The Murine IL-2 Promoter Contains Distal Regulatory Elements Responsive to the Ah Receptor, a Member of the Evolutionarily Conserved bHLH-PAS Transcription Factor Family

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The Murine IL-2 Promoter Contains Distal Regulatory Elements Responsive to the Ah Receptor, a Member of the Evolutionarily Conserved bHLH-PAS Transcription Factor Family

Myung-Shin Jeon and Charlotte Esser

Signaling through the TCR and costimulatory signals primarily control transcription of the IL-2 gene in naive T cells. The minimal promoter necessary for this expression lies proximal, between −300 and the transcription start site. We had previously shown that activation of the arylhydrocarbon receptor (AHR), a member of the bHLH-PAS family of transcription factors, leads to increased mRNA expression of IL-2 in murine fetal thymocytes. The AHR is abundant in the thymus and may play a role for the development of the immune system. Moreover, its overactivation by chemicals such as dioxins leads to immunosuppression and thymic involution. Binding motifs for the liganded AHR can be identified in the distal region −1300 to −800 of the mouse IL-2 promoter. We show here that these DNA motifs, the so-called dioxin response elements, after binding to the liganded AHR are sufficient to transactivate luciferase expression in a reporter gene system. The IL-2 gene can be induced by the AHR also in thymocytes in vivo after injection of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a potent ligand of the AHR. The AHR mediates the IL-2 induction as shown with AHR-deficient mice. However, in spleen cells in vitro costimulation via the TCR is necessary for optimal IL-2 gene induction. Thus, the IL-2 promoter region contains novel distal regulatory elements that can be addressed by the AHR to induce IL-2 and can cooperate with the proximal promoter in this. The Journal of Immunology, 2000, 165: 6975–6983.
IL-2 DISTAL PROMOTER ELEMENTS REGULATED BY AH RECEPTOR

Materials and Methods

In vivo treatment of mice

Female C57BL/6 mice (Zentralinstitut für Versuchstierkunde, Hannover, Germany) and AHR homozygous and heterozygous offspring of female B6,129AhR+/−/− (AhR+/−/−) and male C57BL6AhR+/−/− (AhR+/−/−) mice (The Jackson Laboratory, Bar Harbor, ME) bred in our animal facility were used. All animals were kept under specific pathogen-free conditions. For in vivo exposure, TCDD (purity >99%; Oekometric GmbH, Bayreuth, Germany) was dissolved in DMSO and diluted in sterile corn oil to 5 μg/ml. Mice were injected i.p. with a single dose of 50 μg TCDD/kg body weight. Control mice received DMSO/corn oil. At posttreatment days 3, 6, and 8, mice were killed by asphyxiation, and RNA was extracted from thymi and spleens with TRIzol according to the manufacturer’s instructions (Life Technologies, Gaithersburg, MD). RNA was reverse transcribed by standard procedures. Before RNA extraction, erythrocytes were depleted from spleen cells by hypotonic lysis.

Primary cell cultures

RPMI 1640 supplemented with 5% FCS, 5 × 10−4 M 2-ME, 100 U/ml penicillin, and 0.1 mg/ml streptomycin was used as medium throughout. TCDD was dissolved in 1,4-dioxane (Merck, Darmstadt, Germany) and added to the medium at a final concentration of 10 nM in 0.1% solvent. Anti-CD3ε was coated onto six-well plates, and 10−13 × 106 thymocytes or 5−7 × 106 spleen cells from adult animals were added in 2 ml medium with 10 nM TCDD or solvent alone. After 4 h, RNA was isolated for RT-PCR. In other experiments, supernatants were taken after the indicated incubation times with anti-CD3ε, and IL-2 content was measured by ELISA (Mouse ELISA kit; Endogen, Woburn, MA). Alternatively, fetal thymus lobes from mice at gestation day 15 were excised, freed of adhering tissue, and cultivated on nitrocellulose filters set in a well with 300 μl medium plus or minus 10 nM TCDD as described before (19−21). Control lobes were cultured in medium plus 0.1% solvent alone. At 4 or 5 days of culture, single-cell suspensions were prepared, and RNA was extracted and reverse transcribed. For analysis of thymocyte subpopulations, CD4−CD8−, CD4−CD8+, CD4+CD8−, and CD4+CD8+ thymocytes were sorted on a FACS440 cell sorter (Becton Dickinson, Mountain View, CA) after staining with anti-CD4 and anti-CD8 Abs (PharMingen, Hamburg, Germany).

RT-PCR assays

Semi quantitative RT-PCR assays were performed as previously described (15). Total RNA (1 μg) was used for cDNA synthesis with murine mammary tumor virus (MMTV) reverse transcriptase. [α-32P]-labeled CTP was added during RT-PCR. HPRRT (hypoxanthine phosphoribosyltransferase; Ref. 22), a housekeeping gene, was amplified together with the cDNA of IL-2 in the same reaction tube. To analyze the amplification of both cDNAs in the linear range, the “primer-dropping” method (23) was used to calibrate for the different expression levels of HPRRT vs IL-2 cDNAs. Briefly, the cDNA for the IL-2 test were first amplified for several cycles (depending on their previously established abundance), and then HPRRT primers were dropped into the reaction tubes followed by an additional 25 cycles. PCR products were separated on 10% polyacrylamide gels. Gels were dried and visualized by autoradiography. The primer sequences, the annealing temperature, and cycles for RT-PCR are shown in Table I. The autoradiographs were analyzed with an OmniMedia scanner and the whole-band quantification was performed on autoradiographs in the linear range of radioactivity to photosensitivity. PCR experiments for each gene expression were performed at least two times with similar results; for data presentation, one representative experiment is shown.

EMSA

Complementary single-stranded oligonucleotides were synthesized, annealed, and end-labeled with [γ-32P]-ATP. Sequences of oligonucleotides are shown in Table II. Nuclear and cytoplasmic proteins from an AhRhomo mouse thymic epithelial cell line, ET (a gift from Ronald Palacios, Basel University of Immunology, Basel, Switzerland; Ref. 24), were prepared (25) and EMSA were essentially performed as described (26). Briefly, 15 μg of nuclear proteins were incubated with 30,000 cpm end-labeled double-stranded oligonucleotides for 20 min in a final volume of 15 μl. Binding reactions were conducted in 25 mM HEPES, pH 7.5; 1 mM EDTA; 0.7 mM DTT; 0.5 mM PMSF; 10% glycerol; and 156 mM KCl. Samples were preincubated for 10 min in the presence of 1 μg poly dI:dC(1:10) and 0.5 μg salmon sperm DNA. A 200-fold excess of unlabeled oligonucleotides was used in specific and nonspecific competition experiments. AhR-DRE complexes were separated on 4% (w/v) polyacrylamide gels in TGE buffer (50 nM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5). Gels were transferred to Whatman 3 MM paper (Bio-Rad, Munich, Germany), dried, and autoradiographed. For supershift analyses, the nuclear protein extract and labeled oligonucleotide were incubated with 1 or 3 μl of anti-AHR Ab (Dianova, Hamburg, Germany) for 10 min at room temperature before gel electrophoresis.

Plasmid constructs, transfection, and luciferase assays

The plasmid constructs used in this study were as follows: pGudLuc 1.0, a reporter plasmid containing the firefly luciferase gene under the control of the MMTV promoter; pGudLuc 1.1, produced by cloning five DREs (−1301 to −819) isolated from the CYP1A1 promoter of mouse into the MMTV promoter of pGudLuc 1.0 (both plasmids were gifts from Michael Denison, University of California at Davis; Ref. 29) and used as positive control in all assays; pMTV-IL-2-DRE(0.5), produced by cloning the Espl−3−SpI fragment spanning −1296 to −756 of the 5′ region of the IL-2 gene and containing the three DREs into the BgII site within the MMTV promoter of the pGudLuc 1.0 vector; pDRE(0.5)MutIII and pDRE(0.5)MutIII, in these plasmids, point mutations of the DREIII or only DREIII had been introduced into the core recognition sites of pMTV-IL-2-DRE(0.5); pDRE I, pDRE II, and pDRE III, complementary single-stranded oligonucleotides (50 bases) encoding two copies of each DRE of IL-2 promoter and were synthesized, annealed, and cloned into the BgIII site of pGudLuc 1.0 (Table III). The DNA sequences of the oligonucleotides are shown in Table III (the core recognition sequence for the DRE motifs is in bold print); pSV-IL-2-DRE(0.5) and pTK-IL-2-DRE(0.5), the IL-2 fragment −1296 to −756 was cloned into the pG3PL vector in front of the SV40 promoter or TK promoter (Promega, Madison, WI), respectively. pSV-IL-2-DRE(0.1), the BstEII to NsiI fragment (400 bp) between DRE I and DRE II of the IL-2 5′ region, was deleted from pSV-IL-2-DRE(0.5). All plasmids were examined by sequencing.

Expression of AHR and its dimerization partner ARNT, both necessary for TCDD-mediated gene transcription, was verified by RT-PCR in the human HepG2 hepatoma cells and in mouse lines Hepa C1, Hepa C12, and Hepa C4 (gifts from Oliver Hankinson, University of California at Los Angeles). Cells were grown in culture medium with 10% FCS. Cells (5 × 106) were cultured overnight in six-well plates. Cells were washed twice with PBS and transfected with 2 μg plasmid DNA (pGudLuc 1.0, pMTV-IL-2-DRE(0.5)), the point mutation derivatives thereof, pDRE I, pDRE II, pDRE III, pSV-IL-2-DRE(0.1), and pGudLuc 1.1. Transfections were performed with 8 μg transfectam (Promega) in 1 ml RPMI 1640. After 8 h, 4 ml complete culture medium were added. Cells were cultured for another 16 h before adding 10 nM TCDD or solvent alone. After 14−16 h, cells were lysed, and luciferase activity was measured in a luminometer (LP96 P-Luminometer; Berthold, Nashua, NH). Results were normalized to the amount of total protein in the cell lysate, measured by the Bradford assay (Bio-Rad).

Results

The TCDD-activated AHR binds to the DREs in the IL-2 distal promoter region

In previous studies we had shown that IL-2, TNF-α, and IL-1β mRNAs are inducible in a dose-dependent fashion by TCDD in

Table I. Oligonucleotide primers used for gene expression analysis by RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>bp</th>
<th>T (°C)</th>
<th>Cycle</th>
<th>Reference</th>
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<tr>
<td>IL-2</td>
<td>5′-AGTCCAGACTGACGTCGATACT-3′</td>
<td>502</td>
<td>59</td>
<td>33−35</td>
<td>15</td>
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<td></td>
<td>5′-GGCTTGGTGATAGATGCCGTTCGA-3′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPRT</td>
<td>5′-GGGCCTGGAAGAACATGCTCT-3′</td>
<td>249</td>
<td>40</td>
<td>25−22</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>5′-CAGCAAGCAGTAGAAGCCCTGC-3′</td>
<td></td>
<td></td>
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</tbody>
</table>

*Annealing temperature.
thymocytes. Except TNF-α, these cytokines contain consensus sequence of DREs in the published upstream region of their respective genes (11). The IL-2 gene has three potential DRE sites at −1261, −851, and −815 bp. We decided to test the three DREs of the IL-2 distal promoter region for direct interaction and inducibility by the liganded AHR. Expression of AHR and its dimerization partner ARNT, both necessary for TCDD-mediated gene transactivation, was verified by RT-PCR in the ET thymic epithelial cell line (data not shown). Also, expression of CYP1A1, a known TCDD-responsive gene (3, 12), was inducible by TCDD in this cell line, indicating the functionality of the endogenous AHR and ARNT proteins (data not shown). Nuclear extracts of ET cells, containing the AHR/ARNT complex, did bind to labeled IL-2-DRE-oligonucleotides only when treated with TCDD but not with solvent alone (Fig. 1A). No specific AHR binding was observed with cytoplasmic protein, indicating that TCDD-dependent dimerization of AHR and ARNT takes place in the nucleus (Fig. 1B).

Specificity of binding was demonstrated by comparison experiments using unlabeled IL-2-DRE oligonucleotides or the DRE of CYP1A1 (Fig. 1A, lanes 3 and 4). Binding was not inhibited with oligo-DNA of an unrelated sequence (Fig. 1A, lane 5). Preincubation of the mixture of nuclear extracts and DREs with anti-AHR Abs led to the expected supershift of the complex (Fig. 1A, lanes 6 and 7). The three DREs differed in their binding capacity. In particular, the binding of DRE I was much weaker than that of DRE II and DRE III. This is in agreement with the deviation from the published consensus sequence 5′-T/GC/G/T CAGGC N C/A-3′ (14), which is three base pair for DRE I, but only two for DRE II and DRE III (see Table II). Still, the core sequence, to which the AHR/ARNT/TCDD complex makes physical contact (Fig. 2A), is unchanged in all three DREs. Note that DRE I binds to proteins of an unknown nature, albeit this complex is not shifted by TCDD treatment.

The IL-2 distal region contains enhancer elements responsive to TCDD

To test whether TCDD leads to transactivation of the AHR on the IL-2-DREs, we transiently transfected hepatoma cells with pMTV-IL-2-DRE, a reporter plasmid containing luciferase under the control of the −1296 to −756 region of the 5′ IL-2 gene. We then measured the induction of luciferase activity (Fig. 2). Hepatoma cells were used because of unresolvable technical difficulties in efficiently transfecting primary T cells or thymocytes; T cell hybridomas lost AHR expression during prolonged cultivation. We observed that this distal IL-2 promoter region induced expression of luciferase in the presence of TCDD. The induction of transcription increased ~13 times compared with the background. In comparison, transcription from pGudluc 1.1 (29), which contains five DREs from CYP1A1, a gene highly sensitive to TCDD, was induced ~20-fold (Fig. 2A). Similar results were obtained when using vectors containing either an SV40 or tk minimal promoter. These vectors yielded different backgrounds, yet always easily detectable TCDD induced luciferase expression (Fig. 2C). Thus, the far distal region of the IL-2 promoter contains DNA sequences highly responsive to TCDD. We then introduced single point mutations into the core recognition sites of the DREs II and III in the pMVT-IL-2-DRE(0.5) plasmid, transfected cells, and measured luciferase activity. When both DRE II and III sites are mutated the luciferase induction by TCDD is abrogated. A mutation in the DRE III only reduced the response but did not completely abrogate it (Fig. 2A). Thus, the DREs are necessary elements and both elements are required for a full response (Fig. 2). There is no other, hidden AHR-responsive element in the 500-bp distal enhancer segment.

However, as shown in Fig. 3, the distal IL-2 promoter region has possible AP-1 binding sites, but they have not been demonstrated to be used for the IL-2 enhancer/promoter. It is known that the liganded AHR may induce c-jun and c-fos, creating AP-1 (30). To supply further evidence that DREs are responsible for the IL-2 induction, we cloned each of the three DREs separately into the pGudluc vector (see Fig. 2B), and tested them for promoter activity by TCDD in HepG2 cells. The results shown in Fig. 2B demonstrate that each of the three isolated IL-2-DREs can drive luciferase transcription in the presence of TCDD, demonstrating the transcription-enhancing capacity of these small DNA elements. The same results were obtained with pSV-IL-2DRE(0.1) in which the putative AP-1 sites between DRE I and DRE II are deleted (Fig. 2C, Table IV).

Again, DRE I exhibits a distinctly weaker response, which agrees with the results obtained from the gel shift assay. Thus, at least DRE II and III do not need the context of other sequences of the IL-2 distal region to be functional as promoter elements.

To verify that the induction is AHR dependent, we performed the same assay in AHR- or ARNT-deficient cell lines Hepa C12 and Hepa C4, and wild-type Hepa C1. The results shown in Table IV demonstrate that functional AHR as well as ARNT are necessary for induction driven by IL-2-DREs. Also, in AHR-deficient mice, IL-2 was not induced by TCDD (see below).

Induction of IL-2 mRNA by TCDD depends on the differentiation stage of T cells

Most eukaryotic promoters have various modules that cooperate adaptively to generate differentiation- and situation-specific responses. Consequently, modules that are inducible in vitro assays could be not accessible or not relevant in vivo. Therefore, we tested induction of IL-2 by TCDD in several maturation stages of

Table II. Oligonucleotides used in bandshift assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 DRE I</td>
<td>5′-TTCTTACTCCTCATCAGCCAAGCCAGTG-3′</td>
<td>11</td>
</tr>
<tr>
<td>IL-2 DRE II</td>
<td>5′-CAGCCATATAACAGCCACATAAAAC-3′</td>
<td>11</td>
</tr>
<tr>
<td>IL-2 DRE III</td>
<td>5′-TGTATGTGCAGGGTTACAGTTGATG-3′</td>
<td>11</td>
</tr>
<tr>
<td>Human CYP1A1 (hDRE12)</td>
<td>5′-GAATATGCAAATACGGTG-3′</td>
<td>27</td>
</tr>
<tr>
<td>NS-4</td>
<td>3′-GATCTCCTGAGTCCTCAGGCCAT-5′</td>
<td>28</td>
</tr>
</tbody>
</table>

* DRE consensus sequences are determined as 5′-TGC G/T CAGGC N C/A-3′. Bold letters designated the actual CAGGC AHR contact site, underlined letters deviate from the consensus sequence (14). For mutation experiments, the last C in CAGGC was changed to a T.
T cells and in response to stimulation of the TCR. All cells described here could respond to TCDD with CYP1A1 induction, which indicates a functional AHR/ARNT system (data not shown). In a first series of experiments, mice were injected with 50 μg TCDD, and their thymocytes were analyzed 3, 6, or 8 days later by RT-PCR. We observed an IL-2 mRNA induction in vivo, suggesting that the DREs in the IL-2 promoter can be addressed (Fig. 4A). To assess whether IL-2 induction is dependent on the differentiation stage of cells, we next set up fetal thymus organ cultures (FTOCs). Fetal thymi were excised and exposed to TCDD for 4 or 5 days. Thymocytes were isolated and sorted to high purity by CD4/CD8 expression for very immature (CD4<sup>−</sup>CD8<sup>−</sup>), immature (CD4<sup>+</sup>CD8<sup>+</sup>), prospective helper T cells (CD4<sup>+</sup>CD8<sup>−</sup>), and prospective cytotoxic T cells (CD4<sup>−</sup>CD8<sup>+</sup>). IL-2 mRNA induction was measured by RT-PCR. As shown in Fig. 4B, TCDD increased IL-2 mRNA in total thymocytes, confirming previously published results (15). The overall increase was attributable to the immature CD4<sup>−</sup>CD8<sup>−</sup> cells and future cytotoxic CD4<sup>−</sup>CD8<sup>+</sup> cells, both of which responded to TCDD exposure with a strong (>3- and 5-fold) increase of IL-2 mRNA. CD4<sup>+</sup>CD8<sup>−</sup> cells displayed a constitutive expression of IL-2. However, they also increased IL-2 mRNA by TCDD. CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, which constitute the majority of thymocytes, remained refractory to TCDD. Moreover, IL-2 induction by TCDD could not be detected in spleen cells, a source of naive, fully mature T cells (data not shown). Thus, IL-2 induction by TCDD is dependent on the maturation stage of thymocytes/T cells, and the responsiveness of the promoter can be switched on and off during maturation of T cells.

**TCDD is not sufficient for IL-2 induction in spleen cells but can be complemented by anti-CD3ε signaling**

Induction of IL-2 in T cells classically requires two signals, one from the TCR, and another from the surface receptor CD28. Their dual triggering leads to a cascade of events, eventually generating and activating components like c-jun and c-fos complex (AP-1), NFAT, and NF-κB, which transactivate the promoter (2). Because TCDD alone was not sufficient to induce IL-2 production in mature splenic T cells, we stimulated thymus and spleen cells in six-well plates with anti-CD3ε Ab, mimicking TCR triggering. Like spleen cells from in vivo exposed mice, spleen cells exposed in vitro could not be induced by TCDD alone (Fig. 5A). IL-2 mRNA became inducible by TCDD in spleen cells only upon cross-linking with anti-CD3ε Ab. Induction was consistently about 4 times over the untreated controls, but there was a threshold of 100 ng of anti-CD3ε necessary for the stimulation. The induction of IL-2 mRNA was readily detectable within 4 h, i.e., no cell division was

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**Table III. Sequence of pIL-2 DREs I, II, and III**

| DRE I | 5′-GATCAGCTCATCACGAGGACGACGTGGAGATCAGTCTTGCATACGAGCCAGCAGTGA-3′ |
| DRE II | 3′-TGAGGAGTGGCGTCGGTACCTTACGGGTTAATGCGGGTCGGTCATCTCAG-5′ |
| DRE III | 3′-TATATGCTGGCGTTAGTTGCAGATAGTGCAGGGTCGGTTAGGCT-5′ |

**Figure 1. Detection of binding activity of the AHR/ARNT-complex to IL-2-DREs.** ET were treated with 10 nM TCDD or 0.1% 1,4-dioxan as a solvent control for 1 h. Nuclear extracts (A) and cytoplasm extracts (B) were prepared, and EMSA with radioactive labeled IL-2-DRE I, II, and III were performed. A, Lanes: 1, solvent control; 2–7, TCDD treatment; 3, competition with 200-fold excess of unlabeled DRE from the murine Cyp1A1 promoter; 4, competition with human DREI2; 5, competition with NS-4, as a nonspecific competitor; 6–7, supershift with anti-AHR. The upper arrow indicates a supershift of the AHR/ARNT/DRE complex by the anti-AHR Ab, the middle arrow indicates the AHR/ARNT/DRE complex, and the lower arrow indicates free DREs. a, b, and c indicate unspecific binding. C, solvent control; T, 10 nM TCDD.
necessary. Also, in thymocytes, where IL-2 mRNA had been induced by TCDD alone in vivo (Fig. 4), additional signaling via the TCR augmented IL-2 mRNA in vitro (Fig. 5).

To address whether IL-2 mRNA induction leads to protein synthesis, we analyzed supernatants from anti-CD3-stimulated cultures by ELISA for IL-2 protein (Fig. 6). Exposure of the cells to TCDD increased IL-2 protein synthesis compared with the solvent controls, but a higher concentration of anti-CD3 costimulation than for mRNA induction was needed to detect this. The IL-2 protein production lagged several hours compared with IL-2 mRNA induction, suggesting new protein synthesis. At least for spleen cells, cell density during stimulation and age of mice seemed to be important parameters for the strength of the response, as IL-2 induction varied depending on culture conditions (data not shown).

The Ah receptor is necessary for IL-2 induction by TCDD in vivo

The reporter assays described above strongly suggested that the IL-2 promoter is activated via the TCDD-ligated AHR at its distal DREs. To rule out a gene-inducing mechanism of TCDD not involving the AHR, we injected AhR−/− mice with 50 μg TCDD/kg body weight. After 6 days, thymus cells were harvested and total RNA was isolated. IL-2 mRNA was measured by RT-PCR. As shown in Fig. 7, IL-2 mRNA induction by TCDD is detected in thymus cells from AhR+/− and AhR+/− but not in thymus cells from AhR−/− mice. Thus, the IL-2 mRNA induction by TCDD is mediated by the Ah receptor.

Discussion

In this paper we present evidence for functional DNA motifs in the distant IL-2 promoter, which confer inducibility by the liganded AHR in mouse T lineage cells. As ligand we used TCDD, which is considered to be one of the most potent man-made toxic substances. The AHR mediates acute and chronic biological effects of TCDD, including immunosuppression and changes in T cell development. However, the mechanisms for AHR-mediated pathological responses are not well understood. Targeting cytokines as central mediators of immune responses could be one such mechanism. Apart from these toxicological considerations, our study was aimed at understanding the role of the AHR in the complex treatment. Fold induction by TCDD is the ratio of the relative luciferase activities in TCDD- vs solvent-treated samples. Results are expressed as mean ± SEM of triplicate samples. Experiments were independently repeated two to six times. A representative result is shown. Luc: luciferase.
regulation of the IL-2 gene. IL-2 promoter activity is regulated by a number of elements, i.e., Oct-1, NFAT, NF-κB, and AP-1, which cooperate and thus confer the lineage specificity and external regulation of IL-2. Others and we had shown that IL-2 expression could be induced by TCDD, i.e., the AHR (15, 31, 32). Extending these findings, our analysis of IL-2 induction in subpopulations of thymocytes showed that TCDD can induce IL-2 mRNA in CD4^+ CD8^−, CD4^+ CD8^+, and CD4^− CD8^+ cells, but not in CD4^+ CD8^− cells. Some transcription factors for IL-2 expression, Oct-1, Sp-1, NF-κB, and cAMP response element binding protein (CREB), can be expressed or induced in thymocytes of all developmental stages. In contrast, two essential transcription factors, AP-1 and NFAT, are absent in CD4^+ CD8^− cells. IL-2 is shut off during this maturational phase and only becomes accessible again later on (33, 34). TCDD cannot override this complete inaccessibility. Note that in the same cells, CYP1A1 mRNA remained inducible by TCDD (data not shown).

In contrast to thymocytes, TCDD alone was not sufficient to induce IL-2 in naive T cells, but needed stimulation via the TCR as an additional signal. Again, this is in agreement with findings in the rat, where TCDD could increase serum levels of IL-2 only after synergistic injections of staphylococcus enterotoxin B injections (31). In contrast to thymus, most spleen T cells are resting and thus are unable to express IL-2. Stimulation via the TCR/CD3 pathway alone triggers the mitogen-activated protein kinase pathways (Raf-1, extracellular signal-related kinase). Together with CD28 costimulation, different pathways are activated and the signal cascades merge and eventually lead to AP-1 formation and IL-2 expression (35). Activation of the AHR can bypass the need for CD28 cosignaling, probably in that an alternative signal transduction pathway downstream of the TCR/CD3 is used. Apart from the direct transcriptional activation shown here, other possibilities of the AHR to interfere with the “normal” cell signaling exist. A recent study by Tian et al. (36) demonstrated physical interaction of the AHR with NF-κB, leading to mutual repression in their system. Upon exposure to TCDD, c-jun, c-fos, and c-Jun N-terminal kinase induction has been observed in hepatoma cell lines (37, 38) and in Jurkat cells (38). Thus, indirect up-regulation of IL-2 by TCDD via these factors is possible. However, we found that in thymocytes c-jun is not inducible by TCDD (data not shown), favoring the direct transcriptional activation of IL-2 in these cells. Thus, the AHR is not a strong transcription factor in itself, but rather a modulator that may need the correct cellular set-up for its function. Although it can act in thymocytes without coactivation, in peripheral T cells it needed additional stimuli, i.e., TCR/CD3 triggering. It is interesting to note that the AHR is particularly abundant in thymus epithelium and thymocytes (39), pointing to a physiological role in this tissue. Also, the fact that TCDD did not induce IL-2 mRNA in AHR-deficient mice clearly demonstrated that the AHR mediated this effect.

### Table IV. Control of luciferase expression by IL-2 distal sequences containing DREs

<table>
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<tr>
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<th>pIL-2-DRE (0.5)</th>
<th>pIL-2-DRE (0.1)</th>
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<tr>
<td><strong>wt/wt</strong></td>
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</tr>
<tr>
<td>C</td>
<td>70 ± 3^e</td>
<td>61 ± 7</td>
</tr>
<tr>
<td>T</td>
<td>110 ± 18^e</td>
<td>109 ± 16^e</td>
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<td>AHR deficient</td>
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</tr>
<tr>
<td>C</td>
<td>172 ± 10</td>
<td>165 ± 10</td>
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<tr>
<td>T</td>
<td>188 ± 26</td>
<td>183 ± 2</td>
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<tr>
<td>ARNT deficient</td>
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<tr>
<td>C</td>
<td>155 ± 23</td>
<td>166 ± 7</td>
</tr>
<tr>
<td>T</td>
<td>172 ± 7</td>
<td>167 ± 18</td>
</tr>
</tbody>
</table>

^a^ −756 to −1296 bp of distal IL-2 sequence, or same sequence with a deletion between DRE I and DRE II, for detailed plasmid description see Materials and Methods.

^b^ Wild-type (wt) and either AHR- or ARNT-deficient Hepa cell lines.

^c^ C, solvent control; T, TCDD treatment of cells.

^d^ Units (×1000) of luciferase expression counted in luminometer.

^e^ Data for control and TCDD treatment groups were evaluated by Student’s t test, p < 0.05 for bold numbers.

### Figure 4. IL-2 mRNA induction by TCDD in fetal thymocyte subpopulations and in adult thymus cells. A, C57BL/6 mice were injected i.p. with 50 µg TCDD/kg body weight or corn oil as solvent control. At the indicated days, IL-2 and HPRT mRNA from adult thymus cells was measured by RT-PCR (n = 2/day/treatment). B, Fetal thymus lobes of gestation day 15 were cultured in FTOC for 4 or 5 days with solvent (0.1% 1, 4-dioxan) or 10 nM TCDD. CD4^+ CD8^− (DN), CD4^+ CD8^+ (DP), CD4^+ CD8^− (CD4^+), and CD4^− CD8^+ (CD8^+) were sorted by FACS. IL-2 and HPRT expression in total thymus (FTOC for 4 days data) and in sorted subpopulations (FTOC for 5 days data) were measured by RT-PCR. Amplified mRNA of IL-2 and HPRT are indicated by arrows. Shown are autoradiographs and the calculated densitometric indices of IL-2 vs HPRT.
DNA motifs, which are targets for the AHR/ARNT complex, have been early identified, and a consensus sequence was inferred from a number of DREs in different genes and species. A core sequence within the consensus sequence, which is indispensable for AHR/ARNT contact, has been mapped (14). The DREs, which we identified in the upstream IL-2 region by computer analysis, were functionally tested with EMSA and a luciferase reporter gene assay. Band shift analysis showed that the TCDD-activated AHR binds to DRE II and III, and only very weakly to DRE I. Likewise, in the transfection assay, luciferase induction was higher with DRE II and DRE III than with DRE I. Most likely this reflects the similarity to the consensus sequence. DRE I differs from this functional consensus sequence in three base pairs, whereas DRE II and III have only two base pair deviations. Moreover, DRE II and III have bidirectional core sequences, which are reportedly particularly responsive to TCDD (14). It is less likely that the broader context of other DNA sequences is important, as our luciferase experiments with the bare DRE sequences imply. Mutation of the DREs abrogated the response, demonstrating that there is no other hidden DRE in the 500-bp distal region we analyzed. Also, both DRE II and III are needed for a full response. Even mutating just one of them stopped the induction of the reporter gene; only a small rest activity remained. Generally, very little is known about synergistic or antagonistic effects of spatial distribution of transcription factor binding elements, which may be crucial in promoter regulation.

Most functionally investigated DREs are located between −1200 and −800 bp in the upstream regions of the CYP1A1 from mice, rats, and humans (40–42). Interestingly, the IL-2-DREs were also located between −1200 and −800 bp in the upstream region, and many potential DREs in a number of genes are located in very distal positions (11). Possibly, the distance is functionally important, such that chromatin structure is changed and serves as a stable nucleation site for enhancer-promoter communication and chromatin remodeling, as has been suggested for other genes (43). Ward and colleagues have analyzed this by mapping of DNaseI hypersensitivity sites for the IL-2 promoter, albeit only until −730. They demonstrated the importance of upstream regions for chromatin remodeling and gene accessibility (44). It would be interesting to perform similar studies on the even further upstream region and to analyze remodeling under the influence of the AHR/ARNT complex in a tissue-specific setting. This could also help to understand data conceived from cat reporter assays in EL4E1 cells, indicating a negative regulatory role of the −700 to −1000 region (Fig. 3). In the context of AHR/ARNT activation, this negative regulation might be inverted. Also in humans a few DRE consensus sequences can be found in the very far 5′ region (e.g., at −7324, −3528, −3398, −2735, −2354); however, no functional data are available. The human and mouse distal IL-2 promoter have many features in common, but expression levels differed in the careful analysis of isolated sequences in chloramphenicol acetyltransferase (CAT) assays done by Novak et al. (2). The 500-bp stretch we analyzed here has only little homology to the human IL-2 upstream region (2). It is impossible to say at this time whether the human IL-2 promoter would also be responsive to AHR.

ARNT is the dimerization partner of the AHR; the complex forms in the nucleus, and both proteins participate in DNA binding.
IL-2 is not induced by TCDD in thymus cells from AhR−/− mice. AhR−/+ mice (C57BL6) and AhR+/+ and AhR−/− from inbred mice (B6,129 AhR−/− × C57BL6 AhR−/−) were injected with 50 μg TCDD/kg body weight or corn oil as control. After 6 days, IL-2 and HPRT mRNA expressions in the thymus were measured by RT-PCR. Shown are autoradiographs and the calculated densitometric indices of IL-2 vs HPRT.

Our data are consistent with this because TCDD cannot induce IL-2 expression in ARNT-deficient cell lines. Interestingly, when we searched for a suitable cell line for the transfection experiments, we could not find any T cell line that expresses high levels of AHR. In contrast, all tested T cell lines constitutively expressed ARNT. Thymocytes and thymic epithelium contained AHR in abundance. ARNT is another member of the bHLH-PAS family, and it can dimerize not only with AHR but also with yet other bHLH-PAS proteins, for instance, hypoxia-inducible factor I α (HIF-1α), a protein shown to be involved in organogenesis by response to oxygen pressure in tissues. Competition for ARNT in a cell may be another control mechanism for transcriptional regulation, i.e., ARNT is the central regulator in this protein family (5, 45).

Is there a physiological or pathological relevance for IL-2 induction by TCDD? TCDD causes thymus atrophy and immune suppression at low doses in many animal species (6–9). The finding that TCDD induces IL-2 production seems paradoxical, as IL-2 is an established growth and survival factor for T cells. However, more recently, the role of IL-2 in T lymphocyte death has been discovered. In mice genetically deficient for IL-2, autoimmune lymphoid cells accumulated due to a failure of activation-induced cell death (18, 46, 47). Also, in FTOC, the addition of exogenous lymphoid cells accumulated due to a failure of activation-induced cell death (18, 46, 47). Thus, it cannot be excluded that IL-2 induction by TCDD may have a physiological or pathological relevance for IL-2 expression or the induction of other genes results in immune suppression has to be investigated in further studies.

In summary, the TCDD-ligated AHR is capable of inducing transcription of IL-2 in mouse thymocytes and T cells. The natural ligand of the AHR is not yet known. We are tempted to speculate that the natural ligand is a player in IL-2 induction and thus important for cell homeostasis.

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