Expression of functional human retinol-binding protein in *Escherichia coli* using a secretion vector

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In order to express human serum retinol-binding protein (sRBP) in *Escherichia coli* in a form that is structurally indistinguishable from the native protein, we placed the coding sequence of the RBP cDNA next to that of the outer membrane protein A (OmpA) signal sequence in the secretion vector, pIN-III-OmpA1. However, this construct did not generate detectable expression of RBP in *E. coli*. When the DNA fragment consisting of the ribosome-binding site and the OmpA–RBP fusion sequence was subcloned downstream to the T7 promoter of pKS-Bluescript, however, the resultant construct (pOmp-RBP2) gave low but detectable secretion of RBP into the periplasm. Deletion of the 3' untranslated region of the RBP cDNA (pOmp-RBP3) further

improved the expression (by approx. 20-fold). After charging with retinol, the secreted RBP was purified from the periplasm on a transthyretin-affinity resin. The purified protein exhibited all the three molecular recognition properties characteristic of sRBP, i.e. it interacted with retinol, transthyretin and its cell-surface receptor. Comparison of the receptor binding properties of the recombinant RBP (rRBP) with those of the serum protein revealed that while the affinity of rRBP is similar to sRBP (50 ± 20 nM), the $B_{\max}$ of the rRBP is about 6–8-fold higher. This indicates that a major proportion of RBP, isolated from serum, is incapable of interacting with the receptor.

INTRODUCTION

Vitamin A is transported in the plasma as a complex with a protein called retinol-binding protein (RBP). It is synthesized mainly in the liver and secreted into plasma bound with retinol (for a review see Goodman, 1984). In plasma, it circulates as a complex with another protein called transthyretin (TTR). Although it was previously believed that formation of this complex occurs in plasma, recent evidence suggests that the RBP–TTR complex can occur in the endoplasmic reticulum (ER) of the liver (Melhus et al., 1991). However, in a subsequent paper (Melhus et al., 1992), using HeLa cells transfected with an eukaryotic expression vector construct, the authors have demonstrated that RBP–TTR assembly is not a requirement for the secretion of RBP, and that apo-RBP accumulates in cells grown under vitamin A deprivation. Addition of retinol to the culture medium stimulated the secretion of retinol-bound RBP. These apparently conflicting observations were made with HeLa cells in a cell culture system *in vitro*. The exact details of the hepatic mechanism of RBP–TTR complex formation, however, remains unclear.

Circulating RBP is recognized by specific receptors on vitamin A-requiring cells, an interaction which facilitates the efficient uptake of retinol into the interior of the cell. Thus, RBP is involved in four molecular recognition events, namely it binds to retinol, to TTR, to a cell-surface receptor, and to the ER.

But for the interaction with retinol, the molecular details of the other recognition properties of RBP are largely unknown. The high-resolution X-ray diffraction analysis (Newcomer et al., 1984; Cowan et al., 1990) of RBP allowed identification of a number of residues involved in interaction with retinol. However, it is not clear what conformational changes induce retinol binding to, or release from, RBP. The difficulty in crystallizing apo-RBP without leaving organic molecules in the binding pocket (Monaco

and Zanotti, 1992) is one of the major reasons for this lack of information. An understanding of these conformational changes is crucial as several physiological functions of RBP, including hepatic secretion, binding to TTR and delivery of retinol to target tissues, all appear to be conformationally dependent.

Although the crystal structure of TTR has been available for quite some time (Blake et al., 1978) that of the complex is not. Very recently, Aqvist and Tapia (1992) modelled the RBP–TTR complex using interactive computer graphics surface docking. The model, however, needs to be supported by experimental data. As regards interaction with the cell-surface receptor, there is no structural information.

It would be possible to address several of these unknowns if RBP could be expressed in a heterologous system. Utilization of hosts such as *E. coli*, which do not contain retinol, could be advantageous since apo-RBP expressed in these cells may not contain organic molecules bound in the pocket. Furthermore, it would allow generation of mutants relatively rapidly to help define the structural elements that specify the diverse interactions of RBP. This paper describes the steps taken to express fully functional RBP in *E. coli*.

EXPERIMENTAL

Bacterial strains

E. coli strain XL-Blue (Stratagene) was used for cloning. BL21 (DE3), which carries in its chromosome the T7 RNA polymerase gene under the *lac* UV promoter was a gift from Dr. F. W. Studier (Brookhaven National Laboratory, Upton, NY, U.S.A.).

Plasmids

pGEM-RBP (a gift from Professor De Witt Goodman, Columbia University, New York, U.S.A.) has the *Eco*RI–*Hind*III fragment

Figure 1 Schematic diagrams of human RBP expression vectors, pOmp-RBP2 and pOmp-RBP3

RBP is the cDNA for human retinol-binding protein, OmpA is the bacterial outer membrane signal sequence, RBS is the ribosome-binding site, Amp^R is the β -lactamase sequence and ϕ 10 (●) is the T7 promoter. The 3' untranslated region of the RBP cDNA, ■■■, is deleted in pOmp-RBP3. The arrow indicates the direction of the coding sequence. Details of construction are described under Methods.

(881 bp) of human RBP (Colantuoni et al., 1983) in pGEM in the same orientation as the β -lactamase gene. pIN-III-OmpA1 (a gift from Professor M. Inouye, University of Medicine and Dentistry of Piscataway, NJ, U.S.A.) is a secretion vector that possesses a tandem *lac-lpp* promoter and the OmpA bacterial outer membrane signal sequence. pLysS (a gift from Dr. F. W. Studier, Brookhaven National Laboratory) carries the T7 lysozyme gene in pACYC 184 (Studier et al., 1990).

Other materials

Restriction endonucleases, Klenow fragment of DNA polymerase I, T4 polynucleotide kinase and T4 DNA ligase were obtained from Boehringer-Mannheim or Pharmacia PL-Biochemicals. *Taq* DNA polymerase was from Perkin-Elmer, Cetus, Norwalk, CA, U.S.A. All-*trans*-retinol, dimethyl sulphoxide, and horseradish peroxidase-conjugated anti-(rabbit IgG) antibody were obtained from Sigma Chemical Co. RBP and TTR were purified from human plasma as described previously (Sivaprasadarao and Findlay, 1988). Rabbit antibody raised to human RBP was a gift from Professor J. Glover (Liverpool University, Liverpool, U.K.).

Construction of expression vector

Construction of the expression vector was carried out using standard cloning techniques (Sambrook et al., 1989). The 0.88 kb *EcoRI*–*HindIII* fragment of the RBP gene in pGEM-RBP was subcloned into the *EcoRI* and *HindIII* sites of pIN-III-OmpA1. The resulting plasmid is referred to as pIN-Omp-RBP. The *XbaI*–*HindIII* fragment of pIN-Omp-RBP, containing the ribosome-binding site, OmpA signal sequence and the RBP gene, was subcloned into the *XbaI* and *HindIII* sites of pKS-Bluescript (pOmp-RBP1) to allow easy manipulation.

The RBP cDNA has a 51 bp-long 5' untranslated region and a 48 bp RBP signal sequence. In order to remove these segments and obtain correct fusion between the last amino acid/nucleotide residue of the OmpA signal sequence and the first amino acid of the mature form of RBP, a PCR-based deletion method was developed. Two synthetic oligonucleotides, 5'-GGCCTGCGCTACGGTACGGTAGCGA-3' and 5'-GAGCGCGACTGCCGAGTG-3', the first corresponding to the C-terminal sequence of OmpA signal peptide (anti-sense strand) and the second to the N-terminal sequence of mature RBP, starting with the first glutamic acid residue (sense strand) were used for deletion PCR. The two oligonucleotides were

phosphorylated using T4 polynucleotide kinase and were then used to amplify the entire recombinant plasmid, except for those sequences which were to be deleted. The PCR mixture (100 μ l) contained 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 1 mg/ml gelatin, 200 μ M of each dNTP, 100 pmol of each phosphorylated primer, 10 ng of supercoiled pOmp-RBP1 and 2.5 units of *Taq* DNA polymerase. Thirty cycles, each consisting of 40 s at 95 °C for denaturation, 1 min at 58 °C for annealing, and 10 min at 72 °C for elongation, with a final extension for 10 min at 72 °C were used. The resulting PCR product was electrophoresed on 1% (w/v) agarose, and purified using DEAE (Na45) membrane (Schleicher and Schuell). The purified product was treated with 2 units of Klenow fragment of DNA polymerase I at room temperature for 10 min in the presence of 0.1 mM dNTPs to remove any nucleotides added to the 3' end of the molecule by *Taq* DNA polymerase. The reaction was terminated by heating for 10 min at 80 °C, followed by extraction with phenol/chloroform/iso-amyl alcohol (24:24:1, by vol.). The DNA was then recovered by precipitation with ethanol in the presence of 2.5 M ammonium acetate, and self-ligated at 16 °C overnight with 1 unit of T4 DNA ligase. After transformation of *E. coli* XL-Blue cells, desired clones were selected using *EcoRI* and *HindIII* digestion of the miniprep DNAs. Deletion mutants, identified by the loss of the *EcoRI* site, were sequenced to confirm the deletion and clones free of PCR errors selected. The method is very rapid and efficient (over 95%) compared with non-PCR-based methods.

The resulting new expression/secretion vector, referred to as pOmp-RBP2 (Figure 1), now had the ribosome-binding site and OmpA signal sequence of pIN-III-OmpA1 vector fused to the coding sequence of the mature form of RBP cDNA downstream of the T7 promoter.

To remove the 3' untranslated region of the RBP cDNA, a *SacI* and *HindIII* restriction fragment was isolated from pOmpA-RBP2, digested with *MaeI* (a unique *MaeI* site is present 12 nucleotides downstream of the stop codon in the RBP gene), the 5' overhangs were filled with the Klenow fragment and then digested with *XbaI*. The resultant fragment containing *XbaI* and blunt ends was purified and subcloned into the *XbaI* and *EcoRV* cut pKS-Bluescript to generate pOmpA-RBP3.

Expression of RBP

E. coli BL21 (DE3) cells transformed separately with pOmp-RBP2 and pOmp-RBP3 were used for the expression of RBP.

Initially, RBP from these cells was detected using [³⁵S]methionine pulse-labelling as described by Tabor and Richardson (1985). For large-scale production, the cells harbouring the plasmid were grown at 37 °C, with shaking at 200 rev./min, in M9 medium supplemented with 2 mg/ml casamino acids, 2 mg/ml of glucose, 10 mM MgCl₂, 1 mM CaCl₂, thiamine (2 µg/ml) and ampicillin (100 µg/ml). When the *A*₆₀₀ of the growing cells reached 0.6, isopropyl-β-thiogalactoside (IPTG) was added to a final concentration of 1.0 mM to induce expression. The growth temperature was then reduced to 30 °C and the cells incubated for a further 4 h to allow RBP biosynthesis. The periplasmic contents were isolated from these cells by scaling up the procedure described by Koshland and Botstein (1980). The culture was centrifuged at 5000 *g* for 10 min and the resulting pellet resuspended in one tenth the volume (relative to the original culture) of ice-cold 20% (w/v) sucrose in 10 mM Tris/HCl, pH 8.0. EDTA (0.5 M) was added to achieve a final concentration of 16 mM and the cells incubated on ice for 10 min. After centrifugation at 10000 *g* for 10 min the pellet was resuspended quickly in ice-cold distilled water, stored on ice for 10 min and then centrifuged at 10000 *g* for 10 min. Expressed RBP in the supernatant was detected by SDS/PAGE, followed by Western blotting using rabbit anti-(human RBP) antibodies and horseradish peroxidase-labelled second antibody.

Purification of expressed RBP

Expressed RBP was purified using a TTR-affinity resin. Human TTR was purified from plasma and coupled to CNBr-activated Sepharose CL-4B using published methods (Vahlquist et al., 1971). The periplasm derived from 2 litres of induced culture was freeze-dried and redissolved in 50 mM Tris/HCl/0.2 M NaCl, pH 7.4, containing 0.2 mM phenylmethanesulphonyl fluoride. To this, all-*trans*-retinol dissolved in dimethyl sulphoxide was added to a final concentration of 0.1 mM. After incubating at 37 °C for 2 h in the dark the mixture was centrifuged at 8000 *g* for 10 min. The clear supernatant was allowed to circulate through the TTR-affinity resin (containing 4 mg of TTR/g of resin) overnight. The column (5 cm × 1.2 cm) was washed with 8 vol. of 50 mM Tris/HCl/0.2 M NaCl, pH 7.4. Elution of bound RBP was achieved with distilled water, which had been adjusted to pH 8.0 with ammonia. RBP in the eluted fraction was detected by measuring the absorbance at 280 and 330 nm and by Western blotting. The purity of RBP was assessed by SDS/PAGE.

Receptor binding assay

Purified RBP was labelled with ¹²⁵I using Enzymobeads (Bio-Rad) according to the instructions of the manufacturer. Binding of radiolabelled RBP to the receptor was determined using an oil centrifugation assay as described previously (Sivaprasadarao and Findlay, 1988).

RESULTS

Construction of expression vectors

The secretion vector, pIN-III-OmpA, developed by Ghrayeb et al. (1984), was chosen as the starting point for construction of the RBP secretion vector for several reasons. First, if the coding sequence of a cloned gene is fused to the bacterial OmpA signal sequence in this vector, the resultant construct is expected to

secrete the expressed protein into the periplasm. The oxidizing environment of the periplasm facilitates the formation of correct disulphide bonds and active conformation. Secondly, authentic protein rather than a fusion protein is obtained. Finally, being secreted, purification of the expressed protein from the periplasm is facilitated.

We have constructed two secretion vectors, pOmp-RBP2 and pOmp-RBP3, for human RBP (Figure 1). The latter differs from the former in lacking the 3' untranslated region of the RBP cDNA. In both these constructs, the coding sequence of mature RBP (i.e. minus the signal sequence) was fused to the C-terminus of the OmpA signal sequence and the fusion sequence was placed under the T7 promoter of pKS-Bluescript. A ribosome-binding site derived from the pIN-III-OmpA vector was present seven nucleotides upstream of the start ATG codon. Both of the constructs, when induced in *E. coli* BL21 (DE3) strain secreted an authentic form of RBP (start amino acid being Glu) into the periplasm. The levels of expression from pOmp-RBP3 was about 20-fold higher than that from pOmp-RBP2, suggesting that the removal of the 3' untranslated region greatly enhanced the level of expression (see below). However, when the OmpA-RBP chimeric sequences from the above constructs were placed under the control of the *lac-lpp* promoter of pIN-III-OmpA vector and the resultant constructs induced in *E. coli* JA221 strain, expression of RBP was not apparent by Western blotting.

Expression of RBP

E. coli BL21 (DE3) was transformed with pOmp-RBP2 or pOmp-RBP3 and expression of RBP was monitored by pulse-labelling of the growing cells with [³⁵S]methionine in uninduced, induced and rifampicin-treated states (Tabor and Richardson, 1985). In this system addition of the inducer, IPTG, to growing BL21 (DE3) cells in mid-exponential phase induces expression of the chromosomal copy of the bacteriophage T7 RNA polymerase from the *lac UV5* promoter. The T7 RNA polymerase then transcribes the gene under the T7 promoter in the plasmid construct. Addition of rifampicin to the induced culture inhibits *E. coli* RNA polymerase and thereby allows the expression only of the gene under the control of T7 promoter. This is manifested in the autoradiogram of the proteins separated by SDS/PAGE, with the expressed protein being the exclusively or predominantly labelled species. Figure 2 shows inducible and rifampicin-insensitive expression of two proteins of apparent molecular masses 24 kDa and 21 kDa from both pOmp-RBP2 (lane F) and pOmp-RBP3 (lane I) vectors. The 24 kDa band would appear to correspond to the OmpA-RBP chimeric protein, i.e. RBP with the OmpA signal sequence still attached, while the 21 kDa band corresponds to the mature form of RBP. These two bands were absent from the parent vector with no RBP insert (lane C).

As can be seen from the intensity of the bands, removal of the 3' untranslated region dramatically improved expression levels. However, the level of expression was still low (200 µg/l). We also noticed that RBP expression was toxic to the host cell, as BL21 (DE3) cells harbouring pOmpA-RBP3 did not grow on Luria broth agar plates containing ampicillin and IPTG. Attempts to improve expression by co-expression of T7 lysozyme from pLysS, a plasmid which was successfully used for improving the expression of a number of toxic proteins (Studier et al., 1990), had little influence on the expression of RBP (lane L). Expression of the OmpA-RBP sequence using other similar vectors such as pET (Studier et al., 1990) and pT7.7 (Tabor and Richardson, 1985) was less efficient in comparison with the construct described above. The secondary structure of RBP mRNA is a probable cause of poor expression. We have not attempted to disrupt

Figure 2 Autoradiogram of SDS/polyacrylamide gel of [^{35}S]methionine pulse-labelled proteins

E. coli BL21 (DE3) cells harbouring pKS-Bluescript (lanes A–C), pOmp-RBP2 (lanes D–F), pOmp-RBP3 (lanes G–I) and both pOmp-RBP3 and pLysS (lanes J–L) were grown at 37 °C with shaking at 200 rev./min in M9 medium supplemented with thiamine (20 $\mu\text{g}/\text{ml}$), 18 amino acids (0.1% minus cysteine and methionine) and ampicillin (50 $\mu\text{g}/\text{ml}$). At an A_{550} of 0.3, 1 ml samples were removed from each incubation (uninduced, lanes A, D, G and J) into separate tubes. To the rest, IPTG was added to 2 mM (induced). After further incubation for 30 min, 1 ml samples were removed into separate tubes (induced, lanes B, E, H and K). To the rest (induced, rifampicin-treated, lanes C, F, I and L) rifampicin (20 mg/ml stock in methanol) was added to a final concentration of 200 $\mu\text{g}/\text{ml}$. Growth of all samples was continued for another 30 min. A sample (1 ml) of each was then pulse-labelled with 5 μCi of [^{35}S]methionine for 5 min. Labelling was terminated by centrifugation of the cells and dissolving the pellets in 120 μl of sample buffer. Each sample (40 μl) was run on a 5–20% SDS/polyacrylamide gel. The dried gel was subjected to autoradiography. The molecular masses of standard proteins are indicated in kDa.

secondary structures by altering the codons as the level of expression was satisfactory for most of our purposes. It is perhaps useful to note that, after induction, growing the cells at 37 °C and for longer than 4 h significantly decreased the yield of the protein.

Purification of expressed RBP

For large-scale expression and purification, RBP was expressed in BL21 (DE3) cells containing pOmpA-RBP3. RBP secreted into the periplasm of these cells did not bind to the TTR-affinity resin as expected from the low affinity of apo-RBP for TTR. The extracts, therefore, were treated with retinol before loading on to the resin. The adsorbed protein was eluted from the column with distilled water, the pH value of which was adjusted to 8.0 with ammonia. The identity of the eluted material was confirmed by Western blotting (Figure 3).

The purified protein was homogeneous on SDS/PAGE with a molecular mass of 21 kDa (Figure 3). Since no unprocessed OmpA-protein band was detectable by Western blotting, it would appear that OmpA-RBP had been completely processed to mature RBP. Moreover, mass spectroscopic analysis of the purified protein showed a single peak with a mass of 21083 Da (results not shown), which corresponds to the full-length (183 amino acids long) RBP.

Functional properties of expressed RBP

The purified RBP showed a u.v.-visible spectrum, similar to holo-RBP, with a $A_{330/280}$ ratio of 1.0 (Figure 4), suggesting

Figure 3 SDS/PAGE of purified rRBP

(a) Coomassie Blue-stained gel; (b) Western blot of the purified rRBP.

that expressed RBP bound retinol in a 1:1 ratio. The fact that the expressed RBP could be retained by the TTR-affinity resin suggests that the recombinant RBP (rRBP) is capable of interacting with TTR. The affinity of rRBP for TTR, however, was not examined in the present study. The third property of RBP, i.e. binding to a cell-surface receptor, was examined by an oil centrifugation assay method using human placental membranes and rRBP labelled with ^{125}I as described previously (Sivaprasadarao and Findlay, 1988). Table 1 shows that the labelled rRBP binds to the receptor about seven times better than serum RBP (sRBP). During the course of these studies, we observed that sRBP loses its binding affinity on storage, even at –20 °C. For instance, sRBP which had been stored for ten months had lost nearly 80% of its binding activity. After a year of storage, hardly any receptor-binding activity was detectable with the stored protein. This protein had been stored in aliquots, and had not been thawed and refrozen between assays. The protein did not show any signs of degradation by SDS/PAGE, nor had it lost its ability to bind to the TTR-affinity resin. It was also observed that certain preparations of RBP, particularly those prepared from very out-dated plasma, showed little or no

Figure 4 U.v.-visible spectrum of purified rRBP

Table 1 Dependence of RBP binding on the age of the protein

The proteins were labelled with ^{125}I -RBP and their binding activities were examined using placental membrane vesicles (1.0 mg/ml protein) using an oil centrifugation assay. Non-specific binding was determined in the presence of a 1000-fold excess of unlabelled RBP. The assays were carried out in triplicate and the average values given. The standard errors were within 5%.

^{125}I -labelled RBP	Input (c.p.m.)	Bound (c.p.m.)		
		Total	Non-specific	Specific
Recombinant	75 000	20 407	7150	13 257
Serum (freshly isolated)	73 500	4145	2135	2010
Serum (10-month-old)	80 500	3460	3025	435

receptor-binding activity, although neither retinol- nor TTR-binding properties of the protein were lost.

The reasons for the difference in the binding activities of sRBP and rRBP were investigated by Scatchard analysis (Figure 5). The equilibrium dissociation constant, K_D , of rRBP for the placental receptor was found to be 50 ± 20 nM ($n = 3$). The calculated B_{max} was 6 ± 2 pmol/mg of membrane protein. The K_D value obtained with the recombinant protein is in close agreement with the values reported by us (Sivaprasadarao and Findlay, 1988) for the placental receptor and more recently, by Bavik et al. (1991) for bovine pigment epithelial cells. However, under the conditions of Scatchard analysis, the high-affinity component ($K_D = 3$ nM), reported for the serum protein in placental membranes (Sivaprasadarao and Findlay, 1988), could not be detected with rRBP. In contrast with the K_D values, the B_{max} value obtained with the recombinant protein (6 ± 2 pmol/mg of protein) is about six to eight times higher than that (0.8 ± 0.08 pmol/mg of protein) reported for placental membranes with sRBP. These results suggest that RBP isolated from the plasma contains a high proportion of RBP in a form which is incapable of binding to the receptor. This is further

Figure 5 Competitive inhibition of ^{125}I -recombinant RBP binding to placental membrane vesicles by rRBP (a) and the Scatchard analysis of the binding data (b)

(a) Membranes (1 mg/ml) were incubated with ^{125}I -rRBP (2.4 nM) at 37°C in a $100\ \mu\text{l}$ volume for 15 min in the absence or presence of various concentrations of rRBP. The binding reactions were terminated by oil centrifugation. (b) The competition data were plotted in Scatchard form as described previously (Sivaprasadarao and Findlay, 1988). The calculated K_D and B_{max} values are 48 nM and 7.8 pmol/mg of membrane protein respectively.

Table 2 Competition of ^{125}I -rRBP binding to placental membranes by rRBP and sRBP

Competing protein	Concentration (μM)	Bound (c.p.m.)
None		20620
rRBP	0.05	10250
rRBP	0.5	8960
sRBP	0.5	17610
sRBP	10.0	11780

confirmed in a competitive displacement experiment in which we followed the concentration dependence of displacement of the receptor-bound ^{125}I -RBP by rRBP as well as by sRBP. The results shown in Table 2 clearly demonstrate that rRBP displaces the receptor-bound ^{125}I -RBP at a much lower concentration than the serum protein. The proportion of the inactive form in a given sRBP preparation appears to depend on the age of the plasma

used for purification, the length of storage of the purified protein and, possibly, on the purification protocol used.

DISCUSSION

To express RBP which is structurally as close to the serum protein as possible, we chose the secretion vector, pIN-III-OmpA (Ghrayeb et al., 1984) which has been successfully used to express a number of proteins in *E. coli* (Ghrayeb et al., 1984; Ray et al., 1988). Being a secretion vector, extracellular proteins expressed using this system are usually properly folded and biologically active. For proteins such as RBP, which has three disulphide bonds, the oxidizing environment of the periplasm is critical for formation of appropriate disulphide bridges. However, we were unable to express RBP in detectable amounts using this system. We constructed, therefore, a new secretion vector (pOmp-RBP2) by incorporating the ribosome-binding site and the OmpA bacterial signal sequence and the RBP cDNA sequence into pKS-Bluescript downstream of the T7 promoter. Using this new vector construct, it was possible to detect expression of RBP in *E. coli* BL21 (DE3) strain, but the level of expression was poor (about 10 µg/litre of culture). The reasons for this improvement are not clear, but the higher copy number of pBluescript-derived expression vector compared with the pBR322-derived pIN-III-OmpA vector may be responsible for the enhanced expression. When we deleted the 3' untranslated region of RBP cDNA we noticed a dramatic improvement in the expression level. Up to 200 µg of rRBP could be purified from 1 litre of culture. This result is surprising since, to our knowledge, there are no reports which suggest that 3' untranslated regions have any influence on the expression of cloned cDNA in prokaryotic expression systems.

Both pulse-labelling and Western-blotting experiments clearly demonstrated that the initial OmpA-RBP chimeric protein had been processed to mature RBP by the bacterial signal peptidase during its transport into the periplasm. Mass spectroscopic studies (results not shown) on rRBP indicated that the protein had a molecular mass of 21083 Da, which corresponds to the full-length protein of 183 amino acids. By contrast, RBP isolated from plasma has been shown to be truncated at the C-terminus by one residue (Sundelin et al., 1985).

Expressed RBP appears to have assumed correct conformation, with the appropriate disulphide bridges, as is evident from its ability to exhibit all the three molecular recognition properties of the native protein. It bound retinol in 1:1 ratio and it interacted with both TTR and the membrane receptor. RBP, isolated from serum, has previously been shown to consist of two forms, one able to bind to the placental receptor with high affinity ($K_D = 3.0$ nM) and the other with low affinity ($K_D = 80$ nM). With the recombinant protein, however, we noticed homogeneous binding with a K_D value of 50 ± 20 nM. The high-affinity component has not been observed under the conditions of Scatchard analysis that we have used. We are not certain at this stage whether the high-affinity component is absent from the rRBP preparation or whether its concentration, in comparison with the low-affinity form, is so low that it escaped detection under the conditions of our assay. It has to be emphasized here that the high-affinity form of sRBP, as has been shown previously (Sivaprasadarao and Findlay, 1988), is highly unstable and undergoes rapid inactivation. Moreover, we found that it is often not detectable in certain preparations of sRBP. The structural differences between the high- and low-affinity forms of RBP are under further investigation as these may have some important functional implications.

Scatchard analysis revealed that rRBP bound to more receptor sites on placental membranes than the serum protein, as reflected in a 6- to 8-fold increase in the B_{max} value. This, together with the fact that rRBP displaces 125 I-RBP from the receptor at a much lower concentration than the serum protein, suggests that a major proportion of RBP isolated from serum is incapable of interacting with the receptor. This failure to recognize the receptor occurs independently of its ability to bind retinol or TTR. An important implication of this finding is that use of sRBP to assay the receptor content of tissues may lead to gross underestimates. Moreover, we noticed that RBP, upon storage, gradually loses its receptor-binding ability, without any detectable loss in retinol- or TTR-binding activities. This means that in receptor-binding assays one has to be aware not only of variations between different batches but also within the same batch.

Several laboratories (Raz et al., 1970 and Peterson, 1971) have reported charge microheterogeneity of RBP. They found that on native gel electrophoresis, sRBP produces four bands, representing two apo- and two holo-forms. The relative amounts of these bands depends upon the age of the plasma, the extent of handling during purification and the length of storage of purified RBP. Raz et al. (1970), for example, indicated that RBP isolated quickly from freshly drawn plasma showed only one form of holo-RBP (designated H1), which migrated slowly on gel electrophoresis. Upon storage, H1 was found to be converted into a fast-migrating holo-form (designated as H2). Thus, storage of RBP, whether in crude or purified form, appears to cause some structural modification of the protein. Although the precise details of these changes remain to be determined, it is tempting to draw a correlation with the loss of the receptor-binding activity we observe. Further studies on the nature of these changes could provide some clues to the physiological mechanism responsible for the inactivation of RBP following the delivery of retinol to target tissues.

There are several reports in the literature on the failure to detect a receptor for RBP using 125 I-labelled RBP (Rask et al., 1980; Creek et al., 1989; Shingleton et al., 1989). We (Sivaprasadarao and Findlay, 1988) and recently Bavik et al. (1991) have shown that, in view of the short half-life of RBP and its receptor complex ($t_{1/2} = 4$ min), it is important to use binding-assay methods which allow rapid separation of membrane-receptor-bound 125 I-RBP from the unbound label. The use of procedures which employ extensive washing of the membrane-bound complex to reduce the background levels results in complete dissociation of the specific complex, thereby giving false negative results. A further reason for the reported failures, revealed in the present study, lies in RBP itself. Accordingly, it is very important to use RBP which has been isolated from relatively fresh plasma, preferably using rapid isolation methods. The use of rRBP, however, is preferable in view of its higher specific receptor-binding activity.

The availability of fully functional rRBP now allows a number of experiments aimed at providing information on the molecular behaviour of RBP. First, it provides an opportunity to crystallize apo-RBP. The crystal data, in conjunction with site-directed mutagenesis, would provide valuable information on the conformational changes involved in retinol binding/release. Secondly, it is possible to identify the regions of RBP involved in binding to TTR and to its membrane-bound receptor. Such studies would also provide valuable information on the mechanism of uptake of retinol by tissues. Finally, rRBP, being homogeneous with high receptor-binding activity, is a superior ligand for purification of the RBP receptor using affinity chromatography. The ability of the present expression system to generate RBP

labelled with a radioactive amino acid (rather than covalent modification with ^{125}I) makes it an attractive choice for studying the metabolism of RBP in whole animals.

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