Aryl hydrocarbon receptor (AhR) nuclear translocator (Arnt) gene has been isolated and characterized from a mouse genomic DNA library. The gene is about 60 kilobases long and split into 22 exons. An unusual exon/intron junctional sequence was found in the 11th intron of the gene that begins with GC at its 5'-end. The exon/intron arrangement of mArnt gene differs greatly from those of the other members of the same basic-helix-loop-helix/PAS family. The gene is TATA-less and has several transcription start sites. The promoter region of the mArnt gene is GC-rich and contains a number of putative regulatory DNA sequences such as two GC-boxes, a CAMP-responsive element, E-box, AP-1 site, and CAAT-box. Deletion experiments revealed that all these DNA elements made substantial contributions to a high level of expression of the gene, except for the CAMP-responsive element. Of all, two GC-boxes displayed the most dominant enhancing effects. It was demonstrated that there exist specific factors binding to these DNA elements in the nuclear extracts of HeLa cells. Among them, Sp1 and Sp3, and CAAT-box binding factor-A were identified to bind the GC-boxes and CAAT-box, respectively. Expression of MyoD in HeLa cells stimulated the Arnt promoter activity by binding to the E-box.

Aryl hydrocarbon receptor (AhR) nuclear translocator (Arnt) is a member of the basic-helix-loop-helix/PAS family of heterodimeric transcription factors, which include AhR, hypoxia-inducible factor 1α (HIF-1α), Drosophila Tracheless (Trh), and single-minded protein (Sim) (1–5). Recent molecular cloning and biochemical studies have demonstrated that in the nucleus, Arnt forms a heterodimer with AhR that is activated by binding with a ligand such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, the most toxic chemical, although the target genes for the heterodimer in these effects remain to be elucidated (6). Arnt also forms a homodimer that binds the E-box and potentially activates the genes driven by the promoter containing the E-boxes (7). It has recently been reported that Arnt forms heterodimeric complexes with HIF-1α and HIF-1α-like factor to regulate genes involved in the response to hypoxic conditions (2), and disruption of the Arnt gene by homologous recombination resulted in embryonic death of mice because of abortive angiogenesis and defective responses to glucose and oxygen deprivation (8). Taken together, these results suggest that Arnt plays a central role in transcriptional regulation by the bHLH-PAS transcription factors as a key partner molecule in the formation of transcription/regulation-competent dimers. It would be interesting to elucidate the structure of the Arnt gene with regard to an evolutionary aspect of a gene family of the bHLH-PAS transcription factors and the regulatory mechanism of its transcription.

In this study, we have cloned and characterized the mouse Arnt gene, whose structure consists of 22 exons and is more complex than that of AhR, and elucidated multiple regulatory DNA elements in the promoter region and their trans-acting regulatory factors.

**EXPERIMENTAL PROCEDURES**

**Library Screening and Sequencing**—A 129/SV mouse genomic library (Stratagene) was screened by using a full-length mouse Arnt cDNA and its BamHI/EcoI fragment of mouse Arnt cDNA (about 0.8 kb) as hybridization probes as described (9). Positive clones were isolated and their sequence analyses were performed as described (9). Introns 4 and 8 that were not covered with the cloned DNAs were amplified with PCR by using the LA Taq kit (Takara) and two pairs of 35-mer primers that anneal to the 3'-ends of exons 4 and 8 and the 5'-ends of exons 5 and 10. Genomic DNA from the liver of C57BL/6J mice was used for PCR as templates, and the PCR products were subcloned into pBluescriptII SK(+) for sequencing.

**Determination of the 5'-End of the mArnt Gene**—Total RNA was prepared from C57BL/6J mouse skeletal muscle by the guanidinium method (10), and poly(A)⁺ RNA was purified with oligo(dT) latex (Takara). For RNase protection assay (11), a 296-base pair Ncol/AveI fragment of the genomic clone A18, which was subcloned into the SmaI site of pBluescript II SK(+) was used for synthesis of a riboprobe by using [α-³²P]CTP (800 Ci/mmol) and T7 RNA polymerase. The RNase protection experiments were carried out as described (12). The 5'-RACE was performed according to the protocol of Life Technologies, Inc. Poly(A)⁺ RNA was reverse-transcribed by using a gene-specific primer (Gsp1) complementary to the sequence of +243 to +267 of mArnt mRNA and SuperScriptII reverse transcriptase at 42 °C. The first strand mArnt cDNA was amplified by PCR using a poly-GI adaptor primer (AP) and a nested mArnt-specific primer (Gsp2) complementary to the sequence of +218 +242 of mArnt mRNA. The amplified products were subjected to gel electrophoresis, and the elongated band was subcloned into the SmaI site of pBluescript II SK(+) for sequencing.
with the dye terminator method (Applied Biosystems). The oligonucleotide primers used are given as follows in the 5’ to 3’ direction: AP, GGCAGGCGCTGACTAGTACGGGGHGGGGHG; Gsp1, TGACCGTGCGTATTAGGCCCTCTG; Gsp2, CACACAGTCTCTCCACCTTG; and GATCC.

Deletion Analysis of the mArnt Promoter—The 5’-flanking region (+12 to −1666) of the mArnt gene was inserted into the Smal site of the luciferase reporter plasmid (pGL3 Basic Vector) (Promega). Introduction of various deletions in the 5’-flanking region and transfection experiments using HeLa cells were performed as described (13, 14). The LacZ reporter gene pBos-LacZ (1 µg) was cotransfected with the luciferase reporter genes as an internal control, and the luciferase activities were normalized against the β-galactosidase activity.

DNase I Footprinting Analysis—A 329-base pair SacII/NdeI fragment (+12 to −317 of the mArnt gene) of the genomic clone mA18 was subcloned into the Smal site of pGL3-basic vector. The fragment was 32P-labeled at the XhoI and Asp718 site for the downstream and upstream end-labeling, respectively. Nuclear extracts from HeLa cells were prepared by the method of Schreiber et al. (15). A series of treatments for the DNase I footprinting analysis were performed as described (14).

Gel Mobility Shift Assay (GMSA)—The following double-stranded oligonucleotides were labeled with 32P-ATP by T4 polynucleotide kinase as probes: mAAGC2, 5’-GTTTGGGGCCTCTCTCCGC-3’; mAAGC1, 5’-GACCTACTGTCTTTATGCGGCGCTTC-3’; mAABC, 5’-GACTTTACTGTTCTTATGCGGCGCTTC-3’; mAAP-1, 5’-CCGAGTCACTAGCCCGCTCTAT-3’; mABox, 5’-ATTAGGGAAACGTGGTTGGC-3’; and mACRE, 5’-ATTTCAGTCGACTATACTTG-3’. mAABCm (5’-GATCCTACTGTTCTTATGCGGCGCTTC-3’), mAAP-1 (5’-CCGAGTCACTAGCCCGCTCTAT-3’), mABox (5’-ATTAGGGAAACGTGGTTGGC-3’), and mACREm (5’-ATTTCAGTCGACTATACTTG-3’) are mutated versions of the respective oligonucleotides as described above. All the substituted bases are indicated with small letters. Oligonucleotides of CRE derived from human collagenase gene (16), E-box from the e-subunit of acetylcholine receptor (17), and AP-1 site from the promoter of polyoma virus (18) used for competition assays are: CRE, 5’-gacacaggagagggCAGCT-3’; mAAGC2, 5’-GTTTGGGGCCTCTCTCCGC-3’; mAAGC1, 5’-GACCTACTGTCTTTATGCGGCGCTTC-3’; mAABC, 5’-GACTTTACTGTTCTTATGCGGCGCTTC-3’; mAAP-1, 5’-CCGAGTCACTAGCCCGCTCTAT-3’; mABox, 5’-ATTAGGGAAACGTGGTTGGC-3’; and mACRE, 5’-ATTTCAGTCGACTATACTTG-3’.

Site-directed Mutagenesis—The E-box in the mArnt promoter was mutated in a site-directed manner by the method of splicing by overlap extension (20). The mABox1 and mABox2 were used as primers for introducing the mutagenesis.

FIG. 1. Restriction maps and structural organization of the mouse Arnt gene. For the genomic structure, solid boxes represent coding regions, and open boxes represent the 5’- and 3’-untranslated regions. For cDNA structure, open boxes represent the coding regions, solid lines indicate the 5’- and 3’-untranslated regions, and the number in each box indicates the exon number counted from the 5’-terminus. The 5th exon is an alternative exon marked by an asterisk. The regions of the genomic DNA that are contained in the five mouse genomic DNA clones (mA18, mA9, mA3, mA8, and mA16) are shown at the top. Gaps between isolated genomic DNAs were linked by the PCR method and are indicated by broken lines. The characteristic structural motifs of mArnt are indicated at the bottom. The cleavage sites for the restriction enzyme are indicated by HindIII (H), BamHI (B), EcoRI (E), and EagI.

FIG. 2. Ribonuclease protection mapping of the 5’-end of the Arnt gene. Total RNA of 40 and 60 µg from mouse skeletal muscle cells were used in lanes 5 and 7, respectively. The pBluescript II SK(+) sequence ladder was used as size marker (lanes 1–4). Yeast tRNA (50 µg) was used as control (lane 6). The undigested riboprobe was in lane 8. Arrowheads indicate the sizes of the protected RNA fragments.

Structure and Expression of the mArnt Gene

RESULTS

Characterization of Mouse Arnt Genomic Clones—Sequence analysis of all the clones, mA3, mA8, mA9, mA18, and mA16 isolated from a SV/129 mouse genomic library showed that the Arnt gene is split into 22 exons. Because the mA3, mA8, and mA9 do not overlap with each other, we tried to fill in these
gaps by the PCR method. Unique PCR products of 7- and 4-kb were generated for the respective gaps, and sequence analysis of the bands revealed that the 7- and 4-kb DNA fragments overlapped with the parts of mA3 and mA8 and with those of mA8 and mA9, respectively (data not shown). Consequently, total length of the mArnt gene is about 60-kb long (Fig. 1). Exon 5 is an alternative exon and is omitted in a short form of the mArnt mRNA (3). The two forms of mArnt mRNA encode two Arnt proteins, which have not been known to differ with regard to function and mode of expression. To determine the 5'-end of the Arnt gene, the RNase protection assay was performed to yield three major protected fragments as shown in Fig. 2. Because RNA has a lower mobility in the denaturing polyacrylamide/urea gel than DNA of the same size, we confirmed the result by the 5'-RACE. Among 9 clones analyzed, 5, 3, and 1 started at positions +1, +7, and +9, which correspond to the 139, 133, and 131 nucleotide fragments of the RNase protection experiments, respectively, indicating that +1 is the major transcriptional start site of the Arnt gene. No TATA-like sequence was found in the 5'-flanking region of the 5'-end of the mArnt gene. Instead, the sequence of the promoter region is GC-rich as is often found in the TATA-less genes (Fig. 4).

All splice junctions have the GT/AG consensus except for intron 11 (data not shown), which begins with GC at the 5'-end and ends in AG. A few of the genes were reported to have this unusual GC sequence at the 5'-end of the intron boundary (21–25). The physiological role of this unusual junction sequence has yet to be established.

Exon/Intron Arrangement of bHLH/PAS Gene Family—When the sites of introns of the mArnt gene relative to the amino acid sequence is compared with those of the other members of the bHLH/PAS protein family such as mAhR, dSim, mHIF-1α and mArnt2,2 the Arnt gene shares few sites of splicing junctions with mAhR (Fig. 3), dSim, and mHIF-1α, but has much in common with mArnt2 (data not shown). Even in the gene structure coding for the most conserved bHLH/PAS region, the splicing junction sites of the mArnt gene barely resemble those of mAhR gene and others except for mArnt2 gene. From the similarity of the amino acid sequence, it has been proposed that the bHLH/PAS proteins are mainly divided into the two groups represented by AhR and Arnt, respectively. Because the genes for AhR, HIF-1α, and dSim share many of the splicing junction sites with one another (26), different gene organization between AhR and Arnt gene supports the two divisions of the bHLH/PAS transcription factors (27).

2 Y. Oda, K. Yamamoto, K. Sogawa, and Y. Fujii-Kuriyama, unpublished data.
Transcription Activity of mArnt Promoter—Although the mArnt promoter contains no TATA-box, sequence analysis of the promoter region has revealed several potential cis-acting DNA elements as shown in Fig. 4. To determine how these DNA elements contribute to the mArnt promoter activity, we constructed a fusion gene, pGL3mAPr (−1666), by ligating the promoter sequence (−1666 to −12) of the Arnt gene with the 5′-end of the luciferase gene in the pGL3-Basic vector and transfected it into several cultured cell lines, HeLa, Hepa-1, and 293T cells. Because HeLa cells gave the highest luciferase activity (280-fold over the control level) and are known to express endogenous Arnt mRNA, we chose to use HeLa cells for the transient transfection experiment. Then we introduced various external deletions from the 5′-end of the promoter sequence into the fusion gene and determined the expressed luciferase activity. As shown in Fig. 5, the deletions progressing through the −207 position caused gradual reduction in the promoter activity and finally showed no enhancement in the luciferase expression at the −29 position. The most pronounced reductions were noted when the sequence from −67 to −56 and from −57 to −29, which contain the GC-box1 and GC-box2, respectively (Fig. 4), were deleted. In the deletion process, the promoter activity was reduced concomitantly with the deletion of several DNA fragments, which contain the other putative enhancer elements: E-box (−207 to −139), AP-1 (−139 to −112), and CAAT-box (−112 to −86) sequences.

DNase I Footprinting Analysis of mArnt Promoter—to investigate whether there exist specific DNA binding factors on these regulatory sequences in the nuclear extracts of HeLa cells, DNase I footprinting analysis was performed. When increasing amounts of the nuclear extract were added, five regions from −31 to −62, −88 to −113, −116 to −140, −189 to −210, and −250 to −270 were distinctly protected from DNase I digestion (Fig. 6, A, lanes 3–6 and B, lanes 3–6). As described in Fig. 4, these regions contain the putative GC-boxes, CAAT-
The protected regions from DNase I are bracketed, and the putative regulatory elements are represented with thick bars. Numbering begins at the major transcription initiation site (see Fig. 2). A + G indicates the marker prepared by the Maxam-Gilbert method. A, the probe was incubated with nuclear extracts from HeLa cells (lane 3, 40 µg; lane 4, 30 µg; lane 5, 15 µg; and lane 6, 7 µg) or with buffer C (21) (lanes 1 and 2) and were treated with 0.08 (lane 1), 0.16 (lane 2), and 0.48 (lanes 3, 4, 5, and 6) units of DNase I. B, the probe was incubated with nuclear extracts from HeLa cells (lane 3, 7 µg; lane 4, 15 µg; lane 5, 30 µg; lane 6, 40 µg) or with buffer C (lanes 1 and 2) and were treated with 0.08 (lane 1), 0.16 (lane 2), and 0.48 (lanes 3, 4, 5, and 6) units of DNase I.

**FIG. 6.** DNase I footprinting analysis of the mArnt promoter. The protected regions from DNase I are bracketed, and the putative regulatory elements are represented with thick bars. Numbering begins at the major transcription initiation site (see Fig. 2). A + G indicates the marker prepared by the Maxam-Gilbert method. A, the probe was incubated with nuclear extracts from HeLa cells (lane 3, 40 µg; lane 4, 30 µg; lane 5, 15 µg; and lane 6, 7 µg) or with buffer C (21) (lanes 1 and 2) and were treated with 0.08 (lane 1), 0.16 (lane 2), and 0.48 (lanes 3, 4, 5, and 6) units of DNase I.

**DISCUSSION**

Using the 5’-RACE and RNase protection assay, we mapped transcription start sites of the mArnt gene to three major sites (Fig. 2). In consistence with multiple transcription start sites, we found no TATA sequence in their 5’-upstream proximity as also the case with the AhR gene. Multiple transcription start sites and GC-rich sequences in the promoter region are often reported with the “housekeeping” genes that have a TATA-less promoter (30, 31). Sequence analysis has shown that the Arnt gene is about 60-kb long and is split into 22 exons. The structure of the Arnt gene is very different from and more complex than that of the AhR gene. Even in the gene structure encoding the most conserved amino acid sequence of the bHLH/PAS domain, the exon/intron arrangement is different between the two genes. This conversion of the exon/intron arrangement has been already reported with several gene families and is suggested to be generated by insertion or deletion of introns during the evolutionary process (32). Thus far, several gene structures of the bHLH/PAS superfamily such as AhR, HIF-1α, HIF-1α-like factor, Drosophila Sim and Tracheless have been reported and their exon/intron arrangements are conserved well among them (4, 12, 26). From sequence similarity, the bHLH/PAS transcription factors are mainly divided into two groups represented by AhR and Arnt, respectively (27). The members described above resemble AhR in the amino acid sequence rather than Arnt and they are classified into the AhR group. Different gene structure of Arnt from those of the bHLH/PAS factors of the AhR group indicates that the gene structure has been
differentiated since the division of the two groups. Recently the gene structure of Arnt2, which belongs to the Arnt group, has been found to be highly similar to that of Arnt.

In the upstream region of this TATA-less gene, several interesting potential regulatory sequences such as GC-box, AP-1 site, CAAT-box, E-box, and CRE were found as shown in Fig. 4. Transient DNA transfection experiments using a fusion gene consisting of the upstream sequence of the Arnt gene and the luciferase structural gene demonstrated that the upstream sequence has transcriptional enhancer activity in HeLa cells. Various deletions in the upstream sequence could locate the regulatory DNA sequences whose deletions resulted in a marked reduction in the expressed luciferase activity. These DNA fragments contain the putative regulatory DNA elements described above, indicating that they contribute substantially to the expression of luciferase, except for the CRE whose deletion was apparently not very influential in this expression system. The deletion of one of the two GC-boxes displayed most profound effects on the expression driven by the Arnt promoter, suggesting that the two GC-boxes cooperatively enhance the expression of the gene. DNase I footprinting analysis demonstrated that the nuclear extracts specifically protected those presumed regulatory DNA elements from digesting by DNase I, therefore, indicating the presence of the factors binding to those DNA elements. Although it showed little promoter activity in the transient DNA transfection assay, the CRE sequence was also protected. The putative CRE sequence is a complete 8-base palindromic sequence and could function as a cis-acting regulatory DNA element in other types of cells. Gel mobility shift assay demonstrated the presence of specific factors binding to those regulatory DNA elements in HeLa cells. Experiments using anti-Sp1, anti-Sp3, and anti-CBF-A antibodies.
showed that the two GC-boxes were bound for major part by Sp1 and for minor by Sp3 and that the CAAT-box was bound by CBF-A. It remains to be specified what regulatory factors bind to the putative AP-1 site, CRE, and E-box sequences, because GMSAs using anti-c-Jun, MafK, CREB-1, E12, E47, c-Myc, and human bHLH factor antibodies did not give any supershifted bands. Furthermore, typical oligonucleotides of the AP-1 site and E-box sequences did not compete with probes of the corresponding regulatory sequences of Arnt in GMSA. It is likely that the sequences flanking with the core recognition sequences of Arnt is critical for binding to a nuclear factor of HeLa cells. Further studies are necessary to determine which factors binds these DNA elements including CRE for elucidating the mechanism of the Arnt gene expression.

The members of MyoD family are cell-type-specific trans-acting factors and play an important role in the expression of muscle-specific genes through binding to the E-box in their promoter regions (33, 34). It has been reported that the expression of mArnt mRNA is the highest in muscle among the tissues examined, although mArnt mRNA was expressed ubiquitously (35). Overexpression of MyoD by transfecting the MyoD expression plasmid into HeLa cells enhanced the expression of the luciferase activity driven by the mArnt promoter, suggesting that the Arnt expression is stimulated in the tissues such as muscle, which express MyoD.

Ubiquitous expression of Arnt in various tissues of embryos and adult animals can be generated by cooperative functions of multiple transcription factors bound on the respective regulatory DNA elements. These factors could be changed in different tissues. It would be interesting to identify these regulatory factors in various cultured cells and tissues and clarify how they interact with one another to enhance the expression of the gene.

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**FIG. 8.** Activation of the mArnt promoter activity by transfection with mouse MyoD expression plasmid. A, schematic representations of the expression plasmid for mouse MyoD and the reporter plasmids. B, relative luciferase activities of the mArnt promoters. Values are the means of three independent experiments normalized to α-galactosidase activity used as an internal control. Total DNA used for transfection was adjusted to 10 μg by adding pGL3-basic vector plasmid DNA.