Glucocorticoid Regulation of Mouse and Human Dual Specificity Phosphatase 1 (DUSP1) Genes

UNUSUAL CIS-ACTING ELEMENTS AND UNEXPECTED EVOLUTIONARY DIVERGENCE

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Anti-inflammatory effects of glucocorticoids (GCs) are partly mediated by up-regulation of DUSP1 (dual specificity phosphatase 1), which dephosphorylates and inactivates mitogen-activated protein kinases. We identified putative GC-responsive regions containing GC receptor (GR) binding site consensus sequences that are well conserved between human and mouse DUSP1 loci in position, orientation, and sequence (at least 11 of 15 positions identical) and lie within regions of extended sequence conservation (minimum 65% identity over at least 100 bp). These were located ~29, 28, 24, 4.6, and 1.3 kb upstream of the DUSP1 transcription start site. The homology-based approach successfully identified four cis-acting regions that mediated transnational responses to dexamethasone. However, there was surprising interspecies divergence in site usage. This could not be explained by variations of the GR binding sites themselves. Instead, variations in flanking sequences appear to have driven the evolutionary divergence in mechanisms of regulation of mouse and human DUSP1 genes. There was a good correlation between the ability of cis-acting elements to respond to GC in transiently transfected reporter constructs and their ability to recruit GR in the context of intact chromatin. We propose that divergence of gene regulation has involved the loss or gain of binding sites for accessory transcription factors that assist in GR recruitment. Finally, a novel GC-responsive region of the human DUSP1 gene contains a highly unusual element, in which three closely spaced GR half-sites are required for potent transcriptional activation by GC.

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*1 DUSP1 (dual specificity phosphatase 1), or MKP-1 (mitogen-activated protein kinase phosphatase 1), belongs to a family of around 10 enzymes that inactivate MAPKs by catalyzing the removal of phosphate groups from threonine and tyrosine residues in the activation loops of those kinases. The preferred substrates of DUSP1 are p38α and β MAPKs and JNKs, which play critical roles in the expression of inflammatory mediators at transcriptional and post-transcriptional levels (2, 3). In most cells, the basal expression of DUSP1 is low, but it rapidly increases in response to a wide variety of proinflammatory stimuli, including the cytokines interleukin 1 and tumor necrosis factor, ligands of the Toll-like receptors, environmental stresses like heat shock, oxidative or genotoxic stresses, and UV light (4–6). The induction of DUSP1 by such agonists is dependent on p38 MAPK and/or JNK, therefore creating a negative feedback loop that shapes the signaling response to cell activation and prevents the unconstrained expression of inflammatory mediators (7–10). At first considered to have no phenotype (11), DUSP1−/− mice have subsequently been shown to overrespond to inflammatory insults, such as administration of bacterial lipopolysaccharide (12–15), infection with Gram-positive bacteria (16), ischemia-reperfusion injury (17), and experimental induction of arthritis or anaphylaxis (14, 18). DUSP1 therefore emerges as an important regulator of immune homeostasis, and its level of expression may have a critical impact on the outcome of an inflammatory insult (4–6).

*2 In several cell types, the expression of DUSP1 is also induced by glucocorticoids (GCs), providing a mechanism by which these powerful anti-inflammatory drugs can inhibit JNK and p38 MAPK signaling (4, 19–21). GC-induced DUSP1 expression and consequent inhibition of MAPK signaling have been implicated in the inhibition of proinflammatory gene expression by GCs in macrophages (22, 23), microglia (24), and vascular endothelial cells (25). The induction of DUSP1 also contributes to the cardioprotective effect of GCs (26) and the inhibition by GCs of mucin and β-defensin expression (27, 28). In airway smooth muscle cells, GCs inhibited the expression of CD38, interleukin-6, and the chemokine CXCL1 (CXCR motif ligand 1) at least partly via the induction of DUSP1 (29–31), suggesting that DUSP1 may contribute to therapeutic effects of GCs in asthma. Consistent with this hypothesis, in severe or GC-resistant asthma, GCs were defective in the induction of DUSP1 and failed to inactivate JNK or p38 MAPK (32–34). The mechanism of control of DUSP1 gene expression is fundamental to understanding the anti-inflammatory actions of GCs. Furthermore, it is possible that defects in this regulation may contribute to GC insensitivity, a major problem in the treatment of chronic inflammatory diseases (35).

GCs activate transcription via the GC receptor (GR), a member of the nuclear hormone receptor superfamily of transcription factors. The canonical pathway of transcriptional activa-
tion involves dimerization of GR and binding to inverted repeats of the half-site sequence AGAACA with separation of 3 bp (36). In reality, the DNA binding specificity of GR is somewhat relaxed. Of the 15-bp idealized consensus binding sequence AGAACANNNTGTCTT, only five (underlined) are consistently present at authentic GR binding sites in chromatin (37). Variations in the sequence of individual binding sites are thought to subtly alter the conformation of GR and influence its interactions with transcriptional cofactors (38). GR dimerization is mediated by the D-loop, a short, charged motif at the knuckle of the second zinc finger. Mutation of a single residue of the D-loop (Ala308 → Thr substitution in human GR) created a mutant protein, known as GR308m, that did not efficiently dimerize or activate transcription via consensus GC response elements (39–41). However, some GC-induced genes were not sensitive to this dimerization loop mutation (38, 42, 43), suggesting that either dimerization is dispensable or other protein-protein or protein-DNA interactions might stabilize the binding of GR dimers to certain sites.

In the context of chromatin, GR recognizes only a minute fraction of potential binding sites that contain the relaxed consensus sequence GNCANNNG. It is thought that GR is guided to appropriate sites in chromatin through interactions with other DNA-bound transcription factors in a manner that is dependent on localized chromatin remodeling (20, 37, 39–49). The cis-acting elements that mediate transcriptional responses to GCs tend to be quite extended, containing binding sites not only for GR but also for additional transcription factors with which GR cooperates. Functional GC-responsive regions (GRRs) are very often conserved between orthologous GC-regulated genes of distantly related species, such as mice and humans, and the GR binding sites that they contain tend to be highly similar in sequence (47). Strong evolutionary conservation of the GR binding site and surrounding sequence is presumed to reflect common mechanisms for recruitment of GR to DNA and subsequent activation of transcription. A consequence is that novel, functional GRRs can be discovered by a phylogenetic footprinting approach; in other words, the comparison of putative regulatory regions of orthologous GC-induced genes and the identification of strongly conserved GR binding site consensus sequences (GBS) that lie within regions of high sequence similarity. We used this approach to try to identify GC-responsive cis-acting elements of the human DUSP1 gene.

**EXPERIMENTAL PROCEDURES**

*Plasmids*—Expression vectors for wild-type and dimerization-defective (A458T mutant) GR were a gift of Andrew Cato (Karlsruhe Institute of Technology). A U2OS cell line stably expressing dimerization-defective GR was generously provided by Inez Rogatsky (Cornell University, Ithaca, NY). Human, mouse, rat, and dog DUSP1 promoter fragments were amplified from genomic DNA and subcloned to pGL3b, pGL3p, or pGL4.26 (Promega). Mutations of transcription factor binding sites were introduced using QuikChange site-directed mutagenesis kits (Agilent Technologies). Matrix Inspector (available at the Genomatix Web site) was used during design of mutations to avoid inadvertent introduction of new transcription factor binding sites. All mutations were checked by resequencing.

**Cell Culture, Transfection, and Luciferase Assays**—HeLa, mouse fibroblast, and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere of 5% CO2 at 37 °C. Cells were transiently transfected using Superfect (Qiagen) with 200 ng of firefly luciferase reporters as indicated plus 100 ng of Renilla luciferase expression vector and pBluescript (Agilent Technologies) as carrier to make the total quantity of DNA up to 1 μg. Some experiments included 50 ng of vector expressing wild type GR or GR308m. Following transfection cells were treated with vehicle (0.1% (v/v) EtOH) or Dex (100 nM unless otherwise stated) for 20 h. Cells were harvested, and luciferase activities were measured using the dual luciferase reporter assay kit (Promega) and Microbeta luminometer (PerkinElmer Life Sciences). Firefly luciferase activities were normalized against Renilla luciferase.

**Western Blotting**—COS-7 cells were harvested by lysis in SDS-PAGE loading buffer. Lysates were run on 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride. Western blotting was performed by standard methods. A rabbit polyclonal antibody against GR was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A mouse monoclonal antibody against tubulin was from Sigma.

**Chromatin Immunoprecipitation**—HeLa cells or mouse fibroblasts (3 × 106 cells/10-cm dish) were treated with vehicle (0.1% (v/v) EtOH) or 100 nM Dex for 30 min. A chromatin immunoprecipitation assay was carried out using GR antibody (catalog number sc 8992X, Santa Cruz Biotechnology, Inc.) or rabbit IgG (Santa Cruz Biotechnology, Inc.) as body (catalog number sc 8992X, Santa Cruz, CA). A mouse monoclonal antibody against tubulin was from Sigma.

**Preparation of Nuclear Extracts and Oligonucleotide Pull-down (ABCD) Assay**—HeLa cells were incubated with Dex (100 nM) or vehicle (0.1% (v/v) EtOH) for 1 h. Nuclear protein extract was prepared as described (67). 50 μg of HeLa nuclear extract was incubated with 90 pmol of biotinylated oligonucleotide, 1 μg of binding buffer (5 mM Hepes, pH 7.8, 75 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 10 mM MgCl2, 10% glycerol), 1 μg of...
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RESULTS

Similarities between Human and Mouse DUSP1 Genes—30 kb of the 5' region of the human DUSP1 locus was aligned with the orthologous mouse sequence (Fig. 1). The two sequences were also scanned for matches to the GBS, GNACANNNG (37). Regions of strong sequence similarity between mouse and human DUSP1 loci were identified, extending to at least 30 kb upstream of the transcription start site. The human sequence contained more than 50 and the mouse sequence more than 80 GBS. However, only nine GBS were conserved in position and orientation between the two species. GBS at approximately −13, −24, and −28 kb as well as three closely spaced GBS at −4.6 kb with respect to the transcription start site were located within domains of extended sequence similarity. Several of these GBS were also conserved in rat and dog DUSP1 loci (supplemental Fig. 1) as well as in the DUSP1 loci of other mammalian species. GBS at −2.3 and −16 kb were conserved between mice and humans but were not located within extended domains of sequence conservation and were not consistently present in other mammalian DUSP1 loci (supplemental Fig. 1). This analysis identified regions around −1.3, −4.6, −24, and −28 kb as possible mediators of transcriptional responses to Dex. GBS-29 of the human DUSP1 locus corresponds to the site previously identified at −27 kb of the mouse DUSP1 locus (47). The complex sequence relationships within this region are discussed in more detail below.

Identification of an Unusual GC-responsive Region 4.6 kb Upstream of the Human DUSP1 Gene—To identify regulatory elements of the human DUSP1 gene, we began by studying the region immediately upstream of the transcription start site. Transient transfection experiments were performed using HeLa cells, in which DUSP1 was first identified as a GC-induced gene (7). A fragment extending from −4834 to +211 with respect to the DUSP1 transcription start site mediated a 25-fold response to Dex (Fig. 2). Deletion from −4834 to −2726 strongly impaired but did not ablate the response (compare first and second bars). A second significant decrease in the transcriptional response to Dex was observed on deletion between −1495 and −1095. These results suggested that at least two discrete regions of the human DUSP1 5' region contribute to transcriptional control by Dex.

The distal region was inspected first. Three closely spaced GBS were located −4.6 kb upstream of the human DUSP1 transcription start site, within a region that shows considerable sequence similarity between human and mouse loci (Fig. 1). A multiple species alignment across this region revealed that the first and third GBS (GBS-4.6.1 and -4.6.3) were not strongly conserved between distantly related mammals, whereas the central motif (GBS-4.6.2) was highly conserved (Fig. 3A and supplemental Fig. 1). GBS-4.6.2 is a complex element. It contains not only a match to the consensus GNACANNNG sequence (indicated by asterisks in Fig. 3B) but also an overlapping perfect half-site sequence AGAACA on the antisense strand (indicated by the arrow in Fig. 3B). This unusual structure is well conserved, with no more than 1 nucleotide difference, in the mouse, dog, rat, horse, cow, marmoset, rhesus monkey, chimpanzee, and orangutan DUSP1 loci. At the DUSP1 locus of the armadillo Dasypus novemcintus (Dn.), a 2-nucleotide substitution destroys the overlapping half-site but creates another on the sense strand (Fig. 3B).

A GRR-4.6 fragment, encompassing the region of sequence conservation between human and mouse and containing GBS-4.6.1, −4.6.2, and −4.6.3, mediated a 15-fold Dex response when cloned upstream of the SV40 early promoter in the luciferase expression vector were transiently transfected into HeLa cells, which were then treated for 20 h with vehicle (0.1% ethanol) or Dex (100 nM) before harvesting and measuring luciferase activities. For each reporter construct, firefly luciferase expression was normalized to Renilla luciferase activity, and fold activation in response to Dex was calculated. In this and subsequent figures, mean fold responses ± S.E. from three independent experiments are shown. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., not significantly different. Unless otherwise indicated, statistical comparisons are against the largest construct, pGL3b−4834.

poly(dI-dC), and BSA (0.1 mg/ml) and incubated for 1 h at 4 °C. For competition purposes, the nuclear extracts were incubated with the biotinylated oligonucleotide in the presence of 20-fold molar excesses of non-biotinylated competitor oligonucleotides. Streptavidin-agarose beads (Perbio Science) were preincubated with bovine serum albumin (1 mg/ml) and washed twice in 1X binding buffer. The addition of beads to the samples was followed by 1 h of incubation at 4 °C. Pellets were then washed six times in binding buffer, resuspended in 2X SDS sample buffer, and boiled. The samples were subjected to Western blot. Oligonucleotides used were as follows (top strands only shown): GRR-1.3, 5'-tcccccaggaggggaggaaaccgcagaatgttc-3'; GBScon, 5'-caatctgtcggtaagagatgtttcatcct-3'.

Statistics—Statistical analysis was performed using one-way analysis of variance with Bonferroni post-test or, where appropriate, one-sample t test.

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reporter construct pGL3p (compare first and second bars in Fig. 3C). The response was not impaired by mutation of GBS-4.6.1 or -4.6.3, suggesting that these less well conserved sites are not functional. In contrast, mutation of any of the three ACA triplets of GBS-4.6.2 strongly impaired the Dex response. Although GBS-4.6.2 is well conserved between mammalian species, rat and mouse GRR-4.6 fragments were only weakly responsive to Dex (Fig. 3D). The orthologous dog fragment mediated a very strong response. The 3-nucleotide spacer between half-sites in a conventional GBS is not thought to make specific contacts with GR, yet sequence differences within the spacer can exert allosteric effects on the conformation and activity of the bound receptor (38). We speculated that the single nucleotide difference between human and mouse GBS-4.6.2 might contribute to the difference in activity of the fragments within which these sequences are embedded. However, introduction of the human GBS-4.6.2 sequence into the mouse GRR-4.6 fragment (Fig. 3D, Mm > Hs) caused no gain of activity. Conversely, introduction of the mouse GBS-4.6.2 sequence into the human GRR-4.6 fragment did not impair the response to Dex (Fig. 3D, Hs > Mm).

The SV40 early promoter in pGL3p contains binding sites for Sp1 and AP-1 transcription factors, which may functionally interact with GR (20). In the absence of any subcloned fragment, this construct displayed a weak response to Dex, which has been corrected for in all relevant figures. To test regulatory activities of GBS elements in isolation from other transcription factor binding sites, we used an alternative reporter construct, pGL4.26, which contains only a TATA box upstream of the luciferase open reading frame and has very low basal transcriptional activity or intrinsic Dex responsiveness. In this background, both human and mouse GBS-4.6.2 oligonucleotides were responsive to Dex. If anything, the mouse element responded more strongly than the human. Together, these results clearly show that differential responses of mouse and human GRR-4.6 fragments are due not to the minor sequence differences of the GR binding sites themselves but to differences of the surrounding sequence. Human GRR-4.6 provides a DNA environment that supports and/or amplifies the transcriptional response to Dex, whereas mouse GRR-4.6 does not.

**FIGURE 3.** Characterization of a GC-responsive region 4.6 kb upstream of the human DUSP1 start site. A, Multiz alignment of GGR-4.6 of 28 mammalian species at the UCSC Genome Browser (University of California, Santa Cruz) (66). The positions of GBS-4.6.1, -4.6.2, and -4.6.3 are indicated below the alignment. B, sequences of GBS-4.6.2 of human (Homo sapiens; Hs.), rat (Rattus norvegicus; Rn.), mouse (Mus musculus; Mm.), dog (Canis familiaris; Cfa.), cow (Bos taurus; Bta.), horse (Equus caballus; Eca.), and armadillo (Dasypus novemcinctus; Dn.). The conserved GBS sequence GNACANNNNG is indicated by asterisks, and an additional GR half-site is denoted by an arrow. Differences from the human sequence are highlighted. Sequences of mutated versions of GBS-4.6.2 are also shown. C, a 467-bp human genomic fragment centered on GBS-4.6.2 was cloned upstream of the SV40 early promoter in pGL3p. Mutations at GBS-4.6.1, -4.6.2, or -4.6.3 (as indicated in B) were introduced by PCR. Dex responses were assayed as in Fig. 2 and normalized against the response of the empty vector pGL3p. With the exception of pGL3p-GRR-4.6-GBS-4.6.2-m2, all constructs were significantly different from the parental vector pGL3p. Statistical comparisons against pGL3p-GRR-4.6 are indicated beside each construct, and additional comparisons are as shown. E, short oligonucleotides containing human or mouse GBS-4.6.2 were subcloned into the firefly luciferase reporter pGL4.26 (for simplicity indicated as pGL4 in the figure). Responses to Dex were calculated as in C. Statistically significant differences from pGL4 are indicated beside each construct, and other statistical comparisons are as indicated.
Characterization of a GC-responsive Region 1.3 kb Upstream of the Human DUSP1 Gene—Deletion of residues 1495 to 1095 of the human DUSP1 promoter impaired the transcriptional response to Dex (Fig. 2). An equivalent fragment mediated a 4-fold response to Dex when cloned upstream of the heterologous SV40 early promoter. Deletions from 5’- and 3’-ends led to the identification of a minimal GC-responsive 130-bp fragment extending from −1366 to −1237 with respect to the transcription start site (Fig. 4). The minimal GC-responsive fragment is hereafter referred to as GRR-1.3. On the antisense strand, near the 5’-end of this fragment, is the GBS-1.3 element that was identified on the basis of human/mouse DUSP1 sequence alignment (Fig. 1). GBS-1.3 is very strongly conserved among DUSP1 loci of mammals (Fig. 5, A and B, and supplemental Fig. 1). On the basis of conservation at 15 of 15 positions, this element would be predicted to be functional in the dog, rat, and mouse DUSP1 loci (47). In fact, human and dog GRR-1.3 fragments were similarly responsive to Dex, whereas the rat fragment was very weakly responsive, and the mouse fragment was inert in this assay (Fig. 5C). To confirm that GBS-1.3 is necessary for transcriptional activation of the human reporter by Dex, we introduced two different mutations of this site: a substitution of the three highly conserved ACA residues by GGG (m1) or a substitution of the final conserved G residue by C (m2). The first of these mutations completely abolished and the second severely impaired the response to Dex (Fig. 5C).

The highly conserved element GBS-1.3 was therefore absolutely necessary yet apparently not sufficient for Dex-mediated activation of transcription in the context of the pGL3p reporter.

**FIGURE 4. Identification of a GC-responsive region 1.3 kb upstream of the human DUSP1 start site.** A genomic fragment extending from −1426 to −1096 with respect to the human DUSP1 TSS and various deletion derivatives of this fragment were subcloned into pGL3p. Responses to Dex were calculated as in Fig. 3C. The region −1366 to −1237 was identified as a minimal GC-responsive region and is hereafter referred to as GRR-1.3. Statistical comparisons are first against the parental vector pGL3p and second against the construct containing the minimal GC responsive region −1366 to −1237, which is hereafter referred to as pGL3p-GRR-1.3-Hs.

**FIGURE 5. Characterization of GRR-1.3.** A, Multiz alignment of GRR-1.3 of 28 mammalian species at the UCSC Genome Browser (66). The positions of GBS-1.3 is indicated below the alignment. B, alignment of human (Hs.), dog (Cfa.), and mouse (Mm.) GRR-1.3 sequences. The positions of putative C/EBP and GR binding sites (GBS-1.3) are shown below the alignment. GBS-1.3 (in italic type) is a short oligonucleotide containing the GBS-1.3 element and a few conserved flanking residues. Nucleotide changes introduced into the human GRR-1.3 fragment to create the GBSm1 and GBSm2 are shown. C, dog, rat, mouse, and human GRR-1.3 fragments were subcloned into pGL3p, and responses to Dex were calculated as in Fig. 3C. Differences from the parental vector pGL3p are shown to the right of each bar, and other statistical comparisons are as indicated. D, human and mouse GRR-1.3 fragments or the oligonucleotide GBS-1.3 were subcloned into pGL4.26 (shown as pGL4), and responses to Dex were calculated as in Fig. 3C. Differences from the parental vector pGL4 are shown to the right of each bar, and other statistical comparisons are as indicated.
A 24-bp oligonucleotide that contains GBS-1.3 and the surrounding conserved bases (labeled as GBS-1.3 in Fig. 5B) supported a weak but statistically significant response to Dex when placed upstream of the TATA box in pGL4.26 (Fig. 5D). In this low background reporter construct, a similar weak response to Dex was detected using the mouse GRR-1.3 fragment. The differential responsiveness of mouse and human GRR-1.3 fragments was maintained; human GRR-1.3 gave an almost 3-fold stronger response than either mouse GRR-1.3 or the conserved GBS-1.3 element. Therefore GBS-1.3 is able to mediate a weak response to Dex that can be detected if the signal/noise ratio is sufficiently high. A potent transcriptional response requires sequences that lie outside of GBS-1.3 itself and differ between mouse and human DUSP1 loci.

**Differential Usage of GRR-4.6 and GRR-1.3 in Mouse and Human DUSP1 Promoters**—The contributions of GRR-4.6 and GRR-1.3 were next tested in the context of intact DUSP1 promoters rather than heterologous promoter constructs (Fig. 6). The 5-kb human DUSP1 promoter was activated almost 25-fold by Dex. An internal deletion of 28 base pairs (removing GBS-4.6.2 but leaving GBS-4.6.1 and -4.6.3 intact) reduced the Dex response to ~10-fold, similar to the effect of removing more than 3 kb between positions −4834 and −1495. Therefore, GBS-4.6.2 makes an important contribution to the response of the 5-kb promoter construct, and there are unlikely to be other relevant sites between −4834 and −1495. Mutation of 3 bp of GBS-1.3 (equivalent to m1 in Fig. 5B) impaired the response of the 5-kb promoter even more strikingly, to less than 5-fold. The same mutation also strongly impaired the response of the −1495 promoter construct. In the intact human DUSP1 promoter, GBS-1.3 appears to play a critical role.

**Characterization of a GC-responsive Region 27 kb Upstream of the Mouse DUSP1 Gene**—This raised the question of how the mouse DUSP1 gene is able to respond to Dex. On the basis of genome-wide sequence comparisons, a GC-responsive region was previously identified ~27 kb 5’ to the mouse DUSP1 start site (47). In Dex-treated mouse mesenchymal C3H10T1/2 cells, this region was occupied by GR. The corresponding region of the human DUSP1 locus is ~29 kb 5’ to the start site (supplemental Fig. 1). For consistency, the human, mouse, and other orthologous elements are referred to as GRR-29. Mouse GRR-29 contains a symmetrical sequence element with two nearly perfect matches to the half-site sequence AGAAACA. GBS matches are present on both strands (Fig. 7A). The corresponding rat sequence differs by 1 nucleotide and has only the top strand GBS. The dog sequence differs by 3 nucleotides and has only the bottom strand GBS. The human sequence differs from the mouse by 3 nucleotides and contains a new top strand GBS that is shifted with respect to the background alignment. Although it is true to say that putative GR binding sites within this region are conserved between mammalian species, this statement does not do justice to the complex sequence relationships that exist.

A 500-bp fragment centered on GBS-29 was amplified from mouse genomic DNA and placed upstream of the SV40 early promoter. As has been reported in C3H10T1/2 cells (47), this fragment mediated a strong transcriptional response to Dex in HeLa cells (Fig. 7B). Consistent with previous observations (47), mutation of the ACA triplets of GBS-29 ablated the Dex response of this fragment (Mm-GBSm in Fig. 7B). Corresponding rat, dog, and human fragments were amplified from genomic DNA and tested in parallel. The rat GRR-29 fragment was responsive to Dex, but the human fragment was inert and the dog fragment only weakly responsive. To assess the relative importance of GR binding site sequence and surrounding context, mutagenesis was used to introduce the mouse GBS-29 sequence into the context of human GRR-29. This sequence swap only slightly increased the activity of the human fragment (GRR-29-Hs>Mm in Fig. 7B). The activities of short oligonucleotides containing human and mouse GBS-29 were then tested, using pGL4 to minimize possible contributions from transcription factor binding sites in the vector backbone (Fig. 7C). The human GBS-29 oligonucleotide did not respond to Dex, whereas the mouse oligonucleotide responded very strongly. In this case, differential responses to Dex appear to arise from divergence of both GBS sequence and surrounding context. The GRR-29 region of the human DUSP1 locus con-

**FIGURE 6.** Differential usage of GRR-4.6 and −1.3 in mouse and human DUSP1 promoters. Site-directed mutagenesis was performed to introduce mutations into the human (Hs.) reporter constructs pGL3b−4834 and −1495. The Δ4.6 mutation is a 28-bp internal deletion that removes GBS-4.6.2 but leaves GBS-4.6.1 and -4.6.3 intact. The m1.3 mutation is a 3-bp substitution of GGG for the ACA motif triplet of GBS-1.3 (identical to m1 in Fig. 5B). Corresponding deletion and point mutation constructs were generated for the mouse (Mm.) DUSP1 promoter. The differences in size of mouse and human promoter fragments are due to internal insertions and deletions at the two DUSP1 loci. Responses to Dex were determined as in Fig. 2, n.s., not significant.
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FIGURE 7. Characterization of a GC-responsive region 29 kb upstream of the human DUSP1 start site. A, alignment of GBS-29 sequences of mouse, rat, human, and dog. This element was first described in the mouse DUSP1 locus (47); therefore, the mouse is regarded as the base sequence, and differences from it are highlighted. Matches to the consensus GNACANNNNG on the top or bottom strand are indicated by asterisks. B, dog, rat, mouse, or human GRR-29 fragments were cloned into pGL3p. pGL3p-GRR-29-Mm-GBSm was created by mutagenizing both of the ACA triplets within the mouse GRR-29 fragment to GGG. pGL3p-GRR-29-Hs.>Mm was generated by mutagenizing the sequence GAACTTTCG within human GRR-29 to the corresponding mouse sequence, GAATTTCAAG. This 3-nucleotide change creates a palindromic element with GNACANNNNG matches on both strands. Dex responses were calculated as in Fig. 3C. Differences from the parental vector pGL3p are shown to the right of each bar, and other statistical comparisons are as indicated. C, short oligonucleotides containing human and mouse GBS-29 were cloned into pGL4. Responses to Dex were calculated as in Fig. 3C. Differences from the parental vector pGL4 are shown to the right of each bar, and other statistical comparisons are as indicated. 

FIGURE 8. Species-specific transcriptional responses to Dex in mouse fibroblasts. Dex responses of human and mouse GRR-29, -4.6, and -1.3 fragments were tested in mouse fibroblasts and calculated as in Fig. 3C. Differences from the parental vector pGL3p are indicated above each column, and pairwise comparisons of orthologous mouse and human fragments are also shown. n.s., not significant.

Differences in Regulatory Activity of Mouse and Human Elements Are Related to Sequence and Not Cellular Environment—Human GRR-1.3 and -4.6 were strongly Dex-responsive when tested in HeLa cells, whereas the corresponding mouse sequences were not. In principle, this difference in responsiveness could be influenced by cellular environment. In other words, transcription factors that cooperate with GR to mediate transcriptional responses may have altered in DNA binding specificity during the evolutionary divergence of mice and humans, making mouse cis-acting elements “unfit” for function in the environment of a human cell. Human and mouse elements were therefore assayed in mouse fibroblasts (Fig. 8). Human GRR-4.6 and -1.3 were relatively strongly responsive to Dex, whereas the corresponding mouse fragments were weak or inactive, just as in HeLa cells. Human GRR-29 was unresponsive, whereas the corresponding mouse fragment gave a weak but statistically significant response. Overall, we found exactly the same pattern of species specificity in the mouse fibroblasts as in the HeLa cells, demonstrating that differential responses of mouse and human regulatory elements are at the cis rather than the trans level; in other words, they are explained by sequence differences rather than the cellular environment (human or mouse) in which they are tested.

Recruitment of GR to Functional Elements of Endogenous DUSP1 Genes—Chromatin immunoprecipitation experiments were carried out to assess the recruitment of GR to different regions of human and mouse DUSP1 loci in HeLa and mouse fibroblast cells. In HeLa cells, there was Dex-dependent recruitment of GR to GRR-1.3 and GRR-4.6 but not to GRR-29 (Fig. 9A). Similar recruitment at GRR-1.3 and GRR-4.6 was demonstrated using alternative primer pairs (data not shown). In mouse fibroblasts, there was Dex-dependent recruitment of GR at GRR-29 but none at GRR-1.3 and relatively little at GRR-4.6 (Fig. 9B). In both cases, recruitment of GR to a known binding site of the FKBP51 gene served as a positive control. The ability or inability of isolated cis-acting elements to respond to Dex in transiently transfected cells was therefore in agreement with the ability or inability of the same regions to recruit GR in intact chromatin.

Dependence of Dex Responses on GR Dimerization—There is controversy over whether GR dimerization is necessary for induction of DUSP1 by Dex. In COS-7 cells (which do not express endogenous GR), a mouse DUSP1 promoter was activated by wild type GR but not by the dimerization-defective mutant GRdim (51), yet Dex retained the ability to up-regulate DUSP1 protein in mouse macrophages expressing only GRdim (22). To try to resolve this controversy, we tested whether mutation of the GR dimerization loop influenced the Dex responses of human and mouse DUSP1 promoter and GR reporters in COS-7 cells. First, the pGL3p-Hs.GRR-1.3 reporter
construct was used to establish that transcriptional activation in response to Dex was dependent on GR (Fig. 10A, compare columns 2 and 5) and an intact GR binding site (compare column 5 with columns 4 and 6). It was then shown that GRdim and wild type GR were expressed at similar levels in transfected COS-7 cells (Fig 10B) but differed markedly in their ability to activate a reporter construct (52) that contains two consensus GR binding sites (Fig 10C, top). Next it was shown that activation of a short mouse DUSP1 promoter construct was impaired by the dim mutation (Fig 10C), consistent with a previous publication that used a very similar construct (51). The 5-kb mouse promoter fragment behaved identically (data not shown). Mutation of GBS-1.3 made the reporter even more sensitive to perturbation of GR dimerization. Together with the results presented in Fig. 6, these data suggest that responses of the mouse proximal promoter are largely mediated by a site or sites that are (i) distinct from GBS-1.3 and (ii) strongly dependent on GR dimerization. However, GRR-29, the most powerful GC-responsive region of the mouse DUSP1 promoter that we or others have identified, was insensitive to the dim mutation.

The construct pGL3b-Hs.−4834 contains the major GC-responsive regions that have been identified at the human DUSP1 locus, namely GRR-1.3 and -4.6. In COS-7 cells, wild type GR and GRdim activated this construct equally potently (Fig 10C). The dim mutation did not affect the transcriptional response of GRR-1.3 in isolation and only weakly impaired the response of GRR-4.6. It is likely that transcriptional activation of the endogenous human DUSP1 gene is unaffected by mutation of the GR dimerization motif.
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dimerization loop, but this has not yet been formally demonstrated. We used a GRdim-expressing stable U2OS cell line (53) to investigate transcriptional responses of the dimerization-defective mutant but found the DUSP1 gene to be transcriptionally silenced in these cells (data not shown).

DISCUSSION

A hallmark of many GC-responsive cis-acting elements is the evolutionary conservation of not only GR binding sites themselves but also surrounding sequence and binding sites for accessory transcription factors (37). Across the genome, this conservation can be successfully used to identify novel elements (47). However, the phylogenetic footprinting approach can produce surprising results when applied to individual genes, as illustrated here.

We used the DUSP1 locus to test the hypothesis that “GBS conservation alone is sufficient to predict GR occupancy and... function at individual genes” (47). Human and mouse GRR-1.3 and -4.6 are 70 and 65% identical in sequence and contain GBS elements that are conserved at 15 of 15 or 14 of 15 positions, respectively. The hypothesis predicts that such strong sequence conservation is correlated with conserved function and GR occupancy. The human elements functioned as GC-responsive sequences in either mouse or human cells and were occupied by GR in Dex-treated HeLa cells. Unexpectedly, the corresponding mouse elements were either unresponsive or very weakly responsive in HeLa cells and mouse fibroblasts and recruited GR weakly or not detectably in Dex-treated mouse fibroblasts. A GBS is conserved at ~29 kb upstream of the transcription start site in mouse and human DUSP1 loci, although its exact position with respect to the background sequence alignment differs slightly. Here too, GBS conservation (loosely defined) was predictive of function and GR occupancy in only one of the two species. The mouse element mediated a response to Dex in either HeLa cells or mouse fibroblasts, and was occupied by GR in Dex-stimulated mouse fibroblasts. The human element was unresponsive to Dex in either cell type and not occupied by GR in Dex-stimulated HeLa cells. We also investigated two other putative GC-responsive regions at ~24 and ~28 kb (supplemental Fig. 2). Both human and mouse GRR-28 were unresponsive to Dex and failed to recruit GR in Dex-stimulated HeLa cells or mouse fibroblasts. In contrast, both human and mouse GRR-24 mediated significant transcriptional responses to Dex and recruited GR in Dex-stimulated HeLa cells and mouse fibroblasts. At a single locus, we have therefore found examples of all four possible outcomes where GBS and surrounding sequences were conserved between mouse and human: function in both species (GRR-24), in neither (GRR-28), selectively in mouse (GRR-29), or selectively in human (GRR-1.3 and GRR-4.6).

The single nucleotide difference at GBS-4.6.2 is not sufficient to account for the differential activity of mouse and human GRR-4.6 fragments in mouse or human cells. GBS-1.3 is perfectly conserved between mice and humans, yet GRR-1.3 fragments of the two species displayed very different activities, whether tested in mouse or human cells. Elements that were able to mediate transcriptional activation by Dex in transiently transfected reporter constructs were also able to recruit GR in the context of intact chromatin. We therefore propose that a critical determinant in GC responsiveness is the absence or presence of binding sites for transcription factors that assist in the recruitment of GR. Loss or gain of such accessory sites appears to have been a driving force in the evolutionary divergence of transcriptional responses to GCs.

We have tried to identify sequence differences between mouse and human GRR-1.3 that might account for their different activities. For example, a previous study (54) suggested that in human pulmonary A549 cells, GR regulated DUSP1 expression via indirect tethering to a C/EBPβ site at ~1308 with respect to the TSS (indicated in Fig. 5B). Note that the minimal functional GC-responsive fragment identified in that study contained GBS-1.3. The putative C/EBPβ site was absent from an oligonucleotide that efficiently bound GR in vitro (supplemental Fig. 3), implying that GR recruitment is direct rather than indirect, and consistent with the deleterious effect of mutations within GBS-1.3 (Fig. 5C). C/EBPβ consensus sequences are significantly enriched in the vicinity of GR binding sites in chromatin (37, 48), and there are several documented examples of cooperation between the two transcription factors (20). Therefore, we considered the hypothesis that C/EBPβ assists GR recruitment to GRR-1.3 and that interspecies differences in the vicinity of the C/EBPβ site contribute to differences in function. However, a human to mouse sequence swap across this region did not impair the response of human GRR-1.3 to Dex (m4 in supplemental Fig. 4); nor did a mutation of 5 bp at the core of the C/EBPβ site (m8 in supplemental Fig. 4). In fact, other than mutations of GBS-1.3 itself (m1 and m2 in supplemental Fig. 4), only two of an extensive set of mutations (m6 and m7 in supplemental Fig. 4) significantly impaired the response of human GRR-1.3 to Dex and even then only weakly.

It appears that the sequence differences that underlie the functional differences of human and mouse GRR-1.3 are not discrete or localized. Evolutionary loss or gain of more than one transcription factor binding site may have contributed to the divergence of function.

Closer inspection provides further insight into events that have contributed to the evolution of DUSP1 genes. Approximately half of mammalian DNA is composed of repetitive sequences (55, 56). Much of it is in the form of mobile elements, such as short interspersed elements (SINEs), which are copied and inserted into new genomic locations via reverse transcription from short RNA polymerase III transcripts. They are often regarded as mere genome parasites, but they may contribute to the evolution of gene expression by creating or disrupting regulatory elements (56–60). Like most non-coding DNA regions, the DUSP1 5’ region contains many SINEs and other repetitive elements. In the GBS-4.6.2 sequence TGTTCTGAAAC/CTG-TGTGAC, the last nine residues (after the slash) are derived from a MIR3 SINE, an ancient retrotransposon that predates the divergence of mammalian clades (61) (supplemental Fig. 5). GBS-4.6.2 therefore owes its existence to the insertion of a mobile element. It cannot be known whether that retrotransposition event directly created the GBS or provided the raw material from which the GBS later arose. Whichever of these scenarios is correct, the strong conservation of the SINE sequence across many mammalian species implies that the gen-
eration of a novel regulatory element conferred an advantage and was subject to positive, purifying selection. This is an apparent example of exaptation, the adoption of novel function by a genetic element, which then comes under altered selective pressure. Exaptation of regulatory functions by mobile elements including SINEs is thought to have been a major force driving innovation in gene expression (58, 59). In this case, the process appears to have created a particularly powerful and unusual GR binding site whose function is dependent on three closely spaced or overlapping half-sites. Future studies will aim to discover how GR interacts with this site and which cofactors it is able to recruit when bound.

It has been suggested that the anti-inflammatory effects of GCs are independent of transcriptional activation by GR. This argument is largely based on the properties of a knock-in mouse model in which GCs are independent of transcriptional activation by GR. This unusual GR binding site whose function is dependent on three AGAACA half-sites was actually increased. Although mice and humans employ different cis-acting elements to control DUSP1 expression, the response to GC appears to be insensitive to the dim mutation in both species. DUSP1 is a mediator of anti-inflammatory effects of GCs; therefore, it is unsafe to conclude from the properties of the GRdim knock-in mouse whether or not anti-inflammatory effects of GCs are dependent on activation of transcription.

Transcriptional responses to GCs are highly heterogeneous, and a typical GC-regulated gene is difficult to define. However, the DUSP1 gene is very unusual in many respects. We have shown that, although there is strong evolutionary conservation of several GR binding sites, mouse and human genes employ different sets of cis-acting elements to achieve very similar responses to Dex. Evolution clearly permits a degree of plasticity in the molecular mechanisms of transcriptional regulation, provided that the final outcome is maintained. In both mice and humans, the activation of DUSP1 transcription appears to be independent of GR dimerization or at least insensitive to the mutation of a single residue within the dimerization loop. In the case of the human gene, transcriptional activation is partly mediated by a three half-site motif that is, as far as we are aware, unique. Perhaps reflecting the unconventional nature of its transcriptional control, DUSP1 can be up-regulated by novel GR ligands (63) that are thought to be otherwise poor at mediating transactivation (64). Up-regulation of the DUSP1 gene by GCs is also highly sensitive to cell density, whereas the response of a reporter containing consensus GR binding sites is insensitive to cell density. These observations suggest that it may be possible to selectively modulate the GC response of the DUSP1 gene.

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