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cDNA cloning and structure of mouse putative Ah receptor

M. Ema, K. Sogawa, N. Watanabe, Y. Chujoh, N. Matsushita[1],
O. Gotoh[2], Y. Funae[3] and Y. Fujii-Kuriyama *

Department of Chemistry, Faculty of Science, Tohoku University, Sendai, 980, Japan
[1] Department of Agricultural Chemistry, Nagoya University, Nagoya 464, Japan

[2] Department of Biochemistry, Saitama Cancer Center Research Institute, Inamachi, Saitama 362, Japan

[3] Laboratory of Chemistry, Osaka City University, Medical School, Osaka 545, Japan

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Mouse cDNA clones for a putative Ah receptor have been isolated from a cDNA library of mRNA from Hepa-1 cells by an oligonucleotide probe produced by PCR with a pair of primers which was synthesized according to the reported N-terminal sequence of 26 amino acids. The cDNA clones encode a polypeptide of 805 amino acids with a helix-loop-helix motif and with some similarity to a certain region designated PAS of Drosophila Per and Sim, and human Arnt protein. Cotransfection of an expression vector of the Ah receptor with a reporter plasmid pMC6.3k consisting of CYP1A1 promoter and CAT structural gene into CV-1 cells enhanced the CAT expression in response to added 3-methylcholanthrene.

Halogenated aromatic hydrocarbons usually represented by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are well-known environmental pollutants which produce a variety of biochemical and toxic effects such as induction of various drug-metabolizing enzymes, immune suppression, wasting syndrome, proliferation and / or differentiation of various epitherial tissues and tumor promotion in experimental animals(1, 2). All the effects of these chemicals are thought to be mediated by their binding to a soluble protein designated Ah receptor(2), but the detailed mechanisms have long remained enigmatic, because of extreme scarcity and lability of the Ah receptor(3, 4).

Mechanisms of the inducible expression of P-450c(CYP1A1) gene by the administration of 3-methylcholanthrene(3MC) or TCDD are the best studied case about the role of the Ah receptor. When taken up in the cells, an inducer is recognized and bound by the Ah receptor associated with HSP90 in the cytoplasm(5, 6). Upon association with the inducer, the receptor is considered to be dissociated from HSP90

*To whom correspondence should be addressed.

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and translocated to the nuclei to enhance the transcription of CYP1A1 gene through the interaction with the cis-acting DNA element, XRE(2, 5-10). Although some of them were proved experimentally, the molecular basis of these processes such as organization of the Ah receptor complexes remains unknown.

Recently, the putative ligand-binding subunit of the Ah receptor has been purified from livers of mice to homogeneity to determine the N-terminal sequence of 26 amino acids(11). Taking advantage of this sequence, we have attempted to isolate cDNA clones for the binding protein. Here we report the cloning and structure of a complementary DNA encoding mouse putative Ah receptor.

Materials and Methods

Isolation and analysis of cDNA clones.

Standard methods for manipulating DNA were as described by Sambrook et al.(12). Oligonucleotides used as a pair of primers in PCR were synthesized according to the reported sequence(11) and are shown in Fig. 1a.

PCR was carried out with a cycle of denaturing at 94°C for 1 min, then annealing at 48°C for 2 min and polymerizing at 78°C for 3 min. After 30 cycles of PCR, the products were applied on 1.5% agarose gel electrophoresis. The products of 80 to 100 bp were eluted from the gel and inserted into M13 vector for sequence analysis by the dideoxy chain terminating method(12).

Double-stranded cDNA was prepared from a poly(A) RNA of Hepa-1 cells by the random priming method(12) and inserted into λgt 10 vector. Of the $7X10^6$ primary plaques screened, 4 positives were isolated. The cDNA clone with the longest insert (1.6 kb) was used as a probe in a second screening for full length cDNA clones. cDNAs were subcloned into Bluescript vector (Stratagene), and clones for sequencing were generated by exonuclease III digestion(13).The cDNA were sequenced by the dideoxy chain terminating method(12). All the sequencings were performed on both strands.

Construction of plasmids

Two cDNA clones λmAhR 1 and 3, were joined at a common restriction site (Kpn I). The resultant plasmid with the full-length coding sequence was digested with Xho I and Xba I, after Bbe I site at 928 was changed to Xho I site by using synthetic linkers. The Xho I / Xba I fragment was ligated to pCMSV which had been cleaved by Sal I and Xba I. pCMSV was generously provided by Dr. A. Adachi (Kyoto Univ., Kyoto) and contained a 680 bp fragment of the cytomegalovirus (CMV) promoter and enhancer derived from pCDM8 (14) and a 850 bp fragment of SV40 splicing and poly(A) addition sequence in the Hind III and Xba I sites of pUC18, respectively. In the CMV-AhR chimeric plasmid (pCMAhR), the N-terminal sequence of the Ah receptor, Met-Ser-Ser-Ala-Ala, was converted into Met-Pro-Ala-Gly-Arg-Gly originated from the pUC18 polylinker sequence and the Xho I linker. The construction was confirmed by sequencing.

Transfection and CAT assays

pCMAhR was cotransfected into CV-1 or Hepa-1 mutant (C12)(15, 16) cells with pMC6.3k (7) carrying Ah receptor-recognition sequences (XRE) in the promoter region as a reporter plasmid by the calcium phosphate method. Extracts were prepared from the cells and CAT assays were performed by the method of Gorman et al. (17).

RNA blot analysis

Total RNAs from various rat organs and several cultured cells were subjected to RNA blot analysis according to the method of Thomas(18).

Results and Discussion

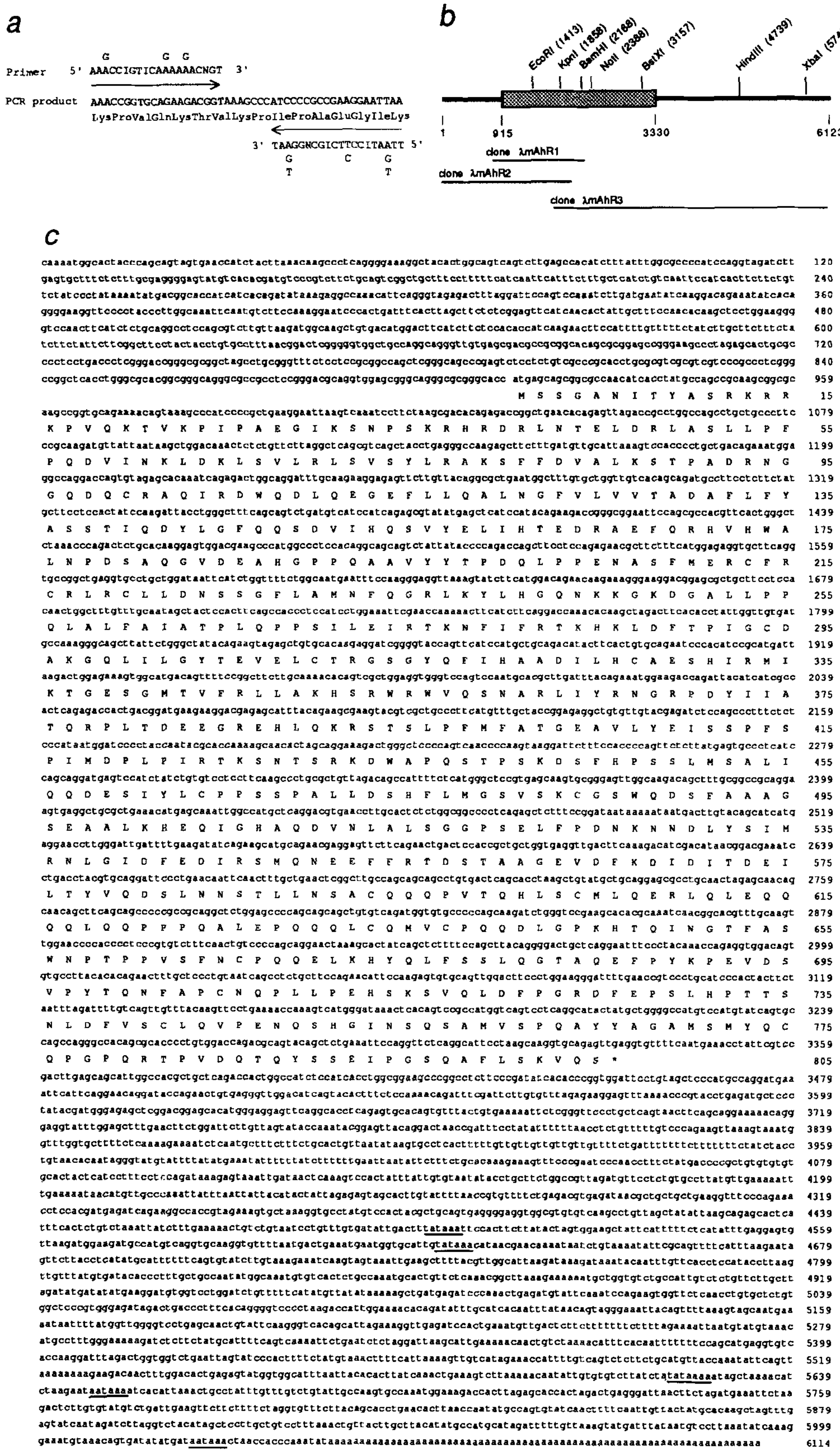
According to the N-terminal sequence of 26 amino acids of the purified Ah receptor published recently(11), we synthesized two oligonucleotides for a pair of primers in the

PCR reaction. PCR using mRNA of Hepa-1 cells gave smeared bands ranging from 80 to 100 nucleotides on an agarose gel electrophoresis (data not shown). These bands were eluted from the gel and cloned into an M13 vector for sequence analysis. The cDNA sequence encoding the determined N-terminal sequence of 16 amino acids of the Ah receptor was found in a frequency of about 1 in 20 cloned sequences and (Fig. 1a) used as a probe for screening of a cDNA library from mRNA of Hepa-1 cells. Four positive cDNA clones were first isolated by screening about $7X10^6$ recombinant plaques. From sequence analysis of a cDNA clone with the longest insert of 1.6 kb, it was found to contain the coding sequence for the reported 26 N-terminal amino acids preceded by the most N-terminal 9 amino acids. Therefore, the purified Ah receptor protein is considered to be a processed product from a precursor protein or to be trimmed off the 9 most N-terminal amino acids during the purification procedure. Physiological significance of the extension peptide is not known.

Since RNA blot analysis using the cloned cDNA as a probe indicated that the mRNA for the Ah receptor was about 6 kb long, further screening for full length cDNA clones was carried out. As shown in Fig. 1b, cloned cDNAs in λmAhR 1, 2 and 3, altogether, seemed to cover the full information of the mRNA. Sequence analysis of these cDNAs revealed that a long open reading frame of 2415 bp, beginning at nucleotide 915 and ending at nucleotide 3329, encodes a polypeptide consisting of 805 amino acids with the calculated molecular weight of 90,380 which is reasonably consistent with that of the purified Ah receptor protein estimated previously(5, 19). The sequence surrounding the putative initiator codon, GGCCACCATGA, fits well with the consensus sequence $GCC^{A}/_{G}CCATGG$ for a translation initiation site(20). A leader and a trailer sequences are 914 and 2713 bases long, respectively. A poly (A) addition signal is present 16 bases upstream of the poly (A) tail (Fig. 1c).

A database search revealed no sequence having similarity to the putative Ah receptor cDNA sequence. But close survey of sequences with similarity to the cDNA-encoded amino acid sequence revealed three regions worthy of note (Fig.2). Firstly, the region starting from amino acid 27 to 82 in the putative Ah receptor protein shows a significant conformity to the consensus sequence for the basic helix-loop-helix motif of certain DNA binding proteins(21) including Drosophila Sim(22), human Arnt (23) and TFEB(24) (Fig.2). Of all basic helix-loop-helix proteins examined, the Sim protein in the region of 11 to 60 amino acid shows the highest similarity to the putative Ah receptor in the region of 33 to 82 amino acid with 44% identity and the Arnt protein is the 2nd highest. The basic helix-loop-helix motif in the DNA binding proteins contains domains for DNA binding and protein-protein interaction for dimer formation(21, 25).

Fig.1. a) A pair of oligonucleotide primers for PCR and the sequence of an amplified product used for screening of cDNA library. b) Cloned cDNAs and outline of the mRNA. Restriction cleavage sites are indicated and numbers indicate the position of nucleotides from the 5'-terminus of the cloned cDNA. A dotted box represents the coding sequence. c) Nucleotide sequence of cloned cDNA and deduced amino acid sequence. Possible poly(A) addition signals are underlined.


a
Primer 5' AAACCIGTICAAAAAACNGT 3'
G G G
PCR product AAACCGGTGCAGAAGACGGTAAAGCCCATCCCCGCCGAAGGAATTAA
LysProValGlnLysThrValLysProIleProAlaGluGlyIleLys
3' TAAGGNCGICTTCCITAATT 5'
G C G
T T
b
EcoRI (1413)
KpnI (1856)
BamHI (2168)
NotI (2388)
BstXI (3157)
HindIII (4739)
XbaI (5743)
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clone λmAhR2
clone λmAhR3
c

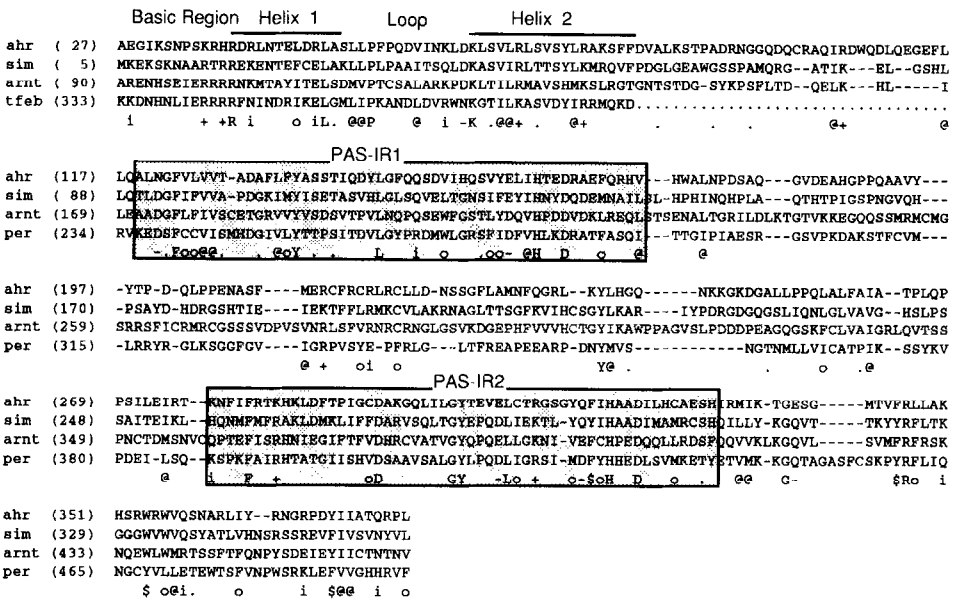

Fig.2. Sequence similarity of the putative Ahr to other proteins in the helix-loop-helix and PAS region. Protein sequences in SEQDB database (Protein Reserch Foundation, Osaka) that are similar to the Ahr were searched for and aligned by the method of Gotoh(45). The internally repeated sequences shared by Ahr, Arnt, Sim, and Per are indicated by shaded boxes. Strictly conserved amino acids are marked by the standard one-letter codes beneath the alignment. Other symbols indicate the sites with conserved physico-chemical properties; small (.), positive (+), negative (-), aromatic ($), large aliphatic(@), polar (i), and apolar (o).

In this context, it is interesting to know whether the Arnt protein directly interacts with the putative Ah receptor through this basic helix-loop-helix motif, because the Arnt protein is proposed to be involved in the nuclear translocation of the Ah receptor from cytoplasm(16, 23). The Ah receptor activated in association with a ligand is considered to translocate from cytoplasm to nucleus and enhance the transcription of the genes by binding to the XRE sequence in their promoter regions(7-10). Secondly, the region from 140 to 340 amino acid of the putative Ah receptor is similar to a part of human Arnt(23), Drosophila Per and Sim proteins(22). Sim is a nuclear protein and functions as a master developmental regulator of the CNS midline lineage(22), whereas Per is found in either the nucleus or cytoplasm, depending on the tissue examined and controls the periodicity of biological rhythms(26). No function has been assigned to this common region designated PAS in the three proteins(22). Thirdly, the region from 594 to 648 amino acid position is a glutamine-rich region which is found in the activator domain of some transcriptional regulatory proteins(27). Taken together, these structural characteristics strongly suggest that the putative Ah receptor is a transcriptional regulatory protein.

In view of biochemical properties(5, 9) and association with HSP90(9, 38), the Ah receptor appears to have some properties in common with a member of steroid / thyroid / retinoic acid (STR) receptor superfamily and was suggested to be a member of that superfamily(29, 30). However, no resembrance is observed in the amino acid

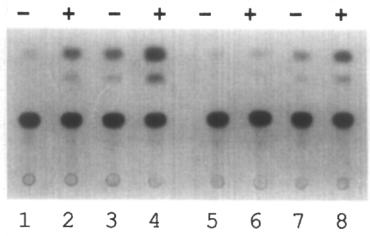

Fig.3. Stimulation of the CAT expression by the AhR in the presense of 3MC. The reporter pMC6.3k (5μg) was cotransfected into C12 Hepa-1 mutant (1 to 4) or CV-1 (5 to 8) cells with 8μg of the expression plasmid pCMSV (1, 2, 5 and 6) or pCMAhR (3, 4, 7 and 8). The control plasmid pCMSV lacks the AhR cDNA sequence, and pCMAhR contains the full coding region of mAhR. Cultures were maintained for 40hr in the absence (–) or presence (+) of 1mM MC.

sequence between the putative Ah receptor and any of those members of the STR superfamily. Analysis of mutants of Hepa-1 cells defective in the Ah receptor function classified them into 3 complementation groups(15, 16), while analogous mutants for glucocorticoid receptor function fall within the same complementation group(31, 32). Photoaffinity labeling techniques showed that the DNA-binding form of the Ah receptor is a heterodimer consisting of 100 and 105 kDa proteins, the smaller being ligand-binding protein or Ah receptor molecule(33). In contrast, several steroid hormone receptors bind to their cognate DNA elements in a homodimer form(30). These results indicate that the mode of action of the Ah receptor function is apparently more complex than that of the steroid receptors and could be different from that of the receptors.

The function of the putative Ah receptor encoded by the cDNA was investigated in cultured cell systems (Fig.3). The expression plasmid (effector gene) of the Ah receptor was transfected into various cell lines with the reporter plasmid (pMC 6.3K). The reporter plasmid carries the inducible enhancer sequence, XRE which expresses the subordinate gene in response to the liganded Ah receptor(7, 8). CV-1 cell which is considered to be defective in the Ah receptor function expressed only a low level of the CAT activity from the transfected reporter gene in the presence of an inducer, 3MC. Cotransfection of the effector and the reporter gene into the CV-1 cells expressed the enhanced CAT activity in the presence of the added 3MC, although the CAT activity in the absence of the inducer seemed to be slightly elevated. When C12 cells, a mutant of Hepa-1 defective in the Ah receptor(2, 15) were transfected with the effector and the reporter genes, the expression of CAT activity was also enhanced in the presence of the inducer. In both cases, cotransfection of the effector plasmid seemed to activate the CAT expression to a certain extent in the absence of the inducer. The reason for this activation of the CAT expression even in the absence of the inducer is not known. RNA blot analysis using the cloned cDNA as a probe revealed that mRNA for the putative Ah receptor protein is detected at about 6 and 4 kb positions mainly in liver and lung, and most abundantly expressed in Hepa-1 cells of all the tissues and cultured cell lines tested (Fig.4). These results are consistent with those reported with immuno-blot

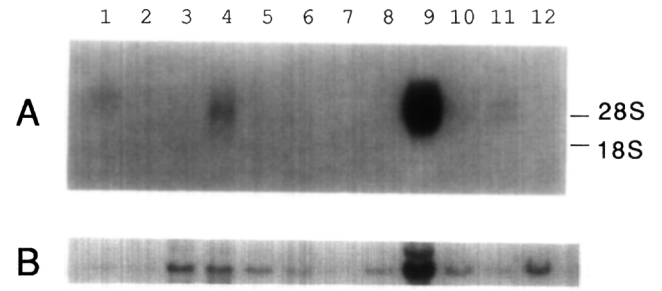

Fig.4. Blot hybridization analysis of total RNA from various rat tissues and cultured cell lines. RNA samples (15μg/lane) were electrophoresed in a 0.8% agarose/formaldehyde gel. RNAs were transferred to a Nylon membrane and hybridized with the [^{32}P]-labeled 0.9kb Eco RV fragment of the cDNA (specific activity, 6.0X10^{8}cpm/μg). Positions of 28S and 18S RNA are shown on the right. Lane 1, liver; 2, kidney; 3, spleen; 4, lung; 5, brain; 6, heart; 7, muscle; 8, testis; 9, Hepa-1; 10, HepG2; 11, HeLa; 12, CV-1. The same membrane was dehybridized three times in 0.1XSSC containing 0.5 % SDS at 95°C for 15 min. and rehybridized with [^{32}P]-labeled cDNA of mouse β-actin (specific activity, 9.2X10^{8}cpm/μg).

analysis(19). The two bands for the mRNA may be due to alternate use of the possible poly (A) signals (underlined in Fig. 1) present upstream of the signal used.

Problems as to whether the putative Ah receptor whose cDNA was cloned here functions by itself as a receptor for the inducer or forms a complex with other proteins such as Arnt protein to activate the expression of CYP1A1 gene should be a next target of our investigation.

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References

1. Poland, A. and Knutson, J. C. (1982) Ann. Rev. Pharmacol. Toxicol. 22:517-554.
2. Whitlock, Jr. J. P. (1990) Ann. Rev. Pharmacol. Toxicol. 30:251-277.
3. Bradfield, C. A., Kende, A. S. and Poland, A. (1988) Mol. Pharmacol. 34:229-237.
4. Bradfield, C. A. and Poland, A. (1988) Mol. Pharmacol. 34:682-688.
5. Cuthill, S., Poellinger, L. and Gustafsson, J.-Å. (1987) J. Biol. Chem. 262:3477-3481.
6. Prokipcak, R. D. and Okey, A. B. (1988) Arch. Biochem. Biophys. 267:811-828.
7. Fujisawa-Sehara, A., Sogawa, K., Yamane, M., Fujii-Kuriyama, Y. (1987) Nucleic Acid Res. 15:4179-4191.
8. Fujisawa-Sehara, A., Yamane, M. and Fujii-Kuriyama, Y. (1988) Proc. Natl. Acad. Sci. USA 85:5859-5863.

9. Wilhelmsson, A., Cuthill, S., Denis, M., Wikström, A.-C., Gustafsson, J.-Å. and Poellinger, L. (1990) EMBO J. 9:69-76.
10. Denison, M. S., Fisher, J. M. and Whitlock, J. P. Jr. (1988) Proc. Natl. Acad. Sci. USA 85:2528-2532.
11. Bradfield, C. A., Glover, E. and Poland, A. (1991) Mol. Pharmacol. 39:13-19.
12. Sambrook, J., Fritsch, E. J. and Maniatis, T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
13. Henikoff, S. (1987) Methods Enzymol. 125:157-165.
14. Aruffo, A. and Seed, B. (1987) Proc. Natl. Acad. Sci. U.S.A. 84:8573-8577.
15. Hankinson, O. (1983) Somat. Cell Genet. 9:497-514.
16. Legraverend, C., Hannah, R. R., Eisen, H. J., Owens, I. S., Nebert, D. W. and Hankinson, O. (1982) J. Biol. Chem. 257:6402-6405.
17. Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) Mol. Cell. Biol. 2:1044-1051.
18. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. USA 77:5201-5205.
19. Poland, A., Glover, E. and Bradfield, C. A. (1991) Molec. Pharmacol. 39:20-26.
20. Kozak, M. (1984) Nucleic Acids Res. 12:857-872.
21. Murre, C., McCaw, P. S. and Baltimore, D. (1989) Cell. 56:777-783.
22. Namba, J. R., Lewis, J. O., Wharton, K. A. and Crews, S. T. (1991) Cell. 67:1157-1167.
23. Hoffman, E. C., Reyes, H., Chu, F.-F., Sander, F., Conley, L. H., Brooks, B. A. and Hankinson, O. (1991) Science. 252:954-958.
24. Carr, C. S. and Sharp, P. A. (1990) Mol. Cell. biol. 10:4384-4388.
25. Davis, R. L., Cheng, P.-F., Lassar, A. B. and Weintraub, H. (1990) Cell. 60:733-746.
26. Saez, L. and Young, M. W. (1988) Mol. Cell. Biol. 8:5378-5385.
27. Mitchell, P. J. and Tjian, R. (1989) Science. 245:371-378.
28. Perdew, G. H. (1988) J. Biol. Chem. 263:13802-13805.
29. Evans, R. M. (1988) Science. 240:889-895.
30. Beato, M. (1989) Cell. 56:335-344.
31. Danielsen, M., Northrop, J. P., Ringold, G. M. (1986) EMBO J. 5:2513-2522.
32. Yamamoto, K. R. (1985) Ann. Rev. Genet. 19:209-215.
33. Elferink, C. J., Gasiewicz, T. A. and Whitlock, J. P. (1990) J. Biol. Chem. 269:20708-20712.
34. Gotoh, O. (1987) Comp. Appl. Biosci. 3:17-20.