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Localization of the estrogen-binding site of α-fetoprotein in the chimeric human–rat proteins

(yeast expression system/recombinant protein)

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ABSTRACT **Rat α-fetoprotein (AFP) has been demonstrated to bind estrogen, whereas human AFP lacks the activity. We constructed four chimeric molecules from cDNAs encoding these AFPs with the use of two restriction sites common to them and expressed them as well as rat and human AFP cDNA in yeast. The recombinant molecules were purified, characterized, and found to have the predicted structures. Analyses of estrogen binding indicated that a rat AFP sequence composed of residues 423–506 that contains 31 rat-specific amino acids is essential for the activity.**

α-Fetoprotein (AFP) is a fetal serum protein that is synthesized mainly by fetal liver and yolk sac. After birth it disappears from the circulation and its reappearance in the blood indicates the presence of hepatocellular carcinoma or a yolk sac tumor. Many studies have been done on the possible biological roles of AFP in ontogeny and malignancies (1, 2). The rat (RAFP) and mouse proteins, but not those of other species such as human, bind estrogens with high affinity and specificity (3–5). In rodents, which have short gestational periods, AFP is a major constituent of fetal blood plasma during gestation as well as for several days after birth (1, 6). It has been postulated that this protein may protect the fetus from the high levels of maternal estrogens by specifically sequestering them (7–11). It has been shown that AFP–estrogen complexes inhibit the growth of the mouse uterus (12, 13). Jacobson *et al.* (14) have also shown that such complexes inhibit the growth of estrogen-dependent tumors. However, in spite of the extensive studies of rodent AFP, the localization and the structural basis of the binding have not been elucidated.

We have recently expressed cDNAs encoding RAFP and human AFP (HAFP) in yeast (15, 16). The rat protein showed the estrogen-binding activity of its natural counterpart. In the present study, we prepared four chimeric cDNAs from the above two cDNAs with the use of common restriction sites and expressed them in yeast. The estrogen-binding site on RAFP was explored by the analysis of the estrogen-binding activity of the recombinant chimeric AFPs.

MATERIALS AND METHODS

Construction of Expression Plasmids. The yeast expression vector used was pAM82 (17), which had been used to construct pAMRA1 (15) and pAMHA3 (16) for the expression of RAFP and HAFP, respectively (Fig. 1). In this study, we constructed four plasmids, designated pAMRHHA, pAMHRRA, pAMRHRA, and pAMHRHA, with the use of *Pvu* II and *Xho* II restriction sites that are present in both rat and human AFP cDNA. The chimeric cDNAs in the plasmids were in turn designated RHH, HRR, RHR, and HRH, respectively. *Pvu* II digestion of pAMHA3 or pAMRA1 generated two fragments, since there is a *Pvu* II site in both AFP cDNA and pAM82. Plasmids pAMRHHA and pAMHRRA were prepared by ligating the two appropriate fragments generated as shown in Fig. 1. pAMHRHA and pAMRHRA were constructed as described below. AFP cDNAs HRR and RHH were isolated from *Xho* I-digested pAMHRRA and pAMRHHA by agarose gel electrophoresis, dephosphorylated with calf intestinal alkaline phosphatase, and digested completely or partially with *Xho* II to generate the desired fragments. The AFP cDNAs RHR and HRH were prepared by ligating the proper combinations of two fragments isolated from the digests and subcloning them at the *Xho* I site of the plasmid pHSG396 (Takara Shuzo, Kyoto) in *E. coli* JM109. The chimeric AFP cDNAs were recovered from the subcloned plasmids and ligated with pAM82. The plasmids thus prepared and propagated in *E. coli* HB101 were confirmed to have the desired structures by restriction mapping. The manipulations of DNA were done by standard methods (19). The amino acid sequences of the AFPs are shown in Fig. 2.

Expression of AFP in Yeast. This was done as previously described (15). Briefly, the expression plasmids propagated in *E. coli* HB101 were introduced into the yeast strain AH22. Transformants grown on Burkholder minimal medium agar plates were initially cultured in the presence of phosphate. To induce AFP production, the acid phosphatase promoter was derepressed by culturing the cells without phosphate. The cells harvested were converted to spheroplasts by Zymolyase 100T digestion and lysed with buffer containing 0.1% Triton X-100.

Purification and Characterization of AFP. HAFP and RAFP were purified by immunoaffinity chromatography using murine monoclonal antibody AFY6 (15, 21). The antibody was prepared against HAFP but was crossreactive with RAFP (21). The sources of these proteins were the serum of a hepatoma patient and the ascitic fluid of rats bearing the transplantable rat hepatoma AH66, respectively. Yeast recombinant AFPs were purified from the yeast cell lysate by DEAE-cellulose and immunoaffinity chromatography by methods similar to those described previously (15, 16). Protein thus purified was analyzed by SDS/PAGE and double immunodiffusion (15).

Estrogen-Binding Assays of AFP. Filter paper disks were activated with CNBr and coupled with antibody to AFP. The amount of the antibody coupled to each disk (diameter, 5 mm) was about 10 μg. The disks were incubated with 100 μl of purified AFP (≈1 μg) at 4°C for 16 hr, washed three times with 1 ml of PBS (0.15 M NaCl, pH 7.2/0.02 M sodium phosphate) and then treated with 100 μl (11,500 dpm) of [2,4,6,7,16,17-^{3}H]estradiol (155 Ci/mmol, TRK.587, Amersham; 1 Ci = 37 GBq) for 4 hr at 4°C. The disks were washed

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Abbreviations: AFP, α-fetoprotein; RAFP and HAFP, rat and human AFP; YRAFP and YHAFP, yeast-expressed RAFP and HAFP.

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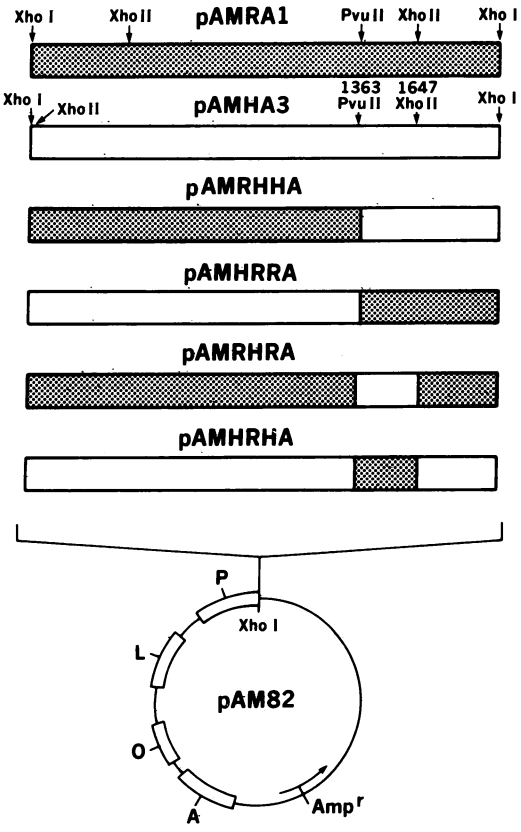

FIG. 1. Construction of expression plasmids. AFP cDNAs were ligated at the *Xho* I site of pAM82. It consists of a DNA fragment of *Escherichia coli* plasmid pBR322 that contains a replication origin and ampicillin-resistance gene (Amp^r), a DNA fragment of yeast that contains an acid phosphatase promoter (P), an autonomous replication origin (A), a replication origin of 2-μm DNA (O) and the *LEU2* gene (L) for insertion into a *leu*$^-$ auxotropic host yeast cell such as AH22 (17). pAMRA1 and pAMHA3 had been constructed to express RAFP and HAFP, respectively, as described (15, 16). pAMRHHA, pAMHRRA, pAMRHRA, and pAMHRHA had chimeric AFP cDNAs that were prepared by ligating cDNAs of RAFP and HAFP at the *Pvu* II and *Xho* II restriction sites indicated. Their positions were reported previously (15, 18).

with PBS, and the radioactivity bound to the disks was measured by liquid scintillation counting. PBS was used for the dilution of AFP and estradiol. The details of the method were described previously (15). Binding constants for reactions of AFP and estradiol were determined by Scatchard analysis (22). AFP (50 ng) was allowed to react with the disks, which were then exposed to various concentrations of estradiol (0.34–3.4 nM).

RESULTS

Expression and Purification of Recombinant AFPs. AFP was purified from lysates of transformed yeast cell cultures. The protein produced in yeast cells harboring plasmids pAMHA3, pAMRA1, pAMRHHA, pAMHHRA, pAMRHRA, and pAMHRHA will be referred to as YHAFP, YRAFP, RHHAFP, HRRAFP, RHRAFP, and HRHAFP, respectively. In one experiment with HRHAFP, 20.2 g of yeast cells were obtained from 4 liters of culture medium, and 200 ml of lysate prepared from them contained 220 μg of AFP and 286 mg of other proteins. The lysate was dialyzed against 0.1 M Tris·HCl (pH 8.0) and loaded on a DEAE-cellulose (DE52, Whatman) column (5 ×10 cm) equilibrated with the same buffer. The column was washed with the same buffer and the material retained was eluted with 0.4 M Tris·HCl (pH 8.0). The eluate, containing 158 μg of AFP, was dialyzed against PBS and loaded on an immunoadsorbent column (1.5 × 10 cm) coupled with 10 mg of AFY6 antibody. The AFP adsorbed was eluted with 0.1 M glycine·HCl (pH 2.1) in a purified form and concentrated into distilled water by Centricon 30 (Amicon) filtration. The amount of AFP obtained in a purified form was 86 μg. The yields of purified chimeric AFPs were essentially the same as those of YHAFP and YRAFP, 50–110 μg from cells from 4 liters of culture. No AFP was detected in the culture medium.

The purified AFPs were subjected to SDS/PAGE (Fig. 3). Hepatoma HAFP, YHAFP, RHRAFP, and HRRAFP migrated as single bands, whereas hepatoma RAFP, YRAFP, HRHAFP, and RHHAFP showed microheterogeneities. The molecular weights of hepatoma RAFP (72,500 and 69,500), YRAFP (69,000), and YHAFP (68,000) were very close to those found previously (15, 16). Those of RHRAFP, HRHAFP, and HRRAFP were about 68,000 and that of RHHAFP was about 69,000.

In double immunodiffusion tests, hepatoma RAFP, YRAFP, and RHRAFP formed a completely fused line with antibody to RAFP (Fig. 4*A*) and hepatoma HAFP, YHAFP, and HRHAFP reacted similarly with antibody to HAFP (Fig.

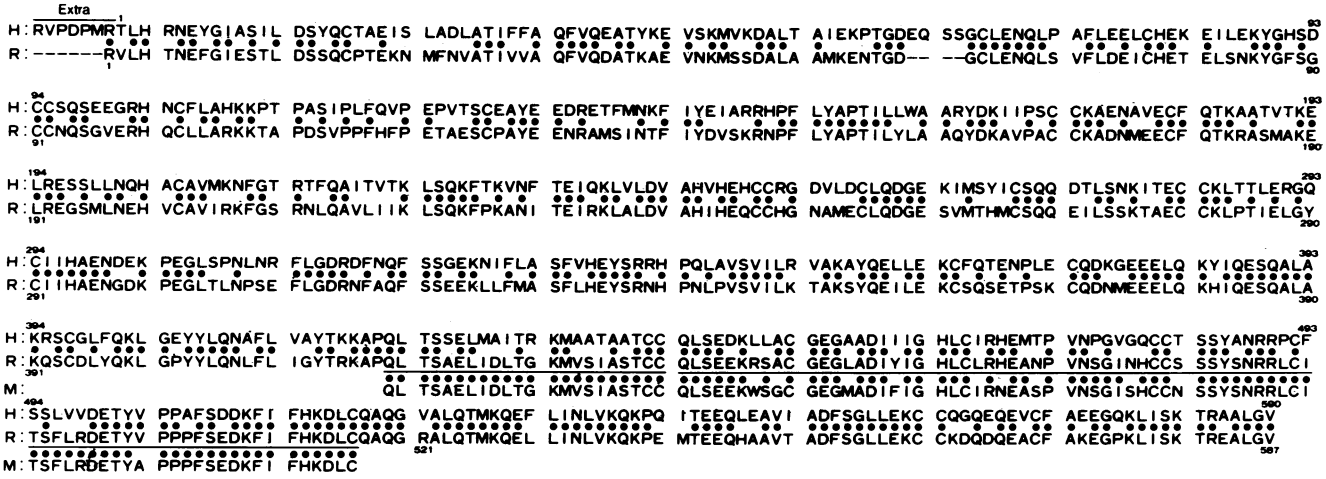

FIG. 2. Primary structures of AFPs encoded by pAMHA3 (H, human) and by pAMRA1 (R, rat), which were composed of 597 and 587 amino acid residues, respectively. The former protein had 7 amino acid residues at the N terminus in addition to those of mature HAFP. The 98 amino acids encoded by the *Pvu* II–*Xho* II fragment of AFP cDNAs are underlined. The corresponding sequence of mouse AFP (20) is also shown (M). Identical residues are indicated (●).

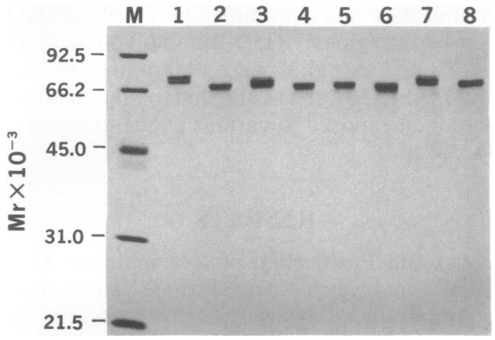

FIG. 3. Analysis of AFPs by SDS/PAGE. The purified AFPs (2 μg of each) were subjected to electrophoresis in a 10% polyacrylamide gel and stained with Coomassie brilliant blue. Apparent molecular weights were 72,000 and 69,500 for hepatoma RAFP and 69,000 for hepatoma HAFP. Molecular weights of chimeric AFPs were intermediate to these. Lanes: M, molecular weight markers (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor); 1, hepatoma RAFP; 2, hepatoma HAFP; 3, YRAFP; 4, YHAFP; 5, RHRAFP; 6, HRHAFP; 7, RHHAFP; 8, HRRAFP.

4*B*). Furthermore, RHRAFP did not react with antibody to HAFP, and HRHAFP did not react with antibody to RAFP (data not shown). These results may indicate that the HAFP sequence in RHRAFP and the RAFP sequence in HRHAFP are not major antigenic sites. HRRAFP showed partial identities both to hepatoma RAFP and to hepatoma HAFP (Fig. 4*C*), and RHHAFP reacted similarly (Fig. 4*D*). These reactions indicated that HRRAFP and RHHAFP were chimeric proteins of RAFP and HAFP.

Estrogen Binding. The results for these studies are summarized in Table 1. Hepatoma RAFP, YRAFP, HRRAFP, and HRHAFP had high activities, binding 26.0–45.8% of the radioactive estradiol employed. No significant activity was observed for hepatoma HAFP, YHAFP, RHHAFP, and RHRAFP. These results indicate that residues 419–516, encoded by the *Pvu* II–*Xho* II fragment of rat AFP cDNA, are essential for estrogen binding.

YRAFP, HRRAFP, and HRHAFP were subjected to Scatchard analysis and their association constants, Ka, determined from the slopes, were essentially the same, 11.4×10^8 M^{-1}, 11.8×10^8 M^{-1} and 9.3×10^8 M^{-1}, respectively (Fig. 5).

DISCUSSION

The yeast expression system was employed in the present studies because RAFP produced in *E. coli* did not show estrogen-binding activity (15). This may have resulted from the failure of the protein to form correct pairs of disulfide bridges that are necessary for estrogen binding (15, 16). We used chimeric molecules of RAFP and HAFP instead of deletion mutants of RAFP because our preliminary experiments of expression of the latter type of molecules yielded very small amounts of products.

Table 1. Estradiol binding by AFPs

AFP	Bound estradiol, dpm (% of input)
Hepatoma RAFP	5271 (45.8)
Hepatoma HAFP	28 (0.2)
YRAFP	4271 (37.1)
YHAFP	27 (0.2)
RHHAFP	38 (0.3)
HRRAFP	2991 (26.0)
RHRAFP	35 (0.3)
HRHAFP	4495 (39.1)

It is clear that AFP molecules having RAFP residues 419–516, encoded by the *Pvu* II–*Xho* II cDNA fragment, can bind estradiol (Table 1 and Fig. 2). Several residues at the N and C termini of the peptide are identical to those of HAFP, and the region essential for the binding activity appears to be limited to residues 423–506 (Fig. 2). These 84 residues of RAFP were compared with those of HAFP and it was found that 53 were identical. This means that the substitutions of 31 residues of RAFP result in a loss of estrogen-binding activity and that, in turn, the similar substitutions enable HAFP to bind estrogen. The crucial 31 residues of RAFP were compared with those of mouse AFP (20), which also binds estrogens, and only 7 differences were noted. All of these variable residues were localized between residues 447 and 480. Assuming that both AFPs have similar structure for the function, this part of the peptide is not essential for the activity and the further localization of the activity may be possible. Since there was no site for suitable restriction enzymes in the cDNAs to construct another chimeric AFP cDNA, this has not been explored further. The use of synthetic DNA will be a powerful approach to a more detailed investigation. That a sequence of RAFP composed of 84 amino acids is essential for estrogen-binding activity suggests that this region probably binds estrogen. However, this region might also cooperate with another segment of the molecule to generate this function. This would mean that this peptide region alters the conformation of the AFP molecule and that a spatially different functional site is allosterically formed. Estrogen binding is restricted to rat and mouse AFPs, whose primary sequences show a homology of 83%, while that of RAFP (15) and HAFP (18) is only 65%. The similar structures of the rodent AFPs further suggest the role of this peptide in estrogen binding. The chimeric AFPs HRRAFP and HRHAFP had association constants for estradiol that were essentially the same as that of YRAFP. This most likely indicates that the structures of their binding sites are very similar and are probably located on a similar or identical peptide segment. Although this suggests that the above postulated allosteric model is highly unlikely, further experiments are needed to rule out the possibility.

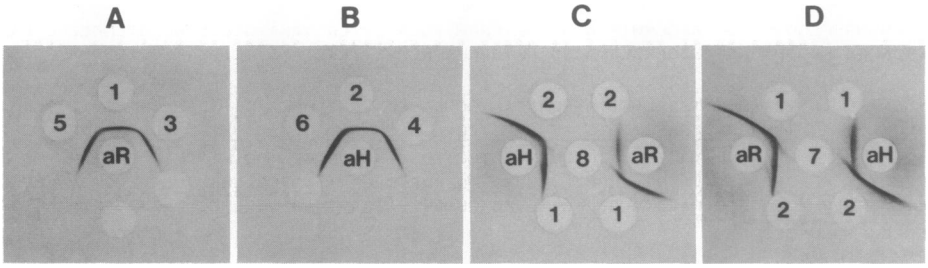

FIG. 4. Immunodiffusion of AFPs. The purified AFPs (1 μg of each) were treated with rabbit antibodies to RAFP (aR) and HAFP (aH) in 1% agarose gels. AFP preparations: 1, hepatoma RAFP; 2, hepatoma HAFP; 3, YRAFP; 4, YHAFP; 5, RHRAFP; 6, HRHAFP; 7, RHHAFP; 8, HRRAFP.

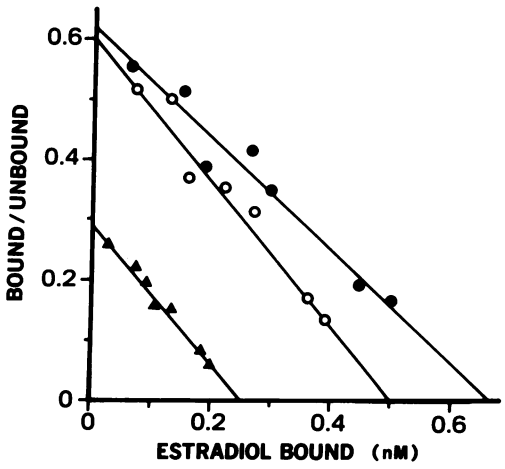

FIG. 5. Scatchard analyses of estradiol binding. Association constants (K_a) obtained from the plots were 11.4×10^8 M^{-1}, 11.8×10^8 M^{-1}, and 9.3×10^8 M^{-1} for YRAFP (●), HRRAFP (○), and HRHAFP (▲), respectively.

RAFP is known to bind not only estrogens but also a number of small ligands such as unsaturated fatty acids, drugs, bilirubin, and retinoids (1, 2, 23). Among them, arachidonic acid (23, 24), tryptophan methyl ester (25), gossypol (26), and phenylbutazone (27) are reported to influence the binding of estrogens to RAFP, and it is postulated that at least some of their binding sites overlap or are spatially close to those of estrogens (24–27). AFP is a three-domain protein that has evolved by triplication of a single primordial domain as proposed for serum albumin by Brown (28), and they have several similar structural and functional characteristics (29). One of the important biological roles of AFP is considered to be the transport of polyunsaturated fatty acids (30). HAFP has three binding sites for these fatty acids (31), one in each domain (32), whereas RAFP has a single high-affinity site, which overlaps with the estrogen-binding site (24). One of the high-affinity fatty acid binding sites of bovine serum albumin is postulated to be located in the middle of domain III (33), which is similar to the estrogen-binding portion described in the present study. Furthermore, crystallographic studies of human serum albumin (34) have shown that there are two binding sites for aspirin and many other compounds within subdomains IA and IIIA. Several compounds such as the diazepines have a higher degree of affinity for subdomain IIIA, which is composed of residues 461–487, and it has been suggested that this portion is the primary fatty acid binding site described previously (33).

The present studies have shown that chimeric AFP molecules can be successfully used for the mapping of biological activity that is localized to a single region of the protein. Such methodology can be applied to other studies, such as the determination of the epitopes recognized by monoclonal antibodies, and should offer valuable approaches to associating various biologic functions with specific segments of AFP.

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