J. Biochem. 128, 1087–1095 (2000)

A Novel *Cis*-Acting Element Regulates *HES-1* Gene Expression in P19 Embryonal Carcinoma Cells Treated with Retinoic Acid[1]

Nobunao Wakabayashi,*[2] Ryoichiro Kageyama,† Toshiyuki Habu,* Toshihide Doi,* Takashi Morita,*[3] Masami Nozaki,* Masayuki Yamamoto,‡[4] and Yoshitake Nishimune*

Department of Molecular Embryology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871; †Institute for Virus Research, Kyoto University, Kyoto 606-8507; and ‡Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577

Received August 15, 2000; accepted October 6, 2000

The regulatory mechanisms of mammalian *hairy* and *Enhancer of split* homologue-1 (*HES-1*) genes were examined in mouse P19 embryonic carcinoma cells (P19 cells). Undifferentiated P19 stem cells expressed a basal level of the *HES-1* gene, whereas the expression of this gene was increased upon induction of the cells to a neural cell lineage using retinoic acid (RA). Reporter co-transfection analysis identified an activating region within the upstream promoter region of *HES-1* from nucleotides −201 to −172. This activating region, called activating region X (ARX), shows a high GC content and contains both an AP-2 binding motif and a CCAAT box. An electrophoretic mobility shift assay using nuclear proteins extracted from P19 cells showed that ARX forms a specific DNA-protein complex. Importantly, ARX-dependent transcription, as well as ARX-binding activity, was significantly increased in P19 cells treated with RA. These results indicate that ARX transduces signals that up-regulate *HES-1* gene expression in response to RA-treatment. Thus, a novel *cis*-acting element involved in *HES-1* gene regulation that plays a role in RA-induced neural differentiation of P19 cells has been identified.

Key words: electrophoretic mobility shift assay, neural differentiation, retinoic acid, P19 embryonic carcinoma cells, transcription factor.

Transcription factors containing a helix-loop-helix (HLH) domain play a crucial role in cell differentiation (*1*, *2*). HES-1 is a member of the mammalian basic region–HLH (bHLH) factors that are homologous to the products of Drosophila *hairy* (*h*) and *Enhancer of split* [*E(spl)*] (*3–9*). The products of *h* and *E(spl)* are known to negatively regulate Drosophila neurogenesis (*10*, *11*). HES-1 is expressed in a variety of cells, including embryonic neural and mesodermal cells (*4*). In the developing nervous system, HES-1 is expressed throughout the ventricular zone where neural precursor cells are located. The expression of HES-1 decreases as neural cell differentiation proceeds and is absent from mature neurons and glial cells (*4*, *12*). This suggests that mouse HES-1 may also act as a negative regulator of neurogenesis, as in the case of the products of Drosophila *h* and *E(spl)*. A down-regulation of *HES-1* gene expression has been suggested to be necessary for precursor cells to undergo differentiation (*12*).

Unlike most bHLH transcription factors that activate transcription *via* the E-box (CANNTG) (*1*), HES-1 represses transcription by binding to the N-box (CACNAG). HES-1 is a unique transcription factor in that it has two different functions. One role is the repression of target gene transcription in a binding site–dependent manner; HES-1 competes with other activating transcription factors for heterodimeric partner molecules required for DNA binding. The other function of HES-1 involves its binding to co-repressor Groucho, whereby it acts as a dominant-negative molecule of target gene transcription (*4*). HES-1 is also known to down-regulate its own expression by binding directly to multiple N-boxes within its promoter (*8*). In addition, as is the case for the dominant-negative HLH protein Id (*13*), HES-1 antagonizes the activity of some bHLH transcription factors through the formation of an inactive heterodimeric complex (*4*). Thus, HES-1 exhibits intriguing functions as a transcription factor in early mammalian development, especially *via* the negative regulation of gene expression.

Recent studies have revealed that HES-1 plays an important role in neural development. A continuous expression of HES-1 blocks neural stem cell differentiation, while an HES-1-null mutation accelerates neuronal differentiation, resulting in severe defects in the morphogenesis of the brain and eye (*12*, *14*, *15*). Perturbation of *HES-1* gene regulation severely disrupts neural development, indicating a necessity for strict transcriptional control of *HES-1*. How-

[1] This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture; the Japanese Society for Promotion of Sciences (JSPS Research for the Future Project). NW was a research fellow of JSPS.

Present addresses: [2] Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577; [3] Department of Molecular Genetics, Osaka-city University Medical School, 1-3-4 Asahi-machi, Abeno, Osaka 545-8585.

[4] To whom correspondence should be addressed. Tel: +81-298-53-6158, Fax: +81-298-53-7318, E-mail: masi@tara.tsukuba.ac.jp

Abbreviations: ARX; activating region X of *HES-1* gene; bp, base pair(s); kb, kilo base.

ever, the mechanism of *HES-1* gene regulation during development is currently unknown. It has previously been proposed that the *HES-1* gene is under the control of the Notch signaling system (*16*), although it has been shown that gene expression does not change in the absence of Notch1 (*17*).

HES-1 plays an important role in the differentiation of P19 cells into neural and myogenic cells upon treatment with different concentrations of RA (*18, 19*). Therefore, P19 cells were chosen as a unique model system in which to study the transcriptional regulation of *HES-1* gene expression during the differentiation of mammalian neural progenitor cells. A reporter gene co-transfection analysis located an activating region, ARX, in the 5′ flanking region of the *HES-1* gene from nucleotide residue −201 to −172. ARX is essential for the RA-induced expression of *HES-1* and acts as a positive regulator of basal *HES-1* gene expression in undifferentiated P19 cells.

EXPERIMENTAL PROCEDURES

Cell Culture and RA Treatment—A monolayer of P19 cells was maintained in α-minimum essential medium (Sigma, USA) containing 10% fetal bovine serum (Hyclone, USA), and incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were treated with 5×10^{-7} M RA (all-trans; Sigma, USA) 12 h after being transferred to a culture dish. Cells were harvested after 24 and 48 h of incubation, and the RNA was extracted. Under these culture conditions, RA did not produce significant alterations in the doubling time of P19 cells or total RNA content per cell.

Isolation of Total RNA and RNA Blot Analysis—Total RNA was prepared from P19 cells by the method of Chomczynski *et al.* (*20*). Twenty micrograms of total RNA was subjected to electrophoresis in a formamide/1.2% agarose gel and transferred to a nylon membrane filter (Hybond N+, Amersham). A *Xho*I fragment containing the complete open reading frame (ORF) of Rat HES-1 cDNA in a pLXSG-HES-1 expression vector (*12*) was radiolabelled with a random primer labelling kit (TAKARA, Osaka) and used as a probe. The filter was hybridized overnight with the probe at 42°C in 50% formamide, 4× SSC, 5× Denhardt's reagent, 0.2% SDS, and 100 μg/ml of heat-denatured salmon sperm DNA. The filter was washed in 1× SSC containing 5% SDS, first at room temperature for 10 min and then at 57.5°C for 1.5 h, and then washed in 0.1× SSC containing 0.5% SDS at 42°C for 0.5 h. The filter was exposed overnight to X-ray film (XAR-5, Kodak) at −80°C using an intensifying screen.

Probes for other markers of differentiation were hybridized as described above, except for the MAP-2 probe (*21*). The filter hybridized with the MAP-2 probe was washed in 2× SSC containing 1% SDS at room temperature for 20 min, in 1× SSC containing 0.5% SDS at 53°C for 1 h, and in 0.5× SSC containing 0.25% SDS at room temperature for 20 min. The intensity of radioactivity was measured with an NIH Image computer software program.

Primer Extension—The −115 to +163 bp region of the *HES-1* gene was isolated from the genomic DNA of a C57BL/6 mouse using the nested-PCR method. The fragment was subcloned into pBluescript II (Stratagene), and the sequence was confirmed. T4 polynucleotide kinase was used to label the 5′ end of the oligonucleotide 5′-GCTTAAGCTTACGTCCTTTTACTTGACTTTC-3′ before hybridization, at 52°C for 2 h with 20 μg of total RNA extracted from cultured P19 cells. Following reverse transcription of the mixture, the primer extension products were subjected to electrophoresis in a 7 M urea/6% polyacrylamide gel.

CAT Reporter Constructs prepared from Various Lengths of the 5′ Flanking Region of the HES-1 Gene—The CAT reporter construct, p-2000CAT, which includes base pairs −2000 to +46 of the 5′-flanking region of the mouse *HES-1* gene, was digested with *Bam*HI, and deletions were made by the *Exo*III-*S1* nuclease deletion method. The 5′-end was filled in and the plasmid was digested with *Eco*RI. Fragments consisting of various lengths of the 5′-flanking region of the mouse *HES-1* gene were subcloned into pUC118 at the *Sma*I and *Eco*RI sites. After sequence confirmation, the fragments were cloned into pEMBL CAT at the *Bam*HI and *Eco*RI sites. Thus, each CAT reporter insert contained the region +1 to +46 bp, in addition to an upstream region of the mouse *HES-1* gene.

For each luciferase reporter gene, the ARX oligonucleotides (shown in Table I) were ligated with *Bam*HI linker and subcloned into pBluescript II for the purposes of sequence verification. The ARX inserts were then digested with *Sac*I and *Xho*I and ligated into pGV-TK-Luci vector, which contains the herpes simplex virus–thymidine kinase (TK) promoter region (*22, 23*) upstream of the firefly luciferase cDNA. For transfection analyses, plasmids were

TABLE I. **Mutant oligonucleotides of ARX.**

Oligonucleotide	Sequence
ARX	5′- G C C C C G G G C T C A G G C G C G C G C C A T T G G C C G -3′ 3′- C G G G G C C C C G A G T C C G C G C G C G G T A A C C G G C -5′
A1	5′- G C C C C G G G C T C A G G C G C G C G A C A T T G G T C G -3′ 3′- C G G G G C C C C G A G T C C G C G C G C T G T A A C C A G C -5′
A2	5′- G C C C C G G G C T C A G G C G C G C T A C A T T G G T C G -3′ 3′- C G G G G C C C C G A G T C C G C G C G A T G T A A C C A G C -5′
C1	5′- G C C C C G G G C T C A G G C G C G C G C C T T A G G C C G -3′ 3′- C G G G G C C C C G A G T C C G C G C G C G G A A T C C G G C -5′
AC1	5′- G C C C C G G G C T C A G G C G C G C G A C A T T T G C C G -3′ 3′- C G G G G C C C C G A G T C C G C G C G C T G T A A A C G G C -5′
AC2	5′- G C C C C G G G C T C A G G C G C G C G C C A T A G A C C G -3′ 3′- C G G G G C C C C G A G T C C G C G C G C G G T A T C T G G C -5′

The CCAAT and AP-2 consensus sequences are underlined and boxed, respectively. Mutations in ARX are indicated by asterisks.

TABLE II. **Oligonucleotides associating with CCAAT box–binding transcription factors or AP-2.**

Oligonucleotide	Sequence
Eα	5′- G C A C T C A A C T T T T A A C C A A T C A G A A A A A T G -3′
	3′- C G T G A G T T G A A A A T T G G T T A G T C T T T T T A C -5′
H1TF2	5′- A G G T G A T G C A C C A A T C A C A G C G C G C C C T A C -3′
	3′- T C C A C T A C G T G G T T A G T G T C G C G C G G G A T G -5′
α-Globin	5′- C G G G C T C C G C G C C A G C C A A T G A G C G C C G C C -3′
	3′- G C C C G A G G C G C G G T C G G T T A C T C G C G G C G G -5′
CP2	5′- G A T C C C A A G T T T T A C T C G G T A G A G C A A G C A C A A A C C A G G -3′
	3′- C T A G G G T T C A A A A T G A G C C A T C T C G T T C G T G T T T G G T C C -5′
hMTII$_A$AP-2	5′- C A G A A C T G A C C G C C C G C G G C C C G T G T G C A G -3′
	3′- G T C T T G A C T G G C G G G C G C C G G G C A C A C G T C -5′

The CCAAT and AP-2 consensus sequences are underlined and boxed, respectively.

purified twice by CsCl density gradient ultra-centrifugation. Ethidium bromide was extracted using an equal volume of water-saturated 1-butanol and CsCl was removed from the plasmid solution by dialysis against TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 24 h.

Transient Transfection and Reporter Assay—P19 cells were plated at a density of 2.0×10^5 cells/ϕ 60 mm dish 8 h before DNA transfection. An equimolar concentration of the DNA of each construct (4.3 pmol of each reporter gene and 2 μg of pSRgal, a normalizing vector) was introduced into the P19 cells by calcium phosphate co-precipitation (*24*). Transfections were repeated three to six times using independent plasmid DNA preparations. To standardize the transfection efficiency, 2 μg of pSRgal plasmid containing the β-galactosidase gene under the control of the RSV-LTR promoter/enhancer was included in each transfection. pBR-322 plasmid DNA was used as a carrier to adjust the total amount of DNA. Cells were harvested 24 h after transfection and the enzyme activities of the cellular extracts were measured using standard procedures (*25*). In the CAT assays, the radioactivities of the reacted products were quantified with a Bioimage Analyzer BAS 2000 (Fuji Film, Tokyo). Luciferase activity was measured according to the manufacturer's instructions (Pica Gene: Toyo Ink, Tokyo). The enzyme activity of each sample was normalized to the β-galactosidase activity (*26*).

Analysis of DNA-Nuclear Factor Binding—Nuclear extracts were prepared from P19 cells that were either treated or untreated with RA according to Screiber *et al.* (*27*), and protein concentrations were determined with a BioRad protein assay using IgG as a standard. Probes were prepared by end labelling 20 ng of primary strand oligonucleotide with [γ-^{32}P]ATP using T4 polynucleotide kinase, and then annealed to the complementary oligonucleotide. In the standard electrophoretic mobility shift assay (EMSA), 25 μl of reaction mixture containing approximately 2×10^4 cpm of nucleotide probe was incubated at 25°C for 30 min with 5 μg of nuclear extract in 10 mM HEPES, pH 7.9, 25 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg poly(dI-dC), 0.05% Nonidet P-40, and 10% glycerol. In the competition experiments, each competing oligonucleotide (as shown in Tables I and II) at a 10- or 50-fold molar excess to the probe was added to the standard EMSA reaction mixture, and the reaction products were loaded onto a 5.0% polyacrylamide gel. All EMSA experiments were repeated three to five times, using nuclear extracts from P19 cells either treated or untreated with RA. To quantify the radioactivity within the shifting bands, the gel was dried and exposed to either X-ray film or a BAS imaging plate (Fuji Film). In the supershift assay, 2 μg of AP-2 antibody (TransCruz; Gel supershift grade) was added to the samples and all reaction mixtures were incubated at 25°C for 30 min. Anti–AP-2 antibody (C-18), an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide, was from Santa Cruz Biotechnology.

RESULTS

Expression of HES-1 mRNA in P19 Cells—To elucidate *HES-1* gene regulation in P19 embryonic carcinoma cells, RNA blotting was used to examine the cellular expression of *HES-1* (Fig. 1A). A basal level of HES-1 mRNA expression was detected in undifferentiated cells, with expression increasing by approximately 3-fold upon RA treatment (Fig. 1B; compare Stem with 24 and 48 h). This induction occurred concomitantly with an alteration in the levels of other differentiation markers (Fig. 1C): the expression of Oct-3 (*28*) decreased while that of tPA (*29, 30*) was induced. The expression of MAP-2 is known to commence upon the differentiation of neural precursor cells (*31, 32*). Indeed, in P19 cells, MAP-2 gene expression was initially detected 24 h after the addition of RA, with expression increasing thereafter (Fig. 1C). Thus, the expression of HES-1 is upregulated during the induction of P19 cells to neuronal progenitors by RA.

To clarify whether basal and inducible *HES-1* gene transcription utilize the same promoter, the transcriptional initiation site of the mouse *HES-1* gene was determined in a primer extension analysis. The results showed that the same initiation site is used in both undifferentiated and RA-induced P19 cells, and also in F9 and BALB3T3 cells (Fig. 2). The transcriptional initiation site coincides with that previously proposed for the HES-1 mRNA in mouse embryo (*8*). These results suggest that *HES-1* gene transcription occurs through a single transcriptional start site in various mouse tissues. Furthermore, the intensity of the HES-1 mRNA band for RA-treated P19 cells was 3-fold stronger than that for undifferentiated P19 cells (Fig. 2; compare 0 with 24 h). Thus, the increase in HES-1 mRNA upon RA treatment is most likely to result from the transcriptional activation of the *HES-1* gene.

Reporter Co-Transfection Transactivation Analysis of the HES-1 Gene—The activity of the *HES-1* gene regulatory region was analyzed by a reporter co-transfection and transactivation method. To this end, a series of CAT reporter constructs was prepared with various lengths of the 5′ flanking region of the mouse *HES-1* gene. The CAT repor-

ter plasmids contained up to nucleotide −2000 of the gene and these constructs were transfected into P19 cells. Deletion of the region from −2000 to −201 did not significantly affect the reporter activity in P19 cells (Fig. 3A). Further deletion to −172, however, reduced the CAT activity to approximately 50%. These results indicate that a positive *cis*-regulatory element exists in the −201 to −172 region. Accordingly, we named this region ARX (activating region X). Initial inspection of the sequence revealed that ARX has two interesting motifs; ARX has an inverted CCAAT box (Fig. 3B, −178 to −174, underlined), which overlaps with an AP-2 consensus motif (−181 to −173, circled) (*33*, *34*).

Since *HES-1* gene expression is induced by RA (Fig. 1), the transactivational activity of ARX was investigated in P19 cells, either with or without RA treatment. Two ARX-containing reporter constructs, −242CAT and −201CAT, showed approximately twofold higher reporter activity in RA-treated P19 cells than in untreated cells (Fig. 4). This increased CAT activity was in accordance with the enhancement in HES-1 mRNA expression by RA in P19 cells (see Figs. 1 and 2). In contrast, no such increase in CAT activity was observed in P19 cells transfected with −172CAT constructs lacking ARX. This indicates that ARX plays an indispensable role in the RA-mediated induction of *HES-1* gene expression.

The CAT reporter activity appeared to be lower in cells transfected with the −172CAT construct than in cells transfected with the ARX-containing constructs −242CAT and −201CAT. In fact, the activity of −172CAT was almost half that of the ARX-containing reporters (Fig. 4). These results indicate that ARX also acts as a positive regulatory region for basal level *HES-1* gene expression in undifferentiated P19 cells.

ARX Binds Transcription Factors in P19 Nuclear Extracts—To test whether ARX actually binds nuclear proteins present in P19 cells, EMSA was performed using ARX as a probe. When a labelled ARX probe was incubated with nuclear proteins extracted from P19 cells, two complex bands were detected (Fig. 5, lanes 1–4). In addition, a minor band with a higher mobility was observed, although

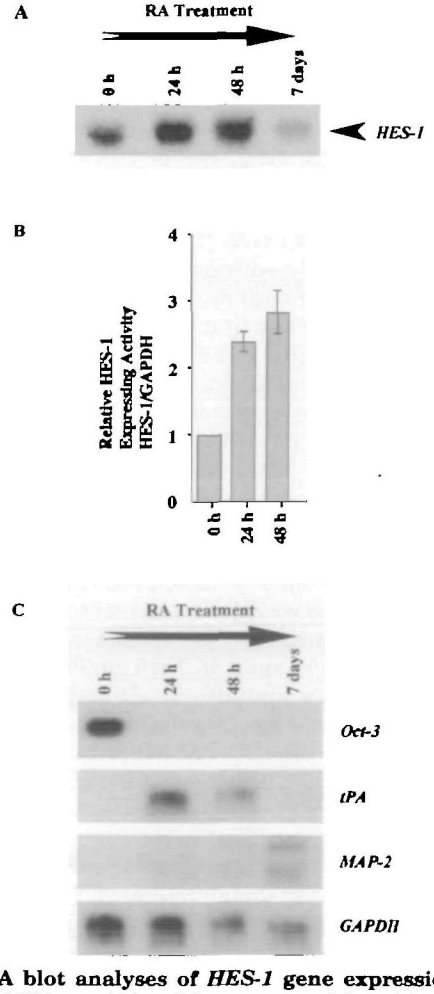

Fig. 1. **RNA blot analyses of *HES-1* gene expression in P19 cells.** Total RNA was isolated from untreated (stem) P19 cells and from cells treated with RA for 24 h or 48 h. A: Total RNA (20 μg per lane) was subjected to electrophoresis, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled rat HES-1 cDNA. B: The expression level of HES-1 mRNA was quantified using a phosphoimager. C: The membrane in A was re-hybridized with ^{32}P-labelled mouse Oct-3, tPA, and MAP-2, and human GAPDH cDNA probes.

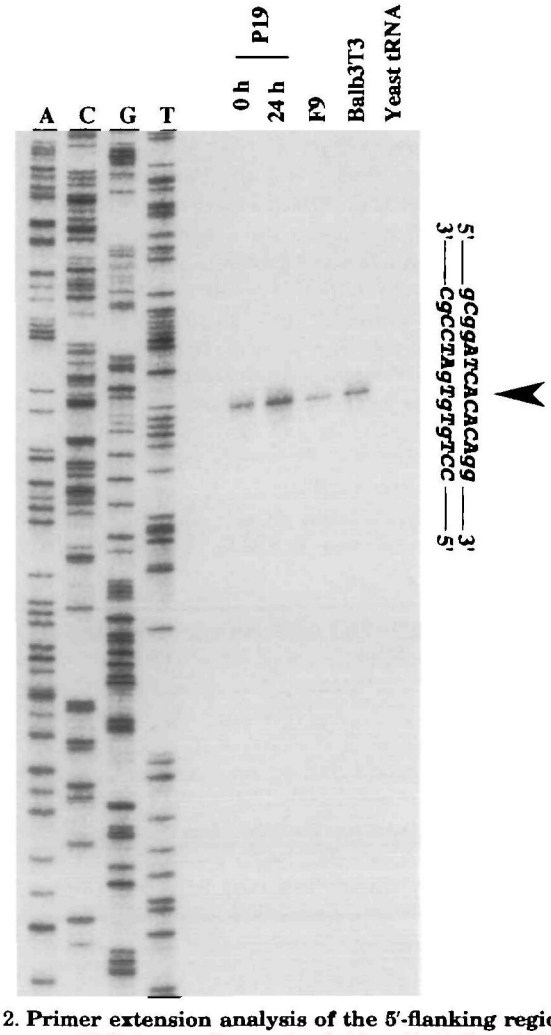

Fig. 2. **Primer extension analysis of the 5′-flanking region of the mouse *HES-1* gene.** Sequencing ladders (A, C, G, and T) were used as molecular size markers. Thirty one-mer complementary oligonucleotides from the region +168 to +137 of the mouse *HES-1* gene were used as primers. The arrowhead indicates the position of the transcriptional initiation site.

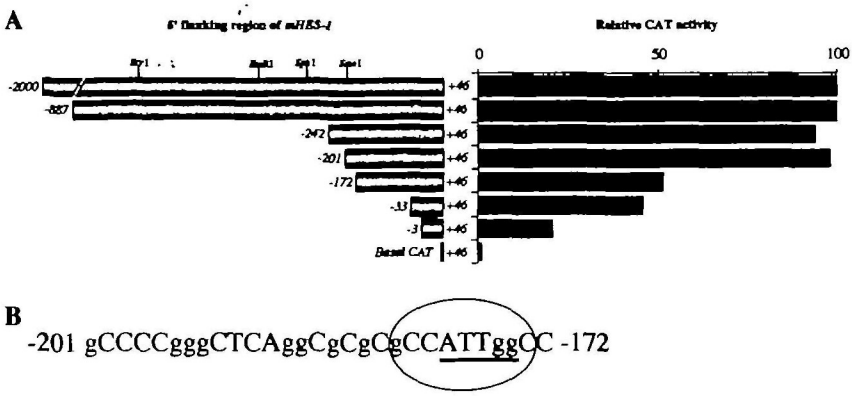

Fig. 3. **Effect of deletions within the *cis*-element on the promoter activity of the mouse *HES-1* gene.** A (left side): CAT reporter plasmids fused with various lengths of the upstream region of the mouse *HES-1* gene were transfected into P19 cells. The 5'- and 3'-end positions of the mouse *HES-1* promoter are labelled. 3A (right side): The CAT activity of the reporter plasmid containing the region between −2000 and +46 was normalized to 100, and the activities of the other plasmids were calculated relative to this value. Each value represents the mean of at least six independent experiments. 3B: The sequence of ARX, an effective up-regulator of *HES-1* gene expression in P19 cells. The AP-2 consensus sequence is outlined by the oval. The complementary CCAAT box is underlined.

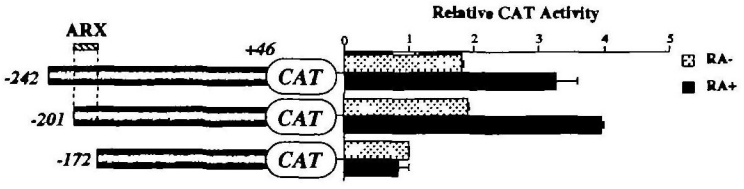

Fig. 4. **Comparison of the promoter activities from P19 cells either treated or untreated with RA.** Reporter plasmids containing three different lengths of the 5'-upstream region of the HES-1 gene (−242CAT, −202CAT, and −172CAT) were transfected into P19 cells. Transfected cells were either left untreated or treated with 5×10^{-7} M RA for 24 h. The CAT activity of the plasmid carrying −172CAT in untreated cells was normalized to 1, and the activities of the other plasmids were calculated relative to this value ±SEM. Each relative CAT activity represents the mean of at least three independent experiments. Untreated and RA-treated P19 cells are represented by dotted and black boxes, respectively.

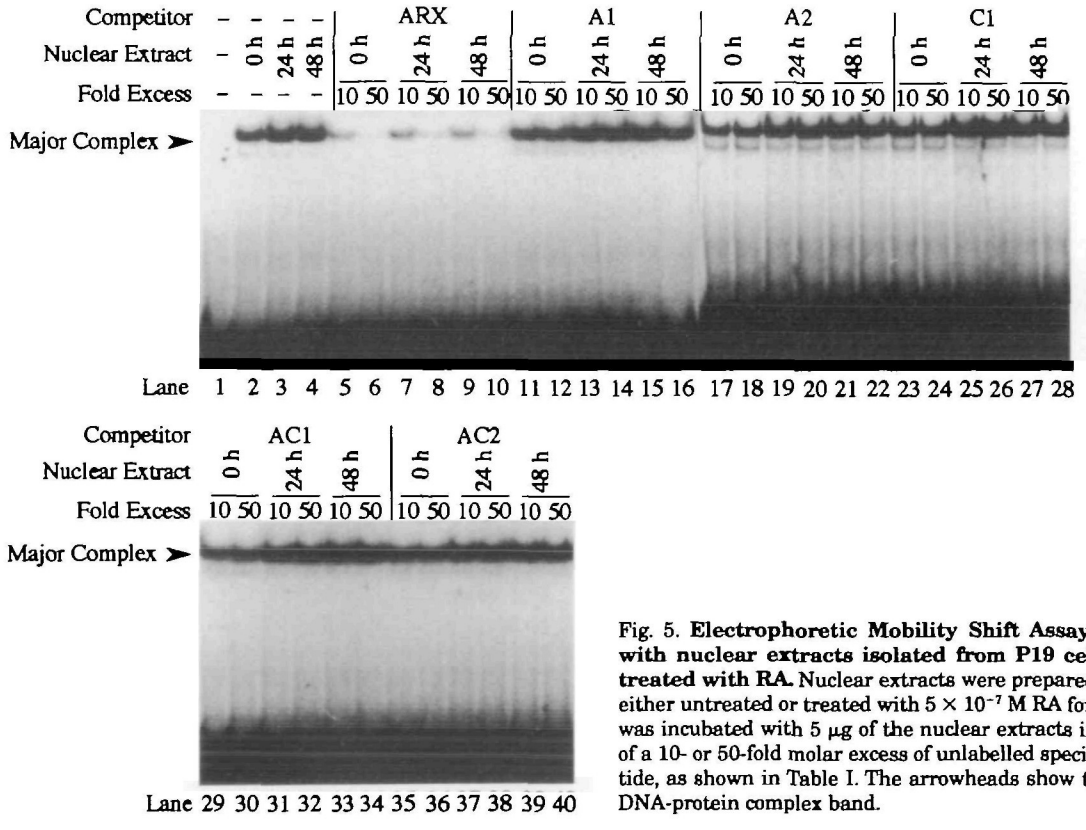

Fig. 5. **Electrophoretic Mobility Shift Assay (EMSA) of ARX DNA with nuclear extracts isolated from P19 cells either untreated or treated with RA.** Nuclear extracts were prepared from P19 cells that were either untreated or treated with 5×10^{-7} M RA for 24 or 48 h. Labelled ARX was incubated with 5 μg of the nuclear extracts in the absence or presence of a 10- or 50-fold molar excess of unlabelled specific competitor oligonucleotide, as shown in Table I. The arrowheads show the position of the specific DNA-protein complex band.

its intensity was weak. Interestingly, the intensity of the major band was enhanced when P19 cells were treated with RA (compare lane 2 with lanes 3 and 4).

The specificity of the complex was examined by competition experiments using an unlabelled ARX probe. When a 10-fold molar excess of competitor was added to the reaction, the major complex was reduced to 10% (lanes 5, 7, and 9). Residual radioactivity largely disappeared in the presence of a 50-fold molar excess of unlabelled probe (Fig. 5, lanes 6, 8, and 10).

ARX contains two overlapping consensus sequences, AP-2 and an inverted CCAAT box (see Fig. 3). Therefore, various competitors with or without point mutations in those regions were prepared (Table I). Oligonucleotides A1 and A2 contain mutations in two and three nucleotides, respectively, within the AP-2 binding motif. Two nucleotide changes exist within the inverted CCAAT box of oligonucleotide C1 (underlined in Table I). Oligonucleotides AC1 and AC2 possess two mutations in both the AP-2 and inverted CCAAT elements. EMSA was performed using these oligonucleotides as competitors (Fig. 5, lanes 11–40). It was found that none of the oligonucleotides could compete with the ARX probe to form the major nuclear extract complex (lanes 11 to 40), regardless of whether the cells were treated with RA. These results demonstrate unequivocally that such mutations in the two binding motifs affect the specific binding of transcription factors to ARX.

Thus, an important binding motif exists in ARX that mediates transduction of the RA signal into HES-1 gene expression. The canonical motifs found in ARX are the CCAAT and AP-2 binding sequences, and previous studies have shown that various CCAAT motifs bind transcription factors in a distinct manner (*35–38*). Oligonucleotides containing the CCAAT motifs of Eα (*35*), the H1TF2-binding motif (*36*), the α-globin promoter (*37*), or the CP-2 binding motif (*38*) were synthesized and their abilities to compete with ARX probe were analyzed (Table II), thus demonstrating the properties required by transcription factors to form

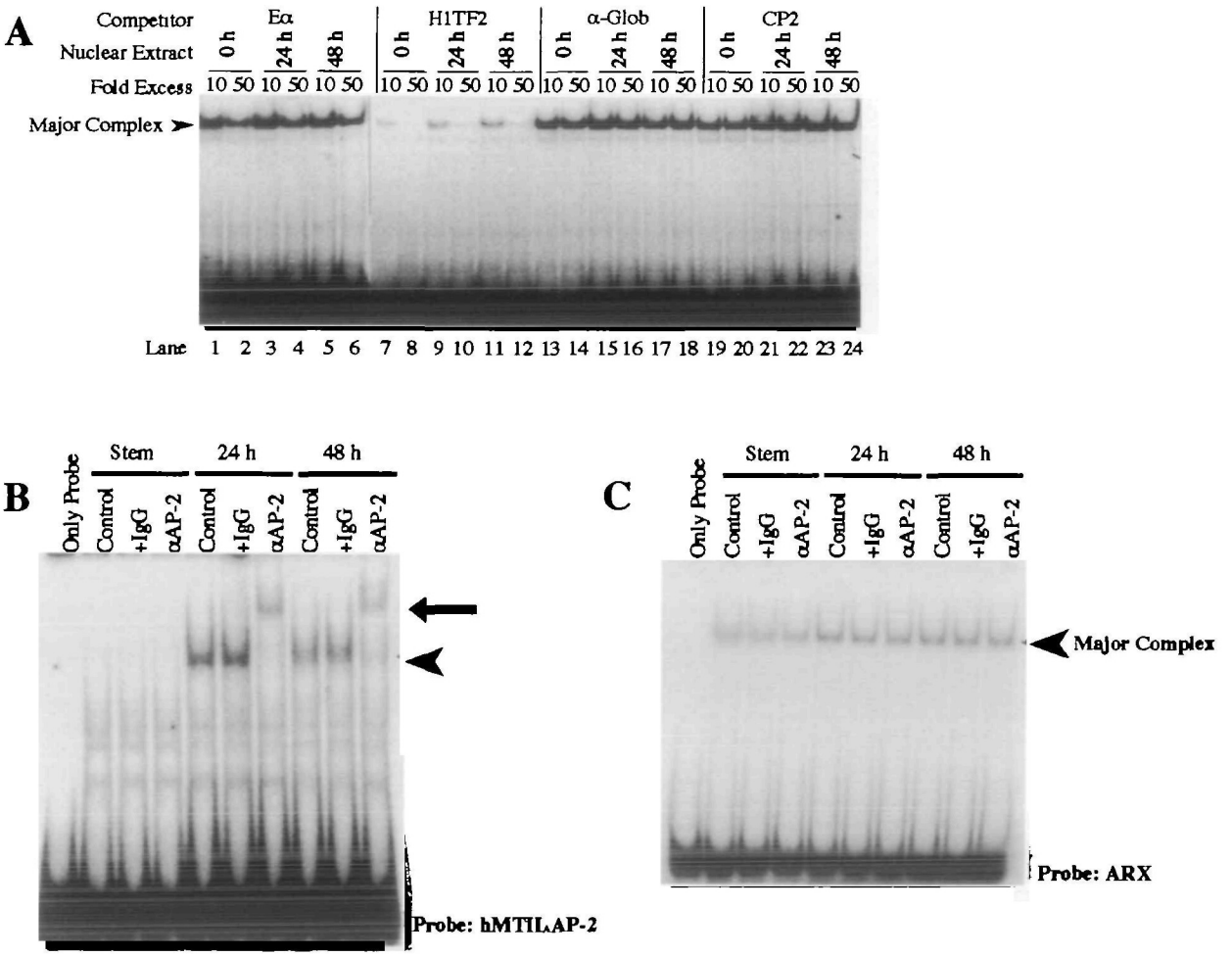

Fig. 6. EMSA of ARX with nuclear extracts isolated from P19 cells that were untreated or treated with RA and αAP-2 antibody. A: Nuclear extracts were prepared from P19 cells that were either untreated or treated with 5×10^{-7} M RA for 24 or 48 h. Labelled ARX was incubated with 5 μg of the nuclear extracts in the absence or presence of a 10- or 50-fold molar excess of unlabelled specific competitor oligonucleotide, as shown in Table II. The arrowhead shows the position of the specific DNA-protein complex band. B: EMSA using labelled human metallothionein IIA enhancer, as shown in Table II, and αAP-2 antibody; the same amount of nuclear extract was used as in A. The arrowhead indicates the specific DNA–AP-2 complex band, and the arrow points out the super shifting complex band. C: EMSA using labeled ARX and the same nuclear extract preparations as used in B. The arrowhead shows the position of the specific DNA-protein complex band.

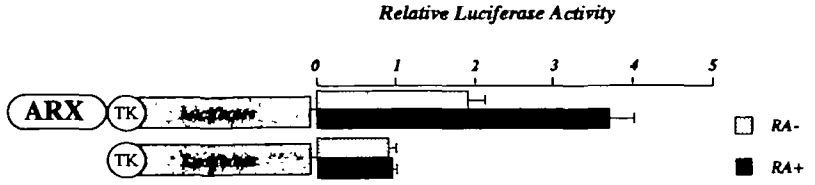

Fig. 7. **Effect of ARX on the expression of luciferase in P19 EC cells that were untreated or treated with RA.** Either HSV-TK luciferase reporter plasmids containing ARX or control HSV-TK luciferase vector were transfected into P19 cells. Transfectants were treated with or without 5×10^{-7} M RA for 24 h. The luciferase activity of P19 cells transfected with control luciferase reporter plasmid (see "EXPERIMENTAL PROCEDURES") in the absence of RA was normalized to a value of 1 and the other activities were calculated relative to this value. The value of each relative luciferase activity represents the mean of at least five independent transfection experiments. The luciferase activities are described as relative luciferase activity ±SEM. The luciferase activities of constructs treated with and without RA are shown by the black and dotted boxes, respectively.

the major complex with ARX. As shown in Fig. 6A (lanes 1 to 24), only the H1TF2 oligonucleotide competed markedly with ARX to form the major complex. Conversely, when the labelled H1TF2 was used as a probe in EMSA, the unlabelled oligonucleotide of ARX clearly competed with the H1TF2 probe to form a complex with nuclear extracts from P19 cells either treated or untreated with RA (data not shown). None of the other oligonucleotides tested competed with ARX in the formation of a complex.

This analysis also included an oligonucleotide with an AP-2–related sequence within the human metallothionein IIA enhancer (*39*) (Table II). Using RA-induced P19 cells and labelled human metallothionein IIA enhancer as a probe, EMSA detected both the AP-2 complex (arrowhead in Fig. 6B) and supershift bands with an anti–AP-2 antibody (arrow). However, the major complex with ARX was unaffected by the anti–AP-2 antibody (Fig. 6C). These results indicate that the transcription factor associated with ARX is most likely to be a CCAAT box binding factor and not an AP-2–related factor.

ARX Is Functional in P19 Cells—In order to examine ARX activity, a chimeric luciferase reporter, TK promoter, ARX construct was made, with ARX preceeding the TK promoter, which in turn preceeded the luciferase gene (ARX-TK-LUC). As expected, RA resulted in a significant increase in the luciferase activity of P19 cells transfected with ARX-TK-LUC, but not in cells transfected with a construct lacking ARX (TK-LUC) (Fig. 7). These results are in excellent agreement with those obtained from the *HES-1* gene deletion study (see Fig. 4). Thus, we conclude that ARX contributes to the RA-induced activation of *HES-1* gene expression in P19 cells.

DISCUSSION

It is well known that embryonic carcinoma cells provide a model for early mammalian embryogenesis. This study revealed that HES-1 is expressed in P19 embryonic carcinoma cells and that *HES-1* gene expression increases upon differentiation of P19 cells along the neural cell lineage by RA treatment. This HES-1 expression profile recapitulates that seen *in vivo* (*4*, *40*), suggesting that our reporter co-transfection analyses using P19 cells reflect the *HES-1* gene regulation that takes place during embryogenesis. Through such analyses, we found a *cis*-acting ARX that transduces an RA signal into *HES-1* gene expression. ARX contains a functional CCAAT element, but no canonical RA response elements. This study thus demonstrates a novel mechanism for the regulation of *HES-1* gene expression by RA that utilizes the CCAAT box.

Activated mammalian Notch is associated with RBP-Jκ (*41*, *42*), a mammalian homologue of the Drosophila *Suppressor of Hairless* gene product (*43*, *44*), and has been reported to function as an activator of *HES-1* gene transcription through RBP-Jκ binding sites within the promoter (*16*). However, HES-1 expression has also been reported to remain unchanged in the absence of either Notch1 or RBP-Jκ (*17*), suggesting the existence of multiple pathways of *HES-1* gene regulation. ARX was discovered in the region from −201 to −172 of the *HES-1* gene and is an effective activator of gene expression in P19 cells. In response to RA, ARX activates reporter gene expression through the TK promoter in P19 cells. ARX was also found to contribute to the basal level of *HES-1* gene expression in these cells. EMSA using nuclear extracts from P19 cells in the stem condition or after RA treatment, revealed the existence of two shifting complexes and showed that the amount of the major complex is increased by RA. These results indicate that ARX contains a positive regulatory element that acts to augment *HES-1* gene expression during the RA-induced differentiation of P19 cells.

Although the precise nature of the transcription factor that binds to the CCAAT core of ARX remains to be clarified, detailed analysis of EMSA has increased our overall knowledge about the factor. Monitoring the radioactivity associated with the shifted bands showed that the amount of transcription factor in the complex increases upon RA-induced differentiation of P19 cells. A multitude of proteins can bind to the CCAAT box, and this diversity is believed to be due to differences in the flanking sequence of the core. For instance, NF-Y binds to the CCAAT box derived from a murine class II MHC Eα gene and a human α-globin gene (*35*), and H1TF2 binds to the CCAAT motif in the histone H1 gene promoter (*36*). In addition, CP2 was found to be a CCAAT binding factor (*37*), although it turned out later that CP2-binding motifs do not always contain a CCAAT box (*38*). Intriguingly, the ARX CCAAT complex band was markedly reduced in the presence of the H1TF2 competitor (*36*). We also carried out the reverse experiment, in which the ARX probe was found to compete significantly with the H1TF2 probe. None of the other competitors in Table II could reduce the intensity of the shifting band with the ARX probe (data not shown).

Our initial analysis of the ARX sequence suggested that AP-2 might be the factor associated with ARX, as AP-2 has been reported to be inducible in P19 cells by RA (*45*). Also, a recently identified member of the AP-2–related gene family, AP-2.2, was found to respond to RA in P19 cells. This factor also binds to the AP-2 consensus sequence (GCCN3/4GGC) (*33*, *34*) defined in the human metallothionein IIA

enhancer (*39*). However, although the AP-2 complex was observed using the human metallothionein IIA enhancer as a probe, AP-2 did not appear to bind to ARX. Thus, a factor with specificity for the H1TF2 binding sequence is expressed in P19 cells and plays important roles in the activation of the *HES-1* gene *via* ARX. Since the classic H1TF2 binding protein is known to be expressed ubiquitously (*36*, *46*), a transcription factor other than H1TF2 that can form a functional complex for binding to the *cis*-element may be expressed in P19 cells. Further analysis is required to elucidate which nuclear proteins bind to the ARX and regulate *HES-1* gene expression, specifically during cellular differentiation to a neural cell lineage.

We thank Drs. Kazuaki Yoshikawa, Toru Nakano, Tomomi Takahashi, Kentaro Yomogida, Nobufumi Kanno, Ken Itoh, and Tania O'Connor for discussion and critical reading of the manuscript. We also thank Dr. Hiroshi Hamada for the generous gifts of pBS-Oct-3.

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