Journal of Chromatography, 452 (1988) 359–367
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 601

PURIFICATION AND PARTIAL AMINO ACID SEQUENCE OF HUMAN URINE PROTEIN 1

EVIDENCE FOR HOMOLOGY WITH RABBIT UTEROGLOBIN

PHILIP J. JACKSON and RONALD TURNER
Unit for Cancer Research, University of Leeds, Leeds LS2 9NL (U.K.)
JEFFREY N. KEEN
SERC Sequence Unit, Department of Biochemistry, University of Leeds, Leeds LS2 9JT (U.K.)
and
ROBERT A. BROOKSBANK and EDWARD H. COOPER*
Unit for Cancer Research, University of Leeds, Leeds LS2 9NL (U.K.)

SUMMARY

We describe the purification of Urine Protein 1 (UP1), a 14–16 kDa protein which occurs in the urine of patients with renal failure, and therefore may originate from the plasma or kidney. Amino acid sequencing shows that UP1 has significant homology with rabbit uteroglobin, a secretory protein of the uterus (during pregnancy) and lungs (both sexes), and previously identified only in lagomorphs (rabbits, hares, pikas). The finding of a human uteroglobin-like protein, which can be purified from a readily available source, may provide further opportunities to elucidate the, as yet, uncertain physiological functions of uteroglobin.

INTRODUCTION

Urine Protein 1 (UP1) is a small protein, with a reported molecular weight of about 20 kDa, which has been found in the urine of patients with renal failure[1]. Consequently, because of its potential clinical application, a commercially produced antiserum to UP1 has been available for several years (Dako, High Wycombe, U.K.).

As is the case for any urinary protein, there are several possibilities for the original source of UP1. A major source of urinary proteins, especially in renal disease, is the plasma. Proteinuria may occur as a result of damage to the glomerular charge-size barrier, indicated by the excretion of plasma proteins >40 kDa, *e.g.* transferrin (80 kDa) and albumin (67 kDa), which would normally be retained in the plasma without entering the glomerulus[2]. Small plasma proteins (<40 kDa) do enter the glomerulus and are normally reabsorbed by the proximal tubules[3]. In renal tubular disease, therefore, the urinary concentration of retinol-binding protein (21 kDa)[4] and α1-microglobulin (27 kDa)[5], for example, is increased. Similarly the excretion of UP1, if it does occur in the plasma, would be by this mechanism.

0021-9673/88/$03.50 © 1988 Elsevier Science Publishers B.V.

Alternatively, UP1 may be released into the urine by the kidney itself, reaching detectable concentrations where pathological damage is present. This is exemplified by the increased excretion of the distal tubular component, Tamm-Horsfall glycoprotein during kidney transplant rejection[6].

A recent study suggesting that UP1 is a plasma protein[7,8] has been corroborated in our laboratory[9] by the finding that its excretion can be induced in healthy volunteers by experimentally inhibiting proximal renal tubular function[10]. However, the possibility that UP1 also occurs in the kidney cannot be ruled out since it has been detected in (a) tissues such as liver and prostate[7,8] in addition to the plasma and (b) renal failure urine where tubular disease is not evident[9].

Clearly, UP1 merits further study, and for this purpose we describe a protocol for the isolation of UP1 from the urine of patients with renal failure. Subsequent amino acid sequencing shows that UP1 is homologous with rabbit uteroglobin[11–13], a secretory protein found in high concentrations in the uterus (during pregnancy)[11–13] and lungs[14]. A uteroglobin-like protein has not been previously found in any species other than lagomorphs (rabbits, hares and pikas)[15,16].

EXPERIMENTAL

Materials and apparatus

Urine from patients with chronic renal failure, attending for dialysis at Leeds General Infirmary, was stored at −70°C in the presence of 0.1% (w/v) sodium azide and 5 m*M* disodium EDTA. Chemicals (analytical grade or equivalent) were obtained from BDH (Poole, U.K.) or Sigma (Poole, U.K.). Rabbit antiserum to UP1 was supplied by Dako.

Protein solutions were concentrated in pressure ultrafiltration cells (50 and 180 ml capacity) fitted with YM5 membranes (Amicon, Gloucester, U.K.) and dialysed in SpectraPor 3 membrane tubing (Pierce, Cambridge, U.K.).

Chromatography wax carried out using a Pharmacia FPLC system with an LCC-500 controller, two P-500 pumps and a UV-M absorbance monitor with a 5-mm flow-cell. All columns (Superose 12 prep grade HR 16/50, PD-10, Mono Q HR 5/5 and Mono S HR 5/5) and DEAE Sepharose Fast Flow gel were obtained from Pharmacia (Uppsala, Sweden).

Analytical methods

Protein in concentrated urine was estimated by the trichloroacetic acid–Ponceau S method[17] using the Sigma standard protein solution containing human albumin and γ-globulin.

During the purification, UP1-containing fractions were identified by counter immunoelectrophoresis[18] *vs.* anti-UP1 antiserum in a 1-mm layer of 1% (w/v) agarose gel containing 3% (w/v) poly(ethylene glycol) 6000 (PEG), 73.2 m*M* Tris, 24.4 m*M* diethylbarbituric acid, 0.4 m*M* calcium lactate and 3.0 m*M* sodium azide, pH 8.6. The samples and antiserum were placed in 5-μl wells 1 cm apart, towards the cathode and anode, respectively, and electrophoresed for 2 h at 5 V/cm. The recovery of UP1 at each stage was monitored by radial immunodiffusion[19] in agarose gel with the above composition. The purity and composition of fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on Pharmacia

PhastSystem™ using pre-cast 8–25% acrylamide gradient gels. The electrophoresis and silver-staining methods were as described by Jackson *et al.*[20].

The p*I* of UP1 was determined by isoelectric focusing on Pharmacia PhastSystem, using a pre-cast pH 4–6.5 gel[20].

For amino acid sequencing, UP1 (1 nmol) was dialysed *vs.* 5 m*M* sodium hydrogencarbonate, lyophilised and dissolved in 0.03 ml of 0.2 *M* sodium hydrogencarbonate, 0.25% (w/v) SDS. The remaining procedure was as described by Cavaggione *et al.*[21]. The program QUICKP was used to search the NBRF (PIR) protein sequence database (release 13.0) for UP1 homologues.

Purification of UP1

Urine containing UP1 (detected by counter immunoelectrophoresis, see above) was concentrated by pressure ultrafiltration to give 100 ml of concentrate containing 5–6 g of protein. Then 25 m*M* bis-tris propane was added to the urine protein concentrate and the pH adjusted to 7.0 with 50% (v/v) acetic acid. An equal volume (100 ml) of 50% (w/v) PEG was added slowly with stirring and the mixture incubated on ice for 15 min. Precipitated protein was removed by centrifugation (3500 *g*, 30 min) and the supernatant (containing UP1) diluted with 4 volumes of 50 m*M* Tris–HCl, pH 7.0. This fraction was applied in two separate batches to a DEAE Sepharose Fast Flow column (300 mm × 16 mm) at 1 ml/min, and non-bound material (including PEG) was removed by washing with 50 m*M* Tris–HCl, pH 7.0. Bound proteins (including UP1) were desorbed with 1.5 *M* sodium chloride in Tris buffer, concentrated to $A_{280} \simeq 6$ and chromatographed in separate 2-ml aliquots on a Superose 12 prep grade HR 16/50 gel filtration column in 0.15 *M* sodium chloride, 50 m*M* sodium potassium phosphate, pH 7.0, at a flow-rate of 1 ml/min. UP1-containing fractions were pooled, concentrated and buffer-exchanged into 6.25 m*M* bis-tris propane, pH 7.5, on PD-10 desalting columns, according to the manufacturer's instructions. Aliquots (14 ml) (A_{280} = 0.045) were then applied separately to a Mono Q HR 5/5 anion-exchange column equilibrated with 6.25 m*M* bis-tris propane, pH 7.5. Protein elution was by a 20-ml 0–100% gradient of 0.35 *M* sodium chloride, 6.25 m*M* bis-tris propane, pH 9.5, flow-rate 1 ml/min. Fractions containing UP1 were pooled, buffer-exchanged into 50 m*M* sodium succinate, pH 4.0, and applied to a Mono S HR 5/5 cation-exchange column equilibrated with the same buffer. A 20-ml 0–100% gradient of 50 m*M* sodium succinate, pH 5.5 (flow-rate 1 ml/min), was used for the elution of purified UP1, which was then immediately adjusted to pH 7.5 with sodium hydroxide and dialysed *vs.* 6.25 m*M* bis-tris propane pH 7.5.

RESULTS

Purification of UP1

The protein composition of the UP1-containing fractions at each stage in the purification was determined by SDS-PAGE. Fig. 1a shows a typical pattern for renal failure urinary proteins where both glomerular and proximal tubular lesions are evident. Some of the proteins occurring in this complex mixture are identified in ref. 20. This pattern was considerably simplified in the supernatant after precipitation with 25% (w/v) PEG (Fig. 1b). However, Table I shows that only 52% of the UP1 in the urine protein concentrate was recovered in the supernatant, with the remainder

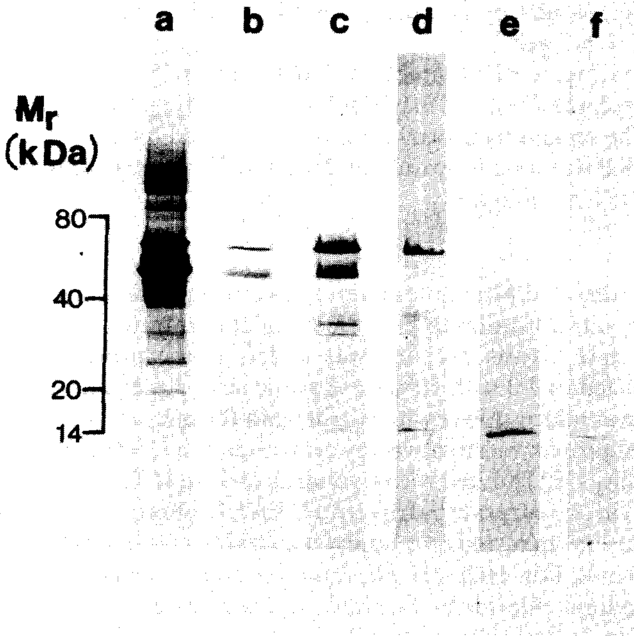

Fig. 1. Stages in the purification of UP1. Samples (1 μl) of UP1-containing fractions were analysed by SDS-PAGE[20] in the absence of 2-mercaptoethanol. (a) Urine protein concentrate (diluted 100-fold); (b) PEG supernatant; (c) DEAE Sepharose-bound proteins; (d) Superose 12 fraction (concentrated 100-fold); (e) Mono Q fraction; (f) Mono S fraction. Protein bands are silver-stained[20].

presumably trapped in the pellet of precipitated protein. A further 48% could be recovered by redissolving and re-precipitating (not accounted for in the remaining results in Table I).

The next step, anion-exchange chromatography on DEAE Sepharose, served to

TABLE I

RECOVERY OF UP1 DURING PURIFICATION

UP1 was estimated by radial immunodiffusion[19] of 5-μl samples into agarose gel containing 0.02 ml/ml anti-UP1 antiserum. Arbitrary units were calculated from the square of the precipitin ring diameter in decimeters multiplied by the appropriate volume factor. No ring was visible in the Superose 12 fraction.

Purification step/fraction	*UP1 recovered (arbitrary units)*	*Percentage of total recovered*
Urine protein concentrate	111	100
Poly(ethylene glycol) supernatant	58	52
DEAE Sepharose	53	48
Superose 12	–	–
Mono Q	11	10
Mono S	9	8

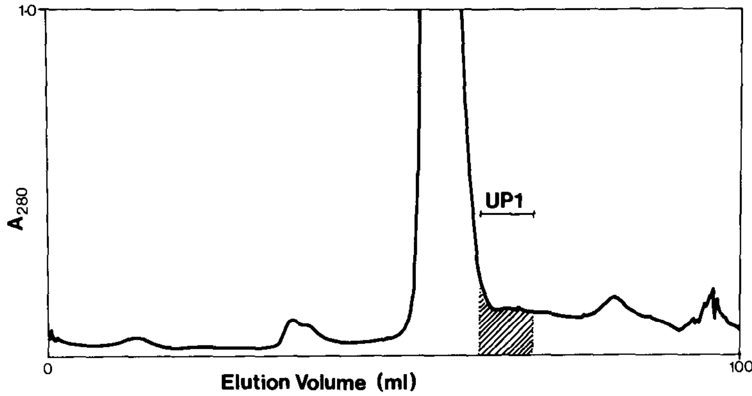

Fig. 2. Gel filtration of DEAE Sepharose-bound proteins on a Superose 12 prep grade HR 16/50 column.

(a) remove PEG, which is non-ionic, from the protein mixture and (b) concentrate the sample. The bound proteins (including UP1) were desorbed in one peak (chromatogram not shown) by using a 1.5 *M* sodium chloride wash. Employing a salt gradient did not fractionate the proteins to any greater extent. The concentrating effect of the DEAE Sepharose is seen in Fig. 1c, in which proteins > 80 kDa become visible (*cf.* Fig. 1b) as diffuse bands. These high-molecular-weight proteins (*e.g.* immunoglobulins) were removed by gel filtration on Superose 12 (Fig. 1d and Fig. 2) and the Mono Q fraction (Fig. 1e and Fig. 3) shows a considerable enhancement in the concentration of UP1 (at *ca.* 14 kDa). The recovery of UP1 over these two steps, however, was only about 20% (Table I) and may be explained by the accumulated loss of material over the seven gel filtration runs (and buffer exchanges) which were necessary to process the whole of the DEAE Sepharose-bound fraction.

A final purification was effected by cation-exchange chromatography on Mono S with an increasing pH gradient (4.0–5.5). UP1 eluted at pH 4.6–4.7 (Fig. 1f; chromatogram not shown) which, as expected, corresponds with its p*I* (see below). Table I shows that the overall recovery of UP1 from a single PEG fractionation of 5.6

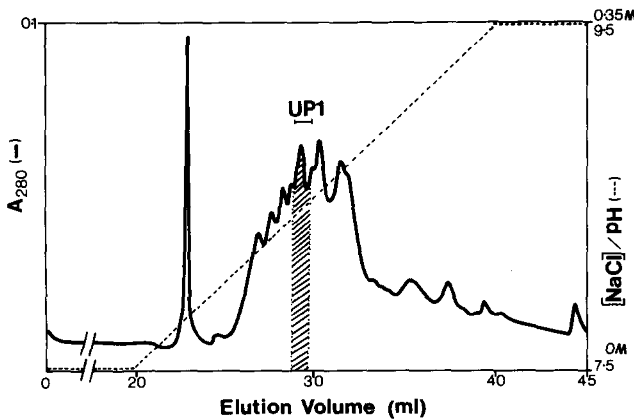

Fig. 3. Anion-exchange chromatography of the UP1-containing Superose 12 fraction on Mono Q HR 5/5.

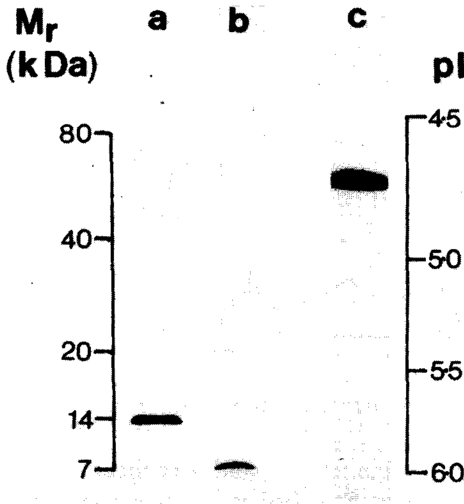

Fig. 4. Molecular weight and p*I* determinations of UP1 by SDS-PAGE (8–25% acrylamide gradient) in the absence (a) and presence (b) of 2-mercaptoethanol, and isoelectric focusing (pH range 4.0–6.5) (c). Protein bands are visualised by immunoblotting *vs.* anti-UP1 antiserum[20].

g of urinary protein was 8%. The mass of UP1 recovered was estimated as 0.3 mg by assuming $A_{280}^{10 g/l} = 1.2$, the extinction coefficient of rabbit uteroglobin[22] (see below).

Molecular weight and pI of UP1

Analysis of UP1 by SDS-PAGE under non-reducing conditions and comparison with molecular weight standards (Pharmacia) indicated an M_r of 14–16 kDa (Fig. 4a). In the presence of 2-mercaptoethanol, the M_r of UP1 decreased to 7–8 kDa (Fig. 4b), indicating that the protein is composed of two subunits of similar or equal M_r bound by disulphide bridges.

Isoelectric focusing and comparison with p*I* standards (Pharmacia) determined the p*I* of UP1 as 4.7 (Fig. 4c).

Amino acid sequence of UP1

Fig. 5 shows the partial amino acid sequence (to residue 47) of UP1. A computerised search of a protein sequence database revealed that UP1 has significant homology with rabbit uteroglobin.

DISCUSSION

The UP1 for commercial antibody production was prepared from the urine of renal failure patients by gel filtration followed by ion-exchange chromatography on DEAE Sephadex and SP Sephadex[23]. UP1 has also been isolated from renal failure urine by affinity chromatography on immobilised anti-UP1 immunoglobulin[7,8]. In our hands, neither method produced UP1 of sufficient purity to give a single residue per cycle during amino acid sequencing. The complexity of the urinary protein mixture used as the source of UP1 therefore merited a different protocol, which is described

										10
UP1:	Gly	Ile	Cys	Pro	Ser	Phe	Gln	Arg	Val	Ile
	**	**	**	**		**		*	**	**
Utg:	Gly	Ile	Cys	Pro	Arg	Phe	Ala	His	Val	Ile
										20
UP1:	Glu	Thr	Leu	Leu	Met	Asp	Thr	Pro	Ser	Ser
	**		**	**	*		**	**	**	**
Utg:	Glu	Asn	Leu	Leu	Leu	Gly	Thr	Pro	Ser	Ser
										30
UP1:	Tyr	Glu	Ala	Ala	Met	Glu	Leu	Phe	Ser	Pro
	**	**			*			**		**
Utg:	Tyr	Glu	Thr	Ser	Leu	Lys	Glu	Phe	Glu	Pro
										40
UP1:	Asp	Gln	Asp	Met	Arg	Glu	Ala	Gly	Ala	Gln
	**	*		**	*	*	**	**	*	**
Utg:	Asp	Asp	Thr	Met	Lys	Asp	Ala	Gly	Met	Gln
										50
UP1:	Leu	Lys	Lys	Leu	Val	Asp	Thr	—	—	—
	*	**	**	*	*	**	*			
Utg:	Met	Lys	Lys	Val	Leu	Asp	Ser	Leu	Pro	Gln
										60
UP1:	—	—	—	—	—	—	—	—	—	—
Utg:	Thr	Thr	Arg	Glu	Asn	Ile	Met	Lys	Leu	Thr
										70
UP1:	—	—	—	—	—	—	—	—	—	—
Utg:	Glu	Lys	Ile	Val	Lys	Ser	Pro	Leu	Cys	Met

Fig. 5. Comparison of the partial amino acid sequence of UP1 with the complete subunit sequence of rabbit uteroglobin (Utg)[13]. Regions of exact homology are indicated by double asterisks and conservative replacements by single asterisks. Residues delineating the progesterone binding site in uteroglobin[28] are underlined.

here. About 0.3 mg of UP1 was recovered from 5.6 g of urinary protein. The overall yield was 8%, but this could be improved by re-extracting the PEG precipitate.

The partial amino acid sequence of UP1 indicates significant homology with rabbit uteroglobin: in the first 47 residues, 55% are identical and a further 23% conservatively replaced (Fig. 5)[11–13]. This finding is supported by the similarities of UP1 and uteroglobin in molecular weight (both 14–16 kDa; Fig. 4a)[12] and subunit composition (both 2 × 7–8 kDa; Fig. 4b)[12]. Their p*I* values, however, are 4.7 (UP1, Fig. 4c) and 5.4[24] (uteroglobin). This difference of 0.7 units may be explained by the substitution of basic residues in uteroglobin with acidic or neutral residues in UP1 (*e.g.* Arg 5 → Ser, Lys 26 → Glu; see Fig. 5). Nevertheless, we present the first evidence for a uteroglobin-like protein in a non-lagomorph species and suggest that this protein may be present in all mammals.

In rabbits, uteroglobin (also called blastokinin) is secreted by the endometrial cells of the uterus on induction with progesterone, either during pregnancy or

experimentally. Secretion is maximal between the fifth and tenth day of pregnancy, when uteroglobin constitutes 50% of the protein in the endometrial fluid[11]. In addition, uteroglobin binds progesterone with high affinity ($K_d = 5 \cdot 10^{-7}\ M$)[25], implying that it acts as a carrier for its inducing steroid.

Uteroglobin has also been isolated in significant quantities from rabbit[14,15,24] and hare[16,26] lungs, where its secretion is induced, not by progesterone but by glucocorticoids[14,24]. With the further finding that uteroglobin inhibits phospholipase A_2 activity[27], this protein also appears to be involved in the regulation of prostaglandin synthesis. Since prostaglandins mediate certain inflammatory responses, it has been suggested that the anti-inflammatory action of glucocorticoids may occur via uteroglobin, in addition to other glucocorticoid-induced proteins (*e.g.* lipomodulin, macrocortin and renocortin)[27].

The physiological function(s) of rabbit uteroglobin are, at present, uncertain. However, it is likely that human UP1 has the same function(s) because of the high degree of sequence homology between the two proteins. This homology even extends to those residues which, according to X-ray crystallographic data on uteroglobin[28], delineate the progesterone binding site (see Fig. 5).

The evidence that UP1 occurs in the plasma[7–9] and certain other tissues, including the liver, prostate[7,8] and possibly the kidney, suggests its widespread location in the body. This also appears to be the case for uteroglobin, which is detectable, albeit in small concentrations, in tissues other than the uterus and lungs[15,29].

Since studies to further elucidate the physiological functions of uteroglobin will depend on obtaining quantities of purified protein, urine from renal failure patients may provide a readily available alternative to laboratory animals.

ACKNOWLEDGEMENTS

We thank Miss Jeanette Gorst, Dako, High Wycombe, U.K., for arranging the generous donation of antiserum to Urine Protein 1. We also thank Miss Katharine Richmond for expert technical assistance.

REFERENCES

1 *Product List*, Dako Ltd., High Wycombe, 1987.
2 R. H. R. White, *Contrib. Nephrol.*, 24 (1981) 63.
3 W. Strober and T. A. Waldman, *Nephron*, 13 (1974) 35.
4 M. D. Poulik, D. Farrah, G. H. Malek, C. J. Shinnick and O. Smithies, *Biochim. Biophys. Acta*, 412 (1975) 326.
5 H. Yu, Y. Yanagisawa, M. A. Forbes, E. H. Cooper, R. A. Crockson and I. C. M. MacLennan, *J. Clin. Pathol.*, 36 (1983) 253.
6 R. H. Schwartz, J. D. Van Ess, A. G. May, E. A. Schenk and R. B. Freeman, *Transplantation*, 16 (1973) 83.
7 J. Ayatse, *Ph.D. Thesis*, University of Surrey, Guildford, 1987.
8 J. W. Wright, University of Surrey, personal communication.
9 P. J. Jackson and E. MacNamara, unpublished results.
10 D. St. J. O'Reilly, E. S. Parry and J. T. Whicher, *Clin. Chim. Acta*, 155 (1986) 319.
11 R. A. Popp, K. R. Foresman, L. D. Wise and J. C. Daniel, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 5516.
12 H. Ponstingl, A. Nieto and M. Beato, *Biochemistry*, 17 (1978) 3908.
13 M. Atger, J.-C. Mercier, G. Haze, F. Fridlansky and E. Milgrom, *Biochem. J.*, 177 (1979) 985.

14 D. Fernandez-Renau, M. Lombardero and A. Nieto, *Eur. J. Biochem.*, 144 (1984) 523.
15 T. K. Torkkeli, K. K. Kontula and O. A. Jänne, *Mol. Cell. Endocrinol.*, 9 (1977) 101.
16 A. Nieto and M. Lombardero, *Comp. Biochem. Physiol.*, 71B (1982) 511.
17 M. A. Pesce and C. S. Strande, *Clin. Chem.*, 19 (1973) 1265.
18 A. Bussard, *Biochim. Biophys. Acta*, 34 (1959) 258.
19 G. Mancini, A. Q. Carbonara and J. F. Heremans, *Immunochemistry*, 2 (1965) 235.
20 P. J. Jackson, C. J. Sampson, E. H. Cooper, D. Heney and J. T. Brocklebank, *Ann. Clin. Biochem.*, 25 (1988) 319.
21 A. Cavaggione, R. T. Sorbi, J. N. Keen, D. J. C. Pappin and J. B. C. Findlay, *FEBS Lett.*, 212 (1987) 225.
22 A. Nieto, H. Ponstingl and M. Beato, *Arch. Biochem. Biophys.*, 180 (1977) 82.
23 J. Gorst, Dako, High Wycombe, personal communication.
24 T. Torkkeli, T. Krusius and O. Jänne, *Biochim. Biophys. Acta*, 544 (1978) 578.
25 M. Beato and R. Baier, *Biochim. Biophys. Acta*, 392 (1975) 346.
26 M. Soledad López de Haro and A. Nieto, *Arch. Biochem. Biophys.*, 226 (1983) 539.
27 S. W. Levin, J. de B. Butler, U. K. Schumacher, P. D. Wightman and A. B. Mukherjee, *Life Sci.*, 38 (1986) 1813.
28 J. P. Mornon, F. Fridlansky, R. Bally and E. Milgrom, *J. Mol. Biol.*, 137 (1980) 415.
29 H. M. Beier, H. Bohn and W. Muller, *Cell Tissue Res.*, 165 (1975) 1.