and Sp1, respectively. Expression of the human IEX-1 gene appears to be regulated by these two regulators. In addition, although individual mutation of the putative elements in the IEX-1 promoter region was not sufficient to show dramatic
carried out by replacing the core sequence of the Sp1 cis present between bp -279 and -200 of the IEX-1 promoter. A, schematic representation of a series of 5’-deletion constructs of the human IEX-1 gene promoter fused to the recombinant luciferase (Luc) reporter gene. B, summary results of luciferase activity. Each deletion construct was transiently transfected into HaCaT (white bars) and primary keratinocyte (gray bars) cells. The cells were harvested 24 h later, and a luciferase reporter assay was performed. pGL3-Basic (which is promoterless) and pGL3-Control (containing the SV40 enhancer and promoter) were used as negative and positive controls, respectively. Transfection efficiencies were normalized to the Renilla luciferase activity from the cotransfected internal control plasmid (pRL-TK). The activities of the luciferase reporter are expressed as -fold relative to the activity of the promoterless pGL3-Basic vector (which was assigned an activity value of 1.0). The data shown are means of three independent experiments in duplicates, with at least two different plasmid preparations.

FIG. 2. Identification of the minimal functional IEX-1 promoter (bp -279 to +1), which contains a repressor element between bp -279 and -200 of the IEX-1 promoter. A, schematic representation of a series of 5’-deletion constructs of the human IEX-1 gene promoter fused to the recombinant luciferase (Luc) reporter gene. B, summary results of luciferase activity. Each deletion construct was transiently transfected into HaCaT (white bars) and primary keratinocyte (gray bars) cells. The cells were harvested 24 h later, and a luciferase reporter assay was performed. pGL3-Basic (which is promoterless) and pGL3-Control (containing the SV40 enhancer and promoter) were used as negative and positive controls, respectively. Transfection efficiencies were normalized to the Renilla luciferase activity from the cotransfected internal control plasmid (pRL-TK). The activities of the luciferase reporter are expressed as -fold relative to the activity of the promoterless pGL3-Basic vector (which was assigned an activity value of 1.0). The data shown are means of three independent experiments in duplicates, with at least two different plasmid preparations.

FIG. 3. Site-directed mutagenesis of the multiple putative cis-acting sites in the human IEX-1 5’-flanking promoter region. The numbers at the ends of the mutated cis-acting elements indicate the template plasmid construct. Each set of site-directed mutants from different templates was transfected into HaCaT cells, and the relative luciferase activity is distinguished by shadowed bars (black, dark gray, medium gray, light gray, and white) according to the templates utilized for the mutations. The luciferase reporter activities were normalized to the activity of the pGL3-Basic plasmid. For detailed experimental conditions, see “Experimental Procedures.” Mutation of the Sp1 cis-acting element using different templates (mSp1/-575, mSp1/-279, and mSp1/-150) was carried out by replacing the core sequence of the Sp1 cis-acting element present between bp -70 to -50 with a SsI restriction enzyme site.

modification of promoter activity, promoter deletion analysis indicated that the combination of these putative elements may exert their effect on overall IEX-1 transcriptional activity.

Apart from these findings, which indicate a role for p53 and Sp1 in the transcriptional control of the human IEX-1 gene, we also mutated a putative helix-loop-helix/E box located in the -185/ -175 region (mE-box/-279). We observed that this mutation up-regulated IEX-1 promoter activity by at least 3-fold, thus raising the possibility of a role for this inhibitory protein in the regulation of IEX-1 gene expression (Fig. 3). Interestingly, however, deletion of the entire E box region (from bp -200 to -150) appeared to be quantitatively neutral for promoter activity (Fig. 2). This finding suggests that a compensatory positively acting factor, which is distinct from the HLH/E box protein, may functionally interact with the -200/ -150 segment of the IEX-1 promoter region. In the remainder of our studies, we focused our investigation on the characterization of p53- and Sp1-related regulatory mechanisms.

The GC Box-binding Protein Sp1, but Not Sp3, Enhances Basal Levels of IEX-1 Gene Transcription—The -110 promoter deletion construct retains significant promoter activity and contains a perfect Sp1 consensus motif. To determine the specific role of Sp1 in the transcriptional regulation of IEX-1 gene expression, we cotransfected Sp1 or Sp3 expression vectors with IEX-1 promoter plasmids and then analyzed the effect on IEX-1 promoter activity. We found that Sp1, but not Sp3, selectively enhanced reporter gene expression (Fig. 4A) and that coexpression of Sp3 and Sp1 did not decrease Sp1-dependent enhancement. The enhancement of IEX-1 promoter activity by Sp1 was observed even in the -110pIEX-1 deletion construct, in which most of the putative elements have been deleted, except for the AP4 and Sp1 motifs. Coexpression of Sp3 and Sp1 also did not show synergistic effects. These results indicate that Sp3 does not function as a dominant-negative inhibitor of Sp1 function, as a cofactor, or as a synergistic enhancer as has been observed for other genes such as the cell growth-regulated dihydrofolate reductase and opioid receptor genes (24, 25). Our results show that Sp1 stimulated IEX-1 promoter activity in a concentration-dependent manner (Fig. 4B), and this transcriptional enhancement was detected with the -279, -200, and -150 promoter deletion constructs. On the other hand, increased amounts of the pcDNA3.1 vector, which contains a cytomegalovirus promoter, failed to enhance the promoter activity of these deletion constructs (data not
We conclude that Sp1 is a rate-limiting element for IEX-1 promoter activity and functions via a site from bp 63 to 55 in the IEX-1 promoter region. Sp1 site-directed mutation experiments demonstrated that this Sp1 mutation not only decreased promoter activity (Fig. 3), but also abolished Sp1-dependent activation of IEX-1 promoter activity (Fig. 4C). The results in Fig. 4 indicate that Sp1 is an important transactivator of IEX-1 gene transcription.

Identification of a Response Element for the p53 Tumor Suppressor Protein in the Human IEX-1 Gene—The inherent inhibitory function of the −279/−200 region, which contains a putative p53 recognition motif, is indicated by transient trans-
Cells were transiently transfected with a DNA segment (Fig. 2) and mutation of the p53 consensus motif. Deletion analyses demonstrating that both deletion of this 79-bp sequence (gray bars) and absence (white bars) of the pCMV-p53 expression vector. The cells were harvested 24 h later, and a luciferase reporter assay was performed. pGL3-Basic (which is promoterless) and pGL3-Null (containing the SV40 enhancer and promoter) were used as negative and positive controls, respectively. Transfection efficiencies were normalized to the Renilla luciferase activity from the cotransfected internal control plasmid (pRL-TK). The activities of the luciferase reporter are expressed as fold relative to the activity of pGL3-Basic (which was assigned an activity value of 1.0). The data shown are means of three independent experiments in duplicates, with at least two different plasmid preparations. For detailed experimental conditions, see "Experimental Procedures."

We found that the -1419, -575, and -279 promoter deletion constructs were all responsive to forced expression of p53, as reflected by a 2-fold decrease in transcriptional activity (Fig. 5). In contrast, the -200, -150, -110, and -70 promoter deletion constructs, which lack the p53 motif, were not responsive (Fig. 5). Furthermore, the p53 site-directed mutant, which exhibited increased basal promoter activity, was not repressed by forced expression of p53. Taken together, these data establish that p53 inhibits IEX-1 promoter activity via a functional p53 response element located in the -279/-200 promoter region of the IEX-1 gene.

**Absence of Direct Molecular Cross-talk between p53 and Sp1 in the Control of IEX-1 Gene Expression**—Based on our findings that the IEX-1 gene contains transcriptional elements for both Sp1 and p53, we examined the possibility that these factors may control IEX-1 gene transcription by molecular cross-talk involving direct protein/protein interactions. We performed transient coexpression experiments with p53 and Sp1 and assessed the effects on IEX-1 promoter activity (reporter construct -279pIEX-1). As shown in Fig. 6, Sp1 enhancement of basal IEX-1 promoter activity was significantly reduced when the cellular levels of p53 were elevated by forced expression. This result is reflected by a 5-fold activation of reporter gene expression by Sp1 in the absence of p53 expression and negligible activation by Sp1 in the presence of p53 expression (Fig. 6). Mutation of the proximal Sp1-binding site (bp -63 to -55) near the TATA box, which reduced basal promoter activity by 70% (Figs. 3 and 4), abolished Sp1-dependent activation; no p53-mediated suppression of Sp1 activity was evident. Mutation of the p53-binding site, which increased the basal promoter activity of the -279pIEX-1 construct, abolished p53-mediated suppression, but did not abrogate Sp1-dependent activation. The double mutation construct mp53/mSp1/-279pIEX-1, in which both the Sp1 and p53 elements are mutated, exhibited a very low basal level of transcription that was only severalfold above that observed for the promoterless plasmid pGL3-Basic and showed no response to forced expression of either Sp1 or p53. Our data indicate that both p53 and Sp1 contribute to the basal promoter activity of the IEX-1 gene.

We performed additional cotransfection experiments by progressively increasing the amounts of p53 and Sp1 factors and assessed the effects on IEX-1 promoter activity. As shown in Fig. 7, Sp1-enhanced IEX-1 promoter activity was significantly reduced to the basal levels observed in the absence of Sp1 when the cellular levels of p53 were elevated by forced expression. The reduction of Sp1-dependent promoter activity corresponded to the amount of p53 that was being overexpressed. Similarly, as concentrations of Sp1 were increased, p53-mediated IEX-1 gene repression was progressively abolished in an Sp1 concentration-dependent manner (Fig. 7). The activities of the luciferase reporter are expressed as fold relative to the activity of the promoterless pGL3-Basic vector (which was assigned an activity value of 1.0). The data shown are means of three independent experiments in duplicates, with at least two different plasmid preparations.

**Fig. 5. Effect of p53 on the transcriptional activity of the IEX-1 promoter in HaCaT cells**. Cells were transiently transfected with a series of IEX-1 promoter deletion or mutant (mp53/-279, with the p53 site replaced with a SalI restriction enzyme site) constructs in the presence (gray bars) and absence (white bars) of the pCMV-p53 expression vector. The cells were harvested 24 h later, and a luciferase reporter assay was performed. pGL3-Basic (which is promoterless) and pGL3-Null (containing the SV40 enhancer and promoter) were used as negative and positive controls, respectively. Transfection efficiencies were normalized to the Renilla luciferase activity from the cotransfected internal control plasmid (pRL-TK). The activities of the luciferase reporter are expressed as fold relative to the activity of pGL3-Basic (which was assigned an activity value of 1.0). The values shown are means of three independent experiments in duplicates, with at least two different plasmid preparations. For detailed experimental conditions, see “Experimental Procedures.”

**Fig. 6. Examination of molecular cross-talk between the p53 tumor suppressor and Sp1 for the regulation of IEX-1 gene expression**. The IEX-1 promoter reporter construct -279pIEX-1 (white bars) or the proximal Sp1 site mutant construct mSp1/-279 was transiently coexpressed with Sp1 (gray bars) or p53 (black bars). Coexpression of both factors (Sp1 and p53) is indicated by hatched bars. The transiently transfected HaCaT cells were harvested 24 h later, and a luciferase reporter assay was performed. pGL3-Basic (which is promoterless) and pGL3-Null (containing the SV40 enhancer and promoter) were used as negative and positive controls, respectively. Transfection efficiencies were normalized to the Renilla luciferase activity from the cotransfected internal control plasmid (pRL-TK). The activities of the luciferase reporter are expressed as fold relative to the activity of the promoterless pGL3-Basic vector (which was assigned an activity value of 1.0). The data shown are means of three independent experiments in duplicates, with at least two different plasmid preparations.

We also performed electrophoretic mobility shift assays to investigate whether p53 and Sp1 interact or interfere with each other through protein/protein interactions. In vitro translated p53 protein was mixed with increased amounts of in vitro translated Sp1 protein (1 and 5 µl) in the binding mixture. Increased amounts of Sp1 protein in the binding mixture did not alter the formation of p53 protein-DNA complexes (Fig. 8A). Similarly, in vitro translated Sp1 protein was mixed with in-
In this study, we have shown that the growth-promoting immediate-early gene IEX-1 is controlled by multiple regulatory elements in the 5′-region of the gene. These elements are necessary for maximal IEX-1 promoter activity. Most interestingly, we found that IEX-1 gene expression is repressed by the p53 tumor suppressor and activated by the Sp1 transcription factor. Consistent with the multiplicity of regulatory elements in the IEX-1 promoter, the IEX-1 gene is regulated by various factors such as x-irradiation (2, 5), ultraviolet radiation and growth factors (e.g. epidermal growth factor) (6), steroid hormones (e.g. 1α,25-dihydroxyvitamin D₃) (7), 12-O-tetradecanoylphorbol-13-acetate (5, 6), and inflammatory stimuli (e.g. cytokines, ceramide, lipopolysaccharide, and lysophosphatidylcholine) (8). Because the sequences and functions of cis-acting elements in the IEX-1 promoter region are highly conserved among different mammalian species (4, 24), it appears that the complexity of the IEX-1 promoter evolved to mediate a transcriptional response under the influence of many distinct types of cell growth-related stimuli.

FIG. 7. Effects of increasing amounts of p53 and Sp1 factors on IEX-1 expression. Sp1 or p53 was cotransfected with IEX-1 promoter constructs together with gradually increased amounts of p53 (A) or Sp1 (B). The wild-type IEX-1 promoter construct −279pIEX-1 (■) showed gradual decreases/increases as the amounts of p53 or Sp1 increased. Mutants of p53 (mp53/−279; ○) and Sp1 (mSp1/−279; □) and the double mutant (mp53/mSp1/−279; ▲) were used for cotransfection as controls in this experiment. The transiently transfected HaCaT cells were harvested 24 h later, and a luciferase reporter assay was performed. The data shown are means of three independent experiments in duplicates, with at least two different plasmid preparations.

FIG. 8. p53 and Sp1 binding to cognate DNA response elements and interactions of p53 and Sp1 proteins in protein-DNA complex formation. A, in vitro translated p53 protein was mixed with increased amounts of in vitro translated Sp1 protein in the presence of the p53 response element of IEX-1 (p53/IEX-1). The p53 consensus sequence and p53/IEX-1 demonstrated a similar intensity of protein/DNA binding, whereas the mutated p53 response element of the IEX-1 promoter (mutp53/IEX-1) showed no binding. B, in vitro translated Sp1 protein was mixed with increased amounts of in vitro translated p53 protein in the presence of the Sp1 response element of IEX-1 (Sp1/IEX-1). The presence (+) or absence (−) of the p53 or Sp1 protein is indicated.

Increased amounts of in vitro translated p53 protein (1 and 5 µl) in the binding mixture. The increased amounts of p53 protein in the binding mixture did not change the amounts of Sp1 protein-DNA complexes formed (Fig. 8B). Competition electrophoretic mobility shift assays using unlabeled specific or non-specific oligonucleotides showed that the protein/DNA interactions were sequence-specific (data not shown). In addition, the mutant oligonucleotides spanning the mutated p53 and Sp1 sites in the IEX-1 promoter (mutp53/IEX-1 and mutSp1/IEX-1, respectively) failed to show binding to either the p53 or Sp1 protein (lane 4). Taken together, these results demonstrate that the Sp1 and p53 transcription factors do not interfere with each other’s binding to DNA and that ternary complexes of Sp1/p53 and DNA are not observed. These findings suggest that p53 and Sp1 exert their biological effects independently in the control of IEX-1 gene expression.

IEX-1 Gene Expression Is Determined by the Ratio of Sp1 and p53—We initiated experiments aimed at understanding how our co-transfection studies involving p53 and Sp1 in HaCaT cells correspond to the natural stimuli regulating IEX-1 gene expression through these transcription factors. We determined whether the ratio of p53 and Sp1 was important for the control of IEX-1 gene expression in vitro. We serum-deprived HaCaT keratinocyte cells for 3 days; restored normal proliferation by supplementing the culture medium with serum for 1 and 4 h; and measured the mRNA expression levels of IEX-1, p53, and Sp1 by Northern analysis (Fig. 9). The levels of each mRNA were quantitated and normalized to the mRNA intensity of GAPDH (Fig. 9, A and B). As shown in Fig. 9A (and plotted after quantitation in Fig. 9B), an Sp1/p53 ratio <1 (lane 1) was associated with a low level of IEX-1 mRNA expression. In contrast, Sp1/p53 ratios >1 (expression ratios following serum stimulation for 1 and 4 h were 1.12 and 7.8, respectively) (lanes 2 and 3) were associated with increased amounts of IEX-1 mRNA. If changes in the Sp1/p53 ratio modulate IEX-1 gene expression at the transcriptional level, then serum restoration of cells would be predicted to increase the activity of the IEX-1 promoter. To test this, we transiently transfected HaCaT cells with the −279pIEX-1 promoter construct, which contains both p53- and Sp1-binding sites. After 24 h, the transiently transfected HaCaT cells were serum-deprived for 16 h, followed by serum restoration for 1, 5, and 8 h. As shown in Fig. 9C, the results of the transient transfections show a significant increase in IEX-1 promoter activity at 5 h after serum restoration. In contrast, the Sp1-binding site mutant construct (mSp1/−279) did not exhibit significantly increased promoter activity following serum restoration. These results are in agreement with the Northern assays (Fig. 9, A and B) and support the concept that changes in the Sp1/p53 ratio regulate IEX-1 gene transcription during serum stimulation. These results demonstrate that the Sp1/p53 ratio is critical for IEX-1 gene expression and that these transcription factors may play a joint role in determining the appropriate expression levels of IEX-1 to maintain cellular homeostasis and to control cell growth.

DISCUSSION

In this study, we have shown that the growth-promoting immediate-early gene IEX-1 is controlled by multiple regulatory elements in the 5′-region of the gene. These elements are necessary for maximal IEX-1 promoter activity. Most interestingly, we found that IEX-1 gene expression is repressed by the p53 tumor suppressor and activated by the Sp1 transcription factor. Consistent with the multiplicity of regulatory elements in the IEX-1 promoter, the IEX-1 gene is regulated by various factors such as x-irradiation (2, 5), ultraviolet radiation and growth factors (e.g. epidermal growth factor) (6), steroid hormones (e.g. 1α,25-dihydroxyvitamin D₃) (7), 12-O-tetradecanoylphorbol-13-acetate (5, 6), and inflammatory stimuli (e.g. cytokines, ceramide, lipopolysaccharide, and lysophosphatidylcholine) (8). Because the sequences and functions of cis-acting elements in the IEX-1 promoter region are highly conserved among different mammalian species (4, 24), it appears that the complexity of the IEX-1 promoter evolved to mediate a transcriptional response under the influence of many distinct types of cell growth-related stimuli.
Our result that p53 represses IEX-1 gene transcription is in agreement with the biological actions of IEX-1 and its status as a downstream target gene for the tumor suppressor p53. The biological actions of IEX-1, which include the induction of cell proliferation and cell cycle entry, are opposite to the known functions of the p53 tumor suppressor protein. The p53-mediated repression of IEX-1 transcription suggests a direct mechanism by which p53 may regulate cell growth in an IEX-1 gene product-dependent manner. Mutations of the p53 tumor suppressor gene are found in a high percentage of human carcinomas (17, 26, 27). We speculate that inactivation of p53 in tumor cells prevents repression of IEX-1 gene expression, which is a downstream target gene of p53. Consequently, genetic inactivation of p53 may result in up-regulation of IEX-1 expression and stimulation of tumor cell proliferation. It will be interesting to investigate the transforming ability of IEX-1 in cells in which p53 is mutated or not expressed.

Schafer et al. (12, 13) have presented data that show increased IEX-1 promoter activity through protein/DNA interactions involving p53 (human and rat) and NF-kB (human). Our data demonstrate that the IEX-1 gene is repressed because inactivation of the p53-binding site increases basal level transcription, and forced expression of p53 reduces IEX-1 gene promoter activity. The differences between our present findings and the observations of Schafer et al. could be due to the different cell types used in the experiments. In our experiments, we used the spontaneously immortalized and non-tumorigenic human skin keratinocyte cell line HaCaT (21), whereas Schafer et al. used Hep3B, HepG2, 818-4 pancreatic carcinoma, and HeLa cells. In agreement with cell type-dependent differences in IEX-1 gene regulation, we found differences in the basal promoter activity of IEX-1 promoter deletion constructs in HaCaT cells (this study) compared with results with analogous constructs transiently
transfected in HeLa cells (3). Taken together, these results suggest that intricate gene regulatory mechanisms may stringently control differential expression of the IEX-1 gene in different cell types.

Apart from the tumor suppressor p53, which functions as a repressor of the IEX-1 promoter in HaCaT cells, we found that Sp1, but not Sp3, is a critical activator of the IEX-1 gene. Sp1 up-regulates IEX-1 promoter activity in a concentration-dependent manner. The specificity of the Sp1-induced enhancement of IEX-1 promoter activity is supported by the absence of a significant increase in transcription following forced expression of Sp3. Deletions and site-directed mutagenesis analysis identified a proximal Sp1 site (bp –63 to –55), although there are three additional putative Sp1 response elements located between bp –1419 and –279 of the IEX-1 promoter. Our data indicate that the proximal Sp1 site located between bp –63 and –55 of the IEX-1 promoter region is sufficient for Sp1 responsiveness and mediates a high basal level of transcription. It has been shown previously that Sp1 and Sp3 can synergize (25, 28) or cooperate (29) to up-regulate target gene expression. In other cases, Sp3 has been shown to interfere with Sp1-dependent transcriptional activation by competition with Sp1 for binding to GC boxes in gene promoters (24, 30, 31). We show here that coexpression of Sp1 and Sp3 does not result in synergism or repression of Sp1-driven transcription by Sp3. This finding is similar to that observed for other cell growth-related genes (e.g. histone H4 and thymidine kinase) (29) and suggests that Sp1 selectively interacts with its cognate response element in the IEX-1 promoter.

Several lines of evidence have been reported that indicate molecular cross-talk between Sp1 and p53, including Sp1/p53-associated reciprocal (32), synergetic (33), and cooperative (34) regulation of target gene transcription and direct interaction of Sp1/p53 protein-mediated gene activation (33, 35). Bargonetti et al. (36) have shown that p53 and Sp1 regulate each other’s DNA-binding activity and that this mutual interference modulates transcription from the human immunodeficiency virus long terminal repeat. The data presented in this study demonstrate that expression of p53 significantly reduces the Sp1-dependent activation of the IEX-1 gene and that Sp1 inhibits the p53-mediated suppression of IEX-1 gene expression. Our gel shift assay showed that neither Sp1 nor p53 interferes with the binding of the other factor to DNA. These results suggest that the mechanism(s) by which Sp1 and p53 exert their biological effects on IEX-1 gene expression are independent. Our findings are consistent with the concept that the ratio of Sp1 and p53 is important for IEX-1 gene expression and that the balance of both gene regulatory proteins dictates physiological levels of IEX-1 gene expression to maintain fidelity of cellular homeostasis, cell proliferation, and/or apoptosis.

Schafer et al. (12) observed tumor necrosis factor-a-induced NF-kB-dependent transactivation via the NF-kB response element present in the human IEX-1 promoter region in Hep3B cells. In our study, NF-kB did not appear to play a rate-limiting role in the basal expression of IEX-1 promoter activity in HaCaT cells because the mutation of this putative site showed only modest reduction of promoter activity (~30% reduction), and forced expression of IxBalpha, a dominant-negative inhibitor of NF-kB, did not show significant changes in IEX-1 promoter activity. It will be of interest to investigate whether the NF-kB motif in the IEX-1 promoter mediates gene expression in response to other growth factors or cytokine-related stimuli in HaCaT cells. Although mutation of p300 and Sox response elements in the IEX-1 promoter reduced reporter gene forced expression of p300 or various Sox factors, including Sox5, Sox6, Sox9, and Sox18. However, IEX-1 promoter deletion analysis showed a dramatic decrease in transcriptional activity upon deletion of the –150/–110 segment of the IEX-1 promoter, which contains the putative binding sites for p300, Sox, and NF-kB. Deletion of a putative E box site (from bp –200 to –150) and an AP4 response element (from bp –110 to –70) resulted in a gradual reduction in IEX-1 promoter activity. For comparison, mutation of the proximal Sp1 site (from bp –63 to –55) significantly decreased IEX-1 promoter activity up to 75–80%, indicating the critical role of the Sp1 factor in IEX-1 gene expression. However, a promoter construct containing only the first 70 bp of the IEX-1 gene promoter exhibited a very modest level of basal promoter activity. Taken together, these results demonstrate that multiple gene regulatory elements are present in the IEX-1 promoter region that cooperatively participate in controlling the physiological levels of IEX-1 gene expression.

In conclusion, we have shown that IEX-1 gene expression is controlled by the tumor suppressor p53, the transcriptional activator Sp1, and multiple regulatory elements in the promoter of the IEX-1 gene. Future studies on the molecular function of IEX-1 and the mechanisms that support molecular interactions of IEX-1 in the nucleus should reveal important insights into IEX-1-dependent control of cell proliferation and responses to various pharmacological and stress-inducing stimuli.

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