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Hepatocyte NF-1 and STAT6 Cooperate with Additional DNA-Binding Factors to Activate Transcription of the Human Polymeric Ig Receptor Gene in Response to IL-4

Hilde Schjerven, Per Brandtzaeg, and Finn-Eirik Johansen

Secretory IgA and IgM, which protect the mucosal surfaces, are generated by selective transport of locally produced polymeric (p)Igs through the epithelial barrier by the pIgR. The expression of this receptor, and hence the generation of secretory Igs, is modulated by numerous extracellular factors. We have previously identified a STAT6 site in intron 1 of the human pIgR gene that is required for the slow and de novo protein synthesis-dependent IL-4-mediated transcriptional activation of the gene. In this study, we show that this intronic IL-4-responsive enhancer is confined to a 250-bp region that is highly conserved in the murine pIgR gene. The enhancer was dependent on the cooperation between the STAT6 site and at least four additional DNA elements. EMSA experiments demonstrated binding by hepatocyte NF-1 to one of these DNA elements. Extensive overlap in the tissue distribution of hepatocyte NF-1 and pIgR suggests that this transcription factor contributes to tissue-specific pIgR expression. Changing the helical phase between the STAT6 site and downstream DNA elements greatly reduced the strength of the IL-4 response, suggesting that the precise organization of this enhancer is important for its proper function. Thus, several transcription factors cooperate in this enhancer to mediate IL-4 responsiveness in HT-29 epithelial cells. The Journal of Immunology, 2003, 170: 6048–6056.

In cooperation with various innate mechanisms, secretory (S)IgA and SLgM Abs constitute the first line of adaptive immune defense of the vulnerable mucosal surfaces. The SLgs are generated by a cooperation between local J chain-expressing plasma cells that produce polymeric (p)Igs (1) and the secretory epithelial cells that express the pIgR, also known as membrane secretory component (SC) (2, 3). The pIgR is expressed at the basolateral surface of the epithelial cells, where it binds the pIgs and transports them by receptor-mediated transcytosis to the luminal surface. Here, the pIgR-pIg complexes are released by specific cleavage of the receptor, which generates SLgs containing the extracellular part of the receptor (SC) bound to the pIgs. This transport mechanism constitutes a sacrificial pathway and therefore requires a relatively high level of constitutive receptor expression. In humans, pIgR expression is restricted to secretory mucosa and serous glandular epithelial cells, with the highest expression occurring in the small and large intestine, but it is also detected in the pancreas, kidney, lung, endometrium, and in lacrimal, salivary, and lactating mammary glands (2, 3). In addition to its constitutive expression, several cytokines can up-regulate pIgR expression (2, 3); it has been demonstrated in cell cultures that such up-regulation by IL-4 and IFN-γ is followed by an increased capacity for pIg transport (4). This reflects how the expression of pIgR can be modulated during immune responses to meet a demand for increased external transport of specific SLgs that can operate as stable Abs in external secretions.

The cytokine-mediated up-regulation of pIgR is regulated at the level of transcription (5), and it is a relatively slow response that depends on de novo protein synthesis (5–8). Both IFN-γ and IL-4-mediated up-regulation of pIgR is inhibited by tyrosine kinase inhibitors (8, 9). Furthermore, we have recently identified an IL-4-responsive enhancer located in intron 1 of the human pIgR gene that is both directly and indirectly dependent on STAT6 activation (10). This transcription factor (TF) is rapidly activated by signaling through the IL-4R (11–13). Upon activation of the IL-4R, latent cytoplasmic monomeric STAT6 is rapidly phosphorylated on tyrosines by the receptor-associated Janus kinases, which leads to dimerization and translocation to the nucleus. Here, it can bind DNA and act as a transcriptional activator. We found that STAT6 has a dual role in up-regulation of the human pIgR gene by IL-4 (10): first, STAT6 binding to its DNA element in intron 1 was required for enhanced pIgR transcription; and second, activation of a conditionally active form of STAT6 in the epithelial cell line HT-29 was sufficient to up-regulate pIgR transcription, suggesting that STAT6 could induce required de novo-synthesized factors (10).

The intronic IL-4-responsive enhancer of the human pIgR gene mediates slow-responsive, cell type-specific cytokine induction of a constitutively expressed gene. The direct binding of STAT6 to the intronic enhancer, together with the slow responsiveness and dependency on both cell type-specific and newly synthesized factors, implies a complex requirement for cooperation among several TFs. In this study, we extend the characterization of this enhancer by detailed deletional and mutational analysis. We demonstrate that at least five distinct DNA elements, including a newly identified binding site for hepatocyte NF (HNF)-1, and the previously identified STAT6 site (10) are required for complete function of the IL-4-responsive enhancer.
Materials and Methods

Plasmid construction

Nucleotide numbering is given relative to the transcription start site (14). The plasmids pRL-PGK, p12, p14, p35, p36, p48, pSC1, and pSC23 have been described previously (10). The 5' and 3' deletions introduced in plasmids denoted p31-p71 (Fig. 3) were constructed with PCR, which introduced restriction enzyme sites for either KpnI (for 5' deletions) or BglII (for 3' deletions), and subsequent restriction enzyme digestion and ligation into the KpnI- and BglII-digested pGL3-promoter vector (Promega, Madison, WI). The 10-bp mutations in p73-p76 (Fig. 2A), and the 4-bp mutations in p42 through p79 (Fig. 3) were introduced either with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), or by conventional PCR-based splicing by overlap extension technique. Point mutations were designed that changed the nucleotide from a purine to the noncomplementary pyrimidine, and vice versa (i.e., A→T, T→A). The 5-bp insertion in pSC72 was introduced by QuickChange site-directed mutagenesis. The 10-bp and 15-bp insertions in pSC73 and pSC74 (Fig. 5) were introduced into pSC1 by multistep subcloning to introduce double-stranded oligonucleotides (listed in Table I) into the Psrl site immediately downstream of the STAT6 site. All mutations, as well as the integrity of the vector-insert boundary of all deletions, were confirmed by DNA sequencing (Medigenomix, Martinsried, Germany).

Expression vectors and Abs

pCDNA3-STAT6/ERα has been described previously (10). The expression plasmids for murine Cdx-1 (15) and Cdx-2 (16), and the Abs against Cdx-1 (17) and Cdx-2 (18), were kindly provided by Dr. P. Traber (University of Pennsylvania, Philadelphia, PA). Expression vectors for murine Helios and Helios-specific Abs were kindly provided by Drs. K. Georgopoulos (Massachusetts General Hospital, Charlestown, MA) (18) and S. T. Smale (University of California, Los Angeles, CA) (19). Abs specific for HNF-1α (C-19), HNF-1β (C-20), HNF-3α (M-20), and HNF-3γ (K-15) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture, transient transfections, and reporter gene analysis

The human colon adenocarcinoma cell line HT-29,m3, previously selected for high expression of pglR (20), was maintained in RPMI 1640 medium supplemented with 50 μg/ml gentamicin, 2 mM l-glutamine, and 10% FCS. Transient transfections were performed with FUGENE6 reagent (Roche Diagnostics, Indianapolis, IN) as previously described (10). Transfected cells were either left untreated or stimulated with 10 ng/ml recombinant human IL-4 for 24 h when indicated or with 3 μM 4-hydroxytamoxifen (4-HT; Sigma-Aldrich, St. Louis, MO) for 24 h (Fig. 6). The luciferase activity of both the reporter gene (firefly luciferase) and the internal control plasmid pRL-PGK (Renilla luciferase) was measured in a luminometer (Victor; Wallac, Turku, Finland; or Luminoskan Ascent; Thermo Labsystems, Helsinki, Finland) with the Dual Luciferase reporter assay system (Promega). Data in Figs. 1, 2A, 3, 5, and 6 show the mean ± SEM of three or more independent experiments.

RT-PCR

Cytoplasmic RNA was isolated from untreated and treated HT-29,m3 cells with the RNeasy Mini kit (Quagen, Hilden, Germany) according to the manufacturer’s protocol. RNA (1 μg), primed with oligdT and reverse transcribed with SuperScript II, was used for a 20-μl cDNA reaction. Gene-specific primers were designed with the Primer3 software (21) and are listed in Table I. Specific cDNA was amplified by PCR, and the PCR product was sequenced (Medigenomix) to confirm the identity of the amplified cDNA.

Preparation of nuclear extracts and EMSA

Preparation of nuclear extracts from HT-29,m3 cells was performed essentially as described (27) with certain modifications detailed previously (10). Approximately 5 μg of nuclear proteins was incubated with 3P end-labeled, double-stranded oligonucleotide probe (0.5 pmol/reaction). The EMSA reactions were performed in buffer containing 1 mM EDTA, 50 mM KCl, 1 mM DTT, 0.1 μg/ml dDTTP (or ssDNA), 0.05% Nonidet P-40, 10 mM Tris (pH 7.9), and 6% glycerol for 30 min at room temperature. Bound and free probes were separated by electrophoresis in a 4 or 5% polyacrylamide gel (0.25× Tris/borate/EDTA) at 150 V for ~1.5 h at room temperature, dried, and visualized on x-ray film overnight. Cold competitors were added in 100-fold excess before addition of labeled probe when indicated. For supershift experiments, 2 μl of specific Ab was added to the reaction mixture and incubated at room temperature for 20 min. The labeled probe was then added, and the reaction was incubated for another 30 min before electrophoresis. The top strands of the oligonucleotide probes used are indicated in each figure (Figs. 2C and 4A–C, top panels), and mutated oligonucleotides used for cold competition contained the mutations indicated in the figures (Fig. 2C and 4A–C). For the different consensus sites used for competition experiments, the oligonucleotide probes used are listed in Table I.

Computer-assisted analysis of DNA sequences

Analysis of DNA sequences was performed with the GenDics Computer Groups package (Genetics Computer Group, Madison, WI) and MatInspector (http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl) (28) or TFSSEARCH (http://molsun1.cbr.aisl.go.jp/research/dbTFSSEARCH.html) (29) software.

Results

The intronic IL-4-responsive enhancer is contained within a 250-bp region

We have previously identified a 554-bp region in intron 1, located 4.1 kb downstream of the transcription start site, that is necessary

Table I. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Application</th>
<th>Sequence</th>
<th>Target</th>
<th>Reference</th>
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</thead>
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<tr>
<td>Cloning of pSC72</td>
<td>5'-CTTCCAAAGAAGACTGCAGCAGAAACAAATACCTTTATATTACAAGG-3'</td>
<td>Cdx (SIF1)</td>
<td>16</td>
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<tr>
<td>Cloning of pSC73</td>
<td>5'-GTGACTGTCGCA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cloning of pSC74</td>
<td>5'-GGGTCGGATATACGAGCCAGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMSA</td>
<td>5'-AGTTTATTAGGAATTTTGTCTAC-3'</td>
<td>HNF-1 (SIF3)</td>
<td>22</td>
</tr>
<tr>
<td>EMSA</td>
<td>5'-ACCTAAAAAGTGTGCCACCC-3'</td>
<td>HNF-3α (SIF4)</td>
<td></td>
</tr>
<tr>
<td>EMSA</td>
<td>5'-AAACAGTTGTTTGTCTTTTCA-3'</td>
<td>HNF-3γ (SIF5)</td>
<td></td>
</tr>
<tr>
<td>EMSA</td>
<td>5'-TTAAGGAGAGGTGAAGGACAG-3'</td>
<td>HNF-3ε (SIF6)</td>
<td></td>
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<tr>
<td>EMSA</td>
<td>5'-AAATATCCCCCAACACACCTTTCC-3'</td>
<td>HNF-3β (SIF7)</td>
<td></td>
</tr>
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<td>RT-PCR</td>
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<td>Human GAPDH</td>
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<tr>
<td>RT-PCR</td>
<td>5'-TTAGCTCTCACCTCAAAGCACC-3'</td>
<td>Human Helios</td>
<td>(GenBank accession no. AF130863) 25</td>
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<tr>
<td>RT-PCR</td>
<td>5'-GCGGCAACACCATGCGGCGGCCG-3'</td>
<td>Human cdx-1</td>
<td>26</td>
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<tr>
<td>RT-PCR</td>
<td>5'-CATTGAGAGGTGAGGCGCAAGG-3'</td>
<td>Human cdx-2</td>
<td>26</td>
</tr>
</tbody>
</table>

* The oligonucleotides used in plasmid construction or in EMSA experiments and as gene-specific primers in RT-PCR are given together with application, primer sequence, target, and reference (where applicable).

* The 5-bp insertion (underlined) was introduced by QuickChange site-directed mutagenesis as described in Materials and Methods.
and sufficient for IL-4 responsiveness of the human plgR gene (10). To define the minimal DNA region encompassing this enhancer, we made sequential deletions from the 5′ and from the 3′ end of the 554-bp enhancer and placed these upstream of a heterologous promoter. We tested these new reporter constructs in transient transfection assays with 24 h of IL-4 stimulation, and used the plgR-derived reporter construct containing 554 bp of the intronic enhancer region (10) as a positive control for full IL-4 responsiveness (Fig. 1; p12). We found that deleting 49 bp from the 5′ end (Fig. 1; p31), or 255 bp from the 3′ end (Fig. 1; p34) did not reduce IL-4 responsiveness, either alone or in combination (Fig. 1; p53). Thus, the entire intronic IL-4-responsive enhancer is located within the 250-bp region from position +4169 to +4418.

Further deletions from either end led to a stepwise reduction in IL-4 responsiveness, suggesting a requirement for cooperation between several DNA elements to confer full IL-4 responsiveness. Previously, we have demonstrated that a STAT6 site located between several DNA elements to confer full IL-4 responsiveness (10). We now found that sequential deletions from the 5′ end of the enhancer region resulted in a gradual loss of responsiveness, with 3-fold reduction retained in the constructs containing only 19 bp upstream of the STAT6 site (Fig. 1; p48 and p54). This result suggested that DNA elements upstream of the STAT6 site located in the region from +4169 to +4314 (Fig. 1; present in p31 but absent in p48) are essential for full responsiveness, although not absolutely required for some enhancer activity in reporter gene assays. Deletion of 33 bp from the 3′ end of the 250-bp fragment reduced IL-4 responsiveness from 6-fold to 2-fold (Fig. 1; compare p34 with p45), and a further 18-bp deletion abolished all responsiveness, despite the presence of 24 bp downstream of the STAT6 site (Fig. 1; p46). This result implied that at least two regulatory DNA elements exist downstream of the STAT6 site, and that one or more of these are absolutely required for IL-4 responsiveness.

**FIGURE 1.** Mapping of the minimal region from intron 1 of the human plgR gene that confers IL-4 responsiveness to a heterologous promoter. Sequential deletions were made from the 5′ end, or 3′ end, or both ends of a 554-bp IL-4-responsive region from the human plgR intron 1 and sub-cloned upstream of pGL3-promoter vector (Promega). HT-29.m3 cells were transiently transfected with the indicated luciferase reporter constructs, and either left untreated or treated with IL-4 for 24 h before harvesting and measurement of luciferase activity. Names and diagrams of the reporter constructs are given on the left, and fold induction after IL-4 stimulation on the right. The position of the previously identified STAT6 site (10) is indicated by a circled S.

Full IL-4 responsiveness depends on an HNF-1 site located 100 bp upstream of the STAT6 site

The regulatory DNA elements within the 250-bp intronic enhancer were further mapped by specific point mutations in the reporter gene that contained 554 bp from the intronic region (p12). These mutants were then analyzed in transient transfection assays. A region of 165 bp upstream of the STAT6 site was required for full enhancer activity. We focused on the sequences between position −114 and −87 relative to the STAT6 site, because deletion of this region reduced the IL-4 responsiveness from −5-fold to −3.5-fold (Fig. 1, compare p69 with p33). We introduced four sequential 10-bp mutations covering this region and found that two of the 10-bp mutations reduced IL-4 responsiveness from 6-fold to −3.5-fold (Fig. 2A). By comparison with previously published consensus DNA elements (22, 30, 31) and searches in TF databases (28), we found that this region contained a putative HNF-1-binding site (Fig. 2B).

To determine whether specific nuclear factors could bind this DNA element, we isolated nuclear extracts from HT-29.m3 cells, untreated or treated with IL-4 for different time periods, and performed in vitro EMSA experiments with a probe spanning this region (named probe A) (Fig. 2C). We could not detect IL-4-inducible factors binding to probe A (data not shown) but demonstrated binding of four constitutive nuclear complexes with this probe (Fig. 2C, lane 1, complexes A-I to A-IV). A 100-fold excess of unlabelled wild-type (wt) probe A, but not an irrelevant oligonucleotide, competed for binding to all four complexes (Fig. 2C, lanes 1–3).

To determine the nucleotides required for binding of these four complexes, we performed competition experiments with oligonucleotides containing sequential 3-bp mutations spanning probe A (Fig. 2C, lanes 4–12). Mutations that affected the putative HNF-1 site showed reduced or abolished ability to compete for binding of the four complexes (Fig. 2C, lanes 8–10), while oligonucleotides with mutations outside of the putative HNF-1 site could compete for binding to the same degree as the wt probe. Lower exposure of the film revealed that mutations d and h also showed somewhat decreased ability to compete for binding (Fig. 2C, lanes 7 and 11). Furthermore, an oligonucleotide containing a consensus HNF-1 site (hSIF3) from the human sucrase-isomaltase gene (22) competed effectively for binding of all four nuclear complexes (Fig. 2C, lane 13).

Finally, we performed EMSA supershift analysis with specific Abs against HNF-1α and HNF-1β and detected the presence of these two proteins in the different complexes (Fig. 2D). Complexes A-I and A-IV were shifted by the HNF-1α Ab, complex A-II was shifted by the HNF-1β Ab, whereas complex A-III was shifted by both Abs (Fig. 2D). Specificity of the supershifts was determined by addition of epitope-specific peptides (Fig. 2D, lanes 5, 8, and 11–13). However, we noted that the supershift of the lowest mobility complex (complex A-IV) by HNF-1α could not be reversed by addition of the corresponding peptide (Fig. 2D, lanes 4, 5, 10, 11, and 13, complex A-IV).

Three additional DNA elements downstream of the STAT6 site are required for full IL-4 responsiveness

Regulatory DNA elements in the region downstream of the STAT6 site were mapped by introduction of sequential 4-bp mutations (except one 3-bp mutation) of the region required for IL-4 responsiveness that had not previously been analyzed (10), covering 63 bp in total. Mutations were introduced into the p12 reporter gene construct, and the effect on IL-4 responsiveness was analyzed in transient transfection assays. Several of the mutations resulted in significantly reduced responsiveness. In particular, we identified
three regions that, when mutated, resulted in only 2- to 3-fold enhancement of reporter gene activity after 24 h of IL-4 stimulation compared with 6-fold induction seen for the wt p12 reporter gene (Fig. 3, sites B, C, and D).

To investigate the binding of nuclear factors to these three DNA elements, we performed in vitro EMSA experiments, with probes that spanned the three respective sites (Fig. 4). Probe B formed a constitutive nuclear complex with nuclear extracts from HT-29 cells (Fig. 4A, complex B), but no IL-4-inducible complexes were detected (data not shown). Competition with oligonucleotides that contained sequential 3-bp mutations revealed the nucleotides required for binding of this complex (Fig. 4A, lanes 5–7). The binding site for complex B overlapped with the functional DNA element identified in the reporter gene assay (Fig. 3), although the in
vitro binding depended more on the downstream nucleotides of this DNA element. By a computer-assisted search in TF binding site databases (28), we found site B to contain a putative Cdx site. However, a consensus Cdx site (hSIF1) from the human sucrase-isomaltase gene (16) could not compete for binding of this specific complex (Fig. 4A, lane 11). Furthermore, complex B was not affected by polyclonal Abs specific for murine Cdx-1 or Cdx-2 (data not shown).

Probe C formed several complexes with nuclear extracts isolated from unstimulated HT-29.m3 cells (Fig. 4B, lane 1); the sequence specificity of these complexes was analyzed by competition with probes that contained sequential 3-bp mutations. All complexes displayed nucleotide specificities that corresponded to one or both of the 4-bp mutations found to reduce IL-4 responsiveness in reporter gene assays (Figs. 3, p65 and p66, and 4B, lanes 4–7). Searches in TF binding site databases (28) revealed that site C contained putative binding sites for several TFs, including HNF-3 and Cdx, which are both known to be expressed by intestinal epithelial cells (17, 32, 33). However, a consensus binding sites for HNF-3 from the human α-1-microglobulin/bikunin precursor gene (23) did not compete with probe C for the formation of any of these complexes (data not shown). Furthermore, addition of specific Abs against HNF-3α, HNF-3β, or HNF-3γ did not supershift or otherwise affect any of the complexes formed with probe C (data not shown). A consensus binding site for Cdx from the human sucrase-isomaltase gene (16) competed with complexes C-IV and C-V (Fig. 4B, lane 9), and recombinant murine Cdx-1 and Cdx-2 could bind to probe C and displayed similar migration in the gel as complexes C-IV and C-V (data not shown). Although we detected cdx-2, but not cdx-1, mRNA expression in HT-29.m3 cells by RT-PCR (data not shown), the addition of Abs specific for murine Cdx-1 (15) or Cdx-2 (17) failed to affect any of the HT-29-derived nuclear complexes seen with probe C (data not shown). Coexpression of Cdx-2 with the p12 reporter gene in HT-29 cells reduced the IL-4 responsiveness of the reporter gene, but also reduced the basal level of expression, whereas coexpression of murine Helios with the pIgR-derived reporter gene strongly reduced responsiveness to IL-4 without affecting the basal level of expression (data not shown). Whether this result was due to displacement of endogenous factors from site D, squelching, or other effects is currently not known.

For probes C and D, we detected a faint IL-4-inducible band that comigrated with STAT6 bound to the STAT6 probe (10) (data not shown). Furthermore, both sites C and D competed for binding of STAT6 to the consensus STAT6 site, although less efficiently than the wt STAT6 site (data not shown). Both probes C and D contain half-sites for STAT factors and imperfect whole STAT6 elements. Repeated adjacent STAT6 binding sites have been shown to enhance binding of STAT6 to DNA (34), suggesting cooperative binding between neighboring STAT6 dimers. Such cooperative binding has previously been demonstrated for other members of the STAT family and has been shown to display a certain permissiveness for imperfect STAT sites (11, 35–37). Therefore, it is possible that sites C and D are functional STAT6 binding sites, but additional experiments are required to determine which factors work through these sites in vivo.

Role of spacing between TF binding sites within the intronic enhancer

We previously reported that introduction of a 4-bp deletion in a PstI restriction enzyme site located immediately 3’ of the STAT6 site greatly reduced the IL-4 responsiveness of the enhancer (10). The reduced IL-4 responsiveness of the mutant with this deletion was greater than that of the mutant with four nucleotide substitutions flanking the STAT6 site, suggesting that the exact distance between the STAT6 site and downstream elements could be important for proper function of the enhancer (10). To test whether changing the helical orientation and/or the distance between the STAT6 site and downstream DNA elements (sites B, C, and D) could affect the IL-4 responsiveness of the enhancer, we introduced either 5-, 10-, or 15-bp oligonucleotides into the PstI site 3’ of the STAT6 site (Fig. 5). Thus, insertions creating half or full helical twists in the DNA between the STAT6 site and site B were introduced into the reporter gene and tested in transient transfections. We found that introduction of 5- or 15-bp spacers significantly reduced the IL-4 responsiveness of the enhancer, suggesting that the exact spacing or the helical orientation between DNA elements in the enhancer is important for its function (Fig. 5, pSC72...
not reduce IL-4 responsiveness significantly, indicating that the linear spacing (distance) introduced by these 10 bp did not significantly affect the function of the enhancer (Fig. 5, p5C73).

We have previously shown that ectopic activation of STAT6, achieved by 4-HT treatment of cells cotransfected with a conditionally active form of STAT6 (STAT6:ER*) was sufficient to activate the plgR intronic IL-4-responsive enhancer in HT-29 cells (10). To test whether activation of this enhancer by ectopic STAT6 activation rather than IL-4 treatment also depended on TF binding to the HNF-1 site, and sites B, C, and D, we cotransfected the STAT6:ER* expression vector with reporter gene constructs mutated in these sites. The wt reporter gene (p12) and a reporter gene with a mutated STAT6 binding site (p36) were used as positive and negative controls, respectively. STAT6 activation enhanced expression of the wt reporter gene ~5-fold (Fig. 6), similar to what we observed previously (10). Mutation of the STAT6 site abolished this enhancement, indicating that the STAT6:ER* protein indeed works through the STAT6 site in our reporter gene. Furthermore, mutations of the HNF-1 site or sites B, C, or D had a similar effect on STAT6:ER* responsiveness and IL-4 responsiveness of these plgR-derived reporter genes (Figs. 2A, 3, and 6).

Discussion

In this study, we characterized the IL-4-responsive enhancer located in intron 1 of the human plgR gene and found it to be contained within a 250-bp region that showed 72% sequence identity with the corresponding region in the murine gene (Fig. 7A). By mutational analysis, we identified four functionally important DNA elements that cooperate with STAT6 to mediate IL-4 responsiveness. Variation of the spacing between the STAT6 site and the downstream elements demonstrated the importance of the precise organization of these DNA elements in the enhancer (Fig. 7B). Furthermore, we characterized the protein-DNA complexes that bind these newly identified DNA elements, and identified HNF-1 as one of the TFs involved in this STAT6-dependent IL-4-responsive enhancer.

A conserved IL-4-responsive enhanceosome

Initial analysis by deletions identified a 250-bp region that was required and sufficient for full IL-4-mediated up-regulation of reporter genes. Although position-dependent effects may occur when such deletions are performed, some of the deletions were confirmed in two different contexts (Fig. 3, compare p54 with p48, and p53 with p34 and p31). Furthermore, the deletion of important DNA elements in p45, p46, and p33 (Fig. 1) was also confirmed by the introduction of point mutations in the 554-bp enhancer fragment (Figs. 2A and 3). Therefore, we believe that the minimal and complete enhancer region is located within the 250-bp DNA fragment present in p53 (Fig. 1).

Focusing on the deletions that most dramatically reduced IL-4 responsiveness of the reporter genes, we have investigated the role of a total of 131 nt within the 250-bp intronic enhancer by mutational analysis (this study and Ref. 10). In this manner, five distinct DNA elements required to cooperate to confer full IL-4 responsiveness of this enhancer have been identified to date. Deletional analysis identified an important DNA element ~100 bp upstream of the STAT6 site. Point mutations in this region, EMSA, and supershift assays demonstrated a functional HNF-1 site required for complete IL-4 responsiveness of the plgR gene. However, we cannot rule out the possibility of additional regulatory DNA elements in this region. In the region downstream of the STAT6 site, all nucleotides were investigated by mutational analysis, except for two gaps of 2 bp flanking the 4-bp mutation of site D (Fig. 3, p58). Four closely situated functional DNA elements (the STAT6 site and pSC74). However, the effect of these insertions was less dramatic than the 4-bp PstI deletion (Fig. 5, p5C23; Ref. 10). Introduction of a 10-bp spacer between the STAT6 site and site B did...
and sites B, C, and D) were identified in this region. We have previously reported conservation of the STAT6 site (10) and a TNF-responsive NF-κB site (38) in intron 1 of the human and murine pIgR genes. We found that the 250-bp human intronic IL-4-responsive enhancer region showed 72% sequence identity with the corresponding region in intron 1 of the murine pIgR gene (39) (Fig. 7A). In site A, the murine sequence had three diverging nucleotides within the 13-bp DNA element (Figs. 2B and 7A), but a probe spanning the murine sequence corresponding to human site A competed for binding of HNF-1 in EMSA experiments (data not shown). Site B was 86% identical (6/7) between the human and murine sequences, whereas sites C and D were 100% conserved (Fig. 7A). Thus, all the identified DNA elements were functionally conserved, and the distance between them were nearly identical in the two species (Fig. 5, A and B).

To investigate the effect of distance and helical phasing between the DNA elements in the enhancer, we introduced 5-, 10-, or 15-bp DNA sequences into a PstI site immediately downstream of the STAT6 site. The inserted DNA was analyzed by a search in TF databases for the presence of known binding sites to avoid introduction of new binding sites for transcriptional regulators. Furthermore, we chose to insert the DNA in the PstI site in a manner that restored the PstI site and thus did not alter the nucleotides flanking the STAT6 site. The nucleotides were thereby introduced into a sequence that, when mutated, only had marginal effect on IL-4 responsiveness (p40; Ref. 10). Changes in the spacing of the DNA elements that altered the helical phase in the enhancer resulted in reduced IL-4 responsiveness—probably due to disruption of the fine-tuned network of protein–protein interactions. A 10-bp insertion was tolerated without significant loss of function, whereas longer spacing (30 bp) reduced IL-4 responsiveness (data not shown), suggesting that the actual distance was also important for function (Figs. 5 and 7B). These data are in agreement with the enhanceosome theory (40, 41), which states that the binding of different TFs to a precisely organized array of DNA elements within an enhancer, creates a protein complex with a specific interaction surface that can recruit coactivators required for enhanced transcription. We found that the 4-bp deletion in pSC23
resulted in a more dramatic reduction in IL-4 responsiveness than the
5-bp insertion in pSC72. This deletion altered the nucleotides
flanking the STAT6 site, and could thereby reduce the binding
affinity for STAT6 (37). In addition, a 4-bp deletion might be more
critical than a 5-bp insertion because of physical constraints be-
tween STAT6 and the factor(s) binding to site B.

We have previously shown that activation of a conditionally
active form of STAT6 (the STAT6:ER fusion protein) was suffi-
cient to activate the IL-4-responsive plgR-derived reporter gene in
HT-29 cells, but not in COS-1 cells (10), suggesting that additional
required factors are not present in COS-1 cells. In this study, we
showed that activation of our reporter genes by the STAT6:ER
fusion protein or by IL-4 stimulation was equally sensitive to mu-
tations in DNA elements surrounding the STAT6 site. Therefore,
appropriate TF binding to several DNA elements in this enhancer
is necessary for STAT6 to activate the reporter gene, supporting
the idea that a number of factors, including STAT6 and HNF-1,
acactivate transcription in this enhanceosome-like structure.

Role of HNF-1 in IL-4-mediated transcriptional activation and
tissue specificity

HNF-1 was initially identified as one of several liver-enriched TFs
(the HNF-family) (31, 42). HNF-1α (also known as LF-B1; en-
coded by the tcf1 gene) and HNF-1β (also known as LF-B3 or
vHNF-1; encoded by the tcf2 gene) both contain an atypical ho-
medomain and a POU A-related domain (31, 43, 44). In addition
to abundant expression in the liver (42), HNF-1 has also been
found to be selectively expressed in the intestine, stomach, kidney,
pancreas (both endocrine and exocrine cells), and lung (HNF-1β
only)—conferring tissue-specific gene regulation (43, 45–47). The
expression pattern of HNF-1 parallels that of plgR, although the
latter is not expressed by human hepatocytes, but by murine and rat
hepatocytes (2). This high degree of overlap and the presence of a
functional HNF-1 site in the plgR intrinsic enhancer imply that HNF-1
contributes to appropriate tissue specificity of plgR expression.

Several laboratories have studied the constitutive expression of
the plgR gene, but very little is known about the tissue-specific
regulation. The only TFs identified to play a role in the constitutive
expression of plgR are the ubiquitously expressed upstream regu-
ulatory factors 1 and 2, which bind to an E-box in the proximal
promoter (14, 48–50). IFN regulatory factors 1 and 2 have been
implicated in TNF- and IFN-γ-responsive up-regulation of plgR
expression through the exon 1 IFN-stimulated response element
(51–54), but these factors are also widely expressed (55). STAT6
and NF-κB, which function through their target sites in intron 1 for
IL-4 and TNF induction of plgR transcription, are also ubiquitous.
Thus, HNF-1 is the first tissue-restricted TF found to be involved
in the regulation of plgR expression. However, additional tissue-re-
stricted TFs must also be involved in the regulation of plgR expres-
sion, particularly in the exocrine glands and the upper airways.

HNF-1α has been demonstrated to be involved in the regulation
of numerous intestinal epithelial-expressed genes in cooperation
with several different TFs, including HNF-4 in the α1-antitrypsin
gene (56), GATA5 in the lactase-phlorizin hydrolyase gene (22, 57),
and GATA4 and Cdx-2 in the sucrose-isomaltase gene (58, 59).
In lung epithelial cells, which lack expression of HNF-1α (43),
HNF-1β appears to be an important regulator of the α1-antitrypsin
gene (46). HNF-1 has been reported to be involved in both acti-
vation and repression of transcription (44), and the ability to ac-
tivate transcription has been linked both to recruitment (60, 61)
and activation (62) of different cofactors with intrinsic histone
acetyltransferase activity. Furthermore, based on observations in
HNF-1α knockout mice, HNF-1 has been implicated in tissue-
dependent histone hyperacetylation and increased chromatin ac-
cessibility to some of its target genes (43, 63).

We found that an intact HNF-1 site ~100 bp upstream of the
STAT6 site was dispensable for basal activity of our reporter gene,
but required for full IL-4 responsiveness. Cooperation between
HNF-1 and STAT6 has, to our knowledge, not been reported pre-
viously. However, HNF-1 is reported to cooperate with IL-6-in-
duced STAT3 in the liver (64) and, as an indirect link, the two
coactivators CREB-binding protein and p300 have both been re-
ported to interact with HNF-1 (60, 61) and STAT6 (65–67).

In conclusion, we have characterized the IL-4-responsive en-
hancer of the human plgR gene and demonstrated the requirement
for cooperation between factors binding to at least five different
DNA elements, including a STAT6 site and an HNF-1 site. The
enhancer is contained within a 250-bp region, which is highly con-
served in the murine gene. Further characterization of the factors
binding to these DNA elements and their mode of action is re-
quired to understand the complex regulation conferred by this in-
trinsic enhancer in vivo.

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