A highly conserved surface loop in the C-terminal domain of ovotransferrin (residues 570–584) is remote from the receptor-binding site

Anne B. MASON,* Stephen A. BROWN and William R. CHURCH

Department of Biochemistry, University of Vermont College of Medicine, Burlington, VT 05405, U.S.A.

A peptide corresponding to a surface loop in the C-terminal domain of chicken ovotransferrin (residues 570–584) was made by solid-phase synthesis and used to immunize rabbits. A 15-amino acid-residue disulphide-linked loop occurs in both domains of all five transferrins for which the sequence is available and lies on the opposite side of the iron-binding site from the interdomain cleft. Polyclonal antibodies to the peptide were specific for non-reduced holo-ovotransferrin and the C-terminal domain, as shown by e.l.i.s.a. and immunoblotting. The antibody did not inhibit binding of ovotransferrin to receptors on chick-embryo reticulocytes but was able to bind ovotransferrin bound to the cellular receptors at 0 °C. The loop composed of residues 570–584 appears to be remote from the transferrin receptor-binding site.

INTRODUCTION

The transferrins (TFs) are a group of homologous glycosylated metal-binding proteins that function in the transport of iron to cells or as bacteriostatic agents in a variety of biological fluids (Aisen & Listowsky, 1980; Brock, 1985; Huebers & Finch, 1987). Primary sequence data for human serum TF (MacGillivray *et al.*, 1983), pig serum TF (Baldwin & Weinstock, 1988), chicken ovotransferrin (OTF) (Jeltsch & Chambon, 1982; Williams *et al.*, 1982), human lactotransferrin (LTF) (Metz-Boutigue *et al.*, 1984) and human melano-transferrin (Rose *et al.*, 1986) reveal that, in addition to a duplication of the domains (Greene & Feeney, 1968; Williams, 1982; Park *et al.*, 1985), there has been substitution of amino acid residues in both domains. Nevertheless there is substantial sequence similarity both between TFs and the two iron-binding domains within each protein. Constraints on amino acid substitutions appear to include the requirement to preserve both the amino acid residues involved in binding iron and the obligate anion and those residues involved in binding to specific protein receptors associated with many actively dividing cells (Newman *et al.*, 1982; Trowbridge *et al.*, 1984). In spite of the obvious importance of the interaction, there is little information as to which regions of TF and of the receptor are involved in mutual recognition and binding. Recent studies show that the portion of the receptor that binds TF is contained in a tryptic-digest fragment of M_r 70000 (Turkewitz *et al.*, 1988a,b), although no information is yet available about the amino acid residues within this large fragment that are involved in binding to TF. Information on which regions of TF interact with receptor is almost as limited. In this case the evidence supports the idea that each half-molecule domain contains a receptor recognition site (Brown-Mason & Woodworth, 1984; Mason *et al.*,

1987a,b; Oratore *et al.*, 1989). The sites in the two domains differ but appear to be conserved within species (Bartek *et al.*, 1985; Mason *et al.*, 1988).

A feature of the TF molecule is the presence of a moderately conserved 15-amino acid-residue disulphide-linked loop present in both domains of all the TFs for which the sequence is known. The X-ray-crystallographic structure of diferric rabbit serum TF indicates that the loop lies on the surface at the opposite side of the iron-binding site from the interdomain cleft (Bailey *et al.*, 1988). The location, the highly conserved length and the presence of a tyrosine residue at position 581 suggested that the loop might be involved in binding to the transferrin receptor. Previous work from our laboratory with the proteolytically derived N- and C-terminal half-molecules (FeOTF/2N and FeOTF/2C) of OTF indicated that there may be a tyrosine residue in the region of the C-terminal half-molecule involved in binding to receptor (Mason & Brown, 1987). For these reasons, a linear peptide corresponding to the loop (residues 570–584) was synthesized by solid-phase peptide synthesis and used to immunize rabbits. Antibodies to the synthetic peptide were used to query the possible role of the loop in receptor recognition.

EXPERIMENTAL

Materials

Bio-Gel P-6DG desalting resin and Affigel-15 were obtained from Bio-Rad Laboratories. Na¹²⁵I was from Amersham (carrier-free; 100 mCi/ml in NaOH solution). Iodogen and *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester were from Pierce Chemical Co. Goat anti-(rabbit IgG) antibody and its horseradish peroxidase conjugate came from Organon Teknika-Cappel. Microtitre plates were from Dynatech. Keyhole-limpet haemocyanin was obtained from Calbiochem. Nitrocel-

Abbreviations used: TF, transferrin; LTF, lactotransferrin; OTF, ovotransferrin; Fe₂OTF, iron-saturated OTF; FeOTF/2N and FeOTF/2C, the iron-binding domains from the N-terminal and C-terminal halves of OTF respectively.

* To whom correspondence and requests for reprints should be addressed.

lulose came from Schleicher and Schuell. Earle's salts, bovine serum albumin (fraction V), Freund's adjuvant, *o*-phenylenediamine and Tween 20 were from Sigma Chemical Co. Dimethylformamide and dichloromethane were from American Burdick Jackson. The amino acid derivatives for peptide synthesis were obtained from Advanced ChemTech.

Protein preparation

Ovotransferrin (OTF), the *N*-terminal half-molecule domain, designated FeOTF/2N, and the *C*-terminal half-molecule domain, designated FeOTF/2C, were prepared as described previously (Brown-Mason & Woodworth, 1984; Mason *et al.*, 1987b). Protein concentrations were determined spectrophotometrically by using the following M_r values and absorption coefficients ($A_{1\text{cm}}^{1\%}$) at 280 nm: diferric OTF (Fe_2OTF), M_r 77700 and $A_{1\text{cm}}^{1\%}$ 13.95; FeOTF/2N, M_r 37500 and $A_{1\text{cm}}^{1\%}$ 12.73; FeOTF/2C, M_r 40200 and $A_{1\text{cm}}^{1\%}$ 13.74. Portions of the ovotransferrin samples were subjected to urea/PAGE to confirm that they were iron-saturated and to SDS/PAGE in 5–12%-gradient gels to assess purity (Brown-Mason & Woodworth, 1984).

Radiolabelling procedures

For cell binding studies, iodination by the method of McFarlane was performed with iron-saturated OTF or the half-molecule domains as described previously (Mason & Brown, 1987). All other iodinations employed the Iodogen method as detailed previously (Mason & Brown, 1987) except that 10 μl of a saturated solution of tyrosine was added to stop the reaction.

Peptide synthesis

A pentadecapeptide having the sequence (in one-letter code) CTDGRRANVMDYREC, representing residues 570–584 of OTF (Jeltsch & Chambon, 1982), was synthesized as the peptide amide by using a Bioresearch SAM II peptide synthesizer and 4-methylbenzyl-hydrylamine resin. Crude peptide was desalted by gel filtration on Sephadex G-10 as described previously (Church *et al.*, 1988a) followed by purification on a PepRPC HR 5/5 column using a Pharmacia f.p.l.c. system with 0.1% (v/v) trifluoroacetic acid/water and 0.1% (v/v) trifluoroacetic acid/acetonitrile gradient elution. Samples were monitored by absorbance measurement at 214 nm and the major peaks were pooled and freeze-dried. The composition of the peptide was confirmed by amino acid analysis after acid hydrolysis, and the correctness of the sequence was verified by using an Applied Biosystems gas-phase protein sequencer. Solutions of synthetic peptide were made by weighing portions of the purified freeze-dried material. An M_r value of 1770 was calculated from the amino acid composition.

Production of antiserum to peptide 570–584

Peptide (3 mg) from the Sephadex G-10 column was conjugated to keyhole-limpet haemocyanin (3 mg) through the cysteine residues of the peptide by using *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Church *et al.*, 1983). Peptide (3 mg) was also conjugated to ovalbumin (3 mg) through free amino groups by using glutaraldehyde (Reichlin, 1980). The immunization protocol consisted of subcutaneous injections into rabbits of 100 μg of both conjugates emulsified in Freund's com-

plete adjuvant. Serum was obtained from the rabbits after two subcutaneous booster injections in incomplete Freund's adjuvant given at 21 and 35 days.

Pooled serum was dialysed against phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.4) (PBS) and then added to a resin slurry of approx. 20 mg of Fe_2OTF coupled to 1.5 ml of Affigel-15 according to the manufacturer's instructions. After incubation overnight on a shaker at 4 °C the sample was transferred into a column, eluted with phosphate-buffered saline until the absorbance at 280 nm was less than 0.01, and then washed with 10 ml of 1 M-NaCl. The antibody [designated $\alpha(\text{OTF})_{570-584}$] was eluted with 10 mM-HCl, immediately neutralized with dilute NaOH, dialysed against phosphate-buffered saline and reduced in volume to 2 ml on a Centricon 30 microconcentrator. Approx. 3 mg of purified antibody was obtained from 17 ml of serum.

Production of antisera to TF and OTF

Polyclonal antibodies to both human serum TF and chicken OTF were produced in rabbits by subcutaneous injection of 100 μg of antigen in Freund's complete adjuvant. Serum was obtained after two booster injections of 100 μg of antigen in incomplete adjuvant at 3-week intervals. Antibody was isolated from serum on affinity columns of the appropriate antigen coupled to Affigel-15.

E.l.i.s.a.

An e.l.i.s.a. was used to titre the anti-peptide antibody and to determine its specificity. Briefly, 12.5 pmol of each test antigen in 100 μl of carbonate buffer (14 mM- NaCO_3 /34 mM- NaHCO_3 buffer, pH 9.6) was placed into the wells of a 96-well poly(vinyl chloride) flat-bottom micro-titre plate. After incubation overnight at 4 °C or for 1 h at 37 °C, sites on the plate that had not reacted were blocked by addition of 2% (w/v) bovine serum albumin in carbonate buffer. Serial dilutions of antibody in phosphate-buffered saline containing 1% (w/v) bovine serum albumin were added to the wells, and bound antibody was determined by the indirect peroxidase technique with *o*-phenylenediamine as substrate. All incubations were for 1 h at 37 °C; washings between steps used 200 μl of phosphate-buffered saline containing 0.05% Tween 20 per well and were repeated three times. After the enzymic reaction had been quenched with 50 μl of 2 M- H_2SO_4 , colour development was determined by absorbance measurement at 490 nm.

Immunoblotting

To confirm further the specificity of the anti-peptide antibody, immunoblots were performed on both non-reduced and reduced antigens. After electrophoresis in SDS/5–12% polyacrylamide gels, staining with Coomassie Blue [0.025% in 10% (v/v) acetic acid/25% (v/v) propan-2-ol] and destaining in 10% (v/v) acetic acid, the gel was soaked in electroelution buffer [25 mM-Tris/192 mM-glycine/20% (v/v) methanol, pH 8.3] containing 0.1% SDS for 2 h at room temperature (Baillyes *et al.*, 1987). The protein in the gel was then transferred to nitrocellulose in electroelution buffer (with no SDS) (Towbin *et al.*, 1979) overnight at 4 °C and 200 mA constant current. After non-specific binding sites on the nitrocellulose had been blocked with 3% (w/v) bovine serum albumin in phosphate-buffered saline for

2.5 h at 37 °C, ^{125}I -labelled anti-peptide antibody (2.5×10^6 c.p.m.) was added directly to the blocking solution. After an additional 3 h incubation at 37 °C with shaking, the nitrocellulose was washed three times with phosphate-buffered saline containing 0.05 % Tween 20 at 37 °C, air-dried and placed with X-ray film overnight at –20 °C for autoradiography to locate the positive bands.

Gel filtration

A Superose 6 (Pharmacia HR 10/30) column was equilibrated in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and 0.02 % NaN_3 . Portions (100 μl) of various samples were injected, a flow rate of 0.5 ml/min was maintained and 0.5 ml fractions were collected.

Cell studies

Chick-embryo reticulocytes were obtained from White Leghorn chick embryos after 14–15 days of incubation at 37 °C and 80 % relative humidity (Williams & Woodworth, 1973; Brown-Mason & Woodworth, 1984). Incubation of the cells to exclude as much endogenous OTF as possible and treatment with 20 mM- NH_4Cl to inhibit the removal of iron from the radioiodinated OTF have been described in detail previously (Mason *et al.*, 1987a, 1988). Experiments to assess the ability of the peptide to block binding and internalization of radioiodinated Fe_2OTF to cells were conducted as described previously (Brown-Mason & Woodworth, 1984; Mason *et al.*, 1987b).

Our previous protocol with some modification was followed to examine whether the anti-peptide antibody blocked binding of Fe_2OTF or the combined half-molecule domains to receptors on chick-embryo reticulocytes (Mason *et al.*, 1987a). A limiting amount of ^{125}I -labelled Fe_2OTF (75 pmol) or $\text{FeOTF}/2\text{C}$ (150 pmol) in the presence of $\text{FeOTF}/2\text{N}$ (300 pmol) was incubated with a 2-fold or a 10-fold molar excess of antibody $\alpha(\text{OTF})_{570-584}$ at room temperature for 15 min in a total volume of 100 μl . At this time 200 μl of NH_4Cl -treated chick-embryo reticulocytes (see above) was pipetted into tubes containing the labelled antigens with or without antibody. Controls include rabbit anti-(human TF) polyclonal antibody and the two monoclonal antibodies $\alpha\text{OT} + \text{C}_1$ and $\alpha\text{OT} + \text{C}_2$ (Church *et al.*, 1988b; Mason *et al.*, 1987a).

A protocol that has already been detailed (Mason *et al.*, 1987a) was used to provide further proof that the anti-peptide antibody was binding to OTF bound to its receptor. Cells were saturated with unlabelled OTF at 0 °C, washed to removed unbound ligand and incubated for 30 min at 0 °C with either anti-peptide antibody, anti-OTF antibody or anti-(human TF) antibody (300 pmol/ml). After additional washing to remove unbound antibody, 200 μl of each cell suspension was added to tubes containing ^{125}I -labelled anti-(rabbit IgG) antibody. After a 20 min incubation at 0 °C, samples (3 $\times$ 50 μl) were removed, washed by centrifugation and assayed for radioactivity.

RESULTS AND DISCUSSION

Polyclonal antibody to the synthetic peptide was isolated from an OTF affinity column. Specificity of the antibody [designated $\alpha(\text{OTF})_{570-584}$] as measured in an

Fig. 1. Binding of antibody $\alpha(\text{OTF})_{570-584}$ determined by e.i.s.a.

Antigens were immobilized in the wells of a micro-titre plate (12.5 pmol of each) and allowed to react with various dilutions of antibody $\alpha(\text{OTF})_{570-584}$ isolated from an OTF affinity column. Each point is the average of duplicate determinations. The concentration of antibody giving 50 % binding for each antigen is as follows: chicken Fe_2OTF (●), 0.8 nM; quail Fe_2OTF (□), 2.4 nM; chicken $\text{FeOTF}/2\text{N}$ (▲), 36 nM; chicken $\text{FeOTF}/2\text{C}$ (△), 1.3 nM; peptide 570–584 (○), 5.0 nM. Human TF (■) did not bind.

e.i.s.a. (Fig. 1) demonstrates binding to chicken and quail Fe_2OTF , $\text{FeOTF}/2\text{C}$ and the peptide. Some binding can be seen to $\text{FeOTF}/2\text{N}$, whereas no binding to human TF was observed. Antibody recognition of $\text{OTF}/2\text{N}$ may be a result of the 47 % sequence similarity between the loops of the two domains. The concentration of antibody that gives 50 % binding in the e.i.s.a. is listed for each antigen in the legend to Fig. 1. Further confirmation of specificity comes from immunoblotting. As shown in Fig. 2, the antibody binds to non-reduced chicken and quail OTF and to chicken $\text{FeOTF}/2\text{C}$. It does not bind to non-reduced human TF, LTF or $\text{FeOTF}/2\text{N}$ or the reduced forms of any of the antigens that were tested. The antibody that was used in these studies was isolated from an OTF affinity column in which the disulphide bonds of OTF are presumably intact. It was thus selected for its ability to recognize the disulphide-bonded 'loop'.

Binding of antibody $\alpha(\text{OTF})_{570-584}$ to OTF in solution was demonstrated by Superose 6 gel-filtration chromatography. Portions of radioiodinated $\text{FeOTF}/2\text{C}$ alone and in the presence of antibody at antigen/antibody molar ratios of 2:1, 10:1 and 20:1 were chromatographed on a Superose 6 column. As shown in Fig. 3, the presence of antibody caused a concentration-dependent shift of radioiodinated antigen to earlier elution time. Thus 72, 35 and 20 % of the ^{125}I -labelled $\text{FeOTF}/2\text{C}$ was in the complex at ratios of 20:1, 10:1 and 2:1 respectively. Controls included chromatography of ^{125}I -labelled $\text{FeOTF}/2\text{C}$ on the Superose 6 column after incubation with a 10-fold molar excess of anti-(human TF) antibody. The elution profile was identical with that of the antigen alone, showing no interaction under these conditions. In addition, ^{125}I -labelled $\text{FeOTF}/2\text{N}$ chromatographed in the presence of a 20-fold molar excess of anti-peptide antibody showed no shift in elution volume. These results demonstrate that antibody $\alpha(\text{OTF})_{570-584}$ is specific for the C-terminal domain of

Fig. 2. Binding of antibody $\alpha(\text{OTF})_{570-584}$ determined by immunoblotting

Portions (10 μg) of various antigens were subjected to SDS/PAGE in 5–12% gradient gels. After electrophoresis, the gel was stained with Coomassie Blue, destained, photographed (top panel) and electrophoretically transferred to nitrocellulose as described in the Experimental section. Positive bands were detected by autoradiography following treatment of the blot with radioiodinated antibody $\alpha(\text{OTF})_{570-584}$ (lower panel). Non-reduced samples were as follows: lane 1, human serum TF; lane 2, human LTF; lane 3, quail Fe_2OTF ; lane 4, chicken Fe_2OTF ; lane 5, chicken $\text{FeOTF}/2\text{N}$; lane 6, chicken $\text{FeOTF}/2\text{C}$; lane 7, Bio-Rad low- M_r standards (from top to bottom: phosphorylase b , M_r 97400; BSA, M_r 66200; ovalbumin, M_r 45000; carbonic anhydrase, M_r 31000; soya-bean trypsin inhibitor, M_r 21500; lysozyme, M_r 14400). Reduced samples were as follows: lane 8, quail Fe_2OTF ; lane 9, chicken Fe_2OTF ; lane 10, chicken $\text{FeOTF}/2\text{N}$; lane 11, chicken $\text{FeOTF}/2\text{C}$; lane 12, human LTF.

OTF and that anti-(human TF) antibody does not bind to $\text{FeOTF}/2\text{C}$.

An experiment was conducted to examine whether antibody $\alpha(\text{OTF})_{570-584}$ was able to block binding of ^{125}I -labelled Fe_2OTF or of ^{125}I -labelled $\text{FeOTF}/2\text{C}$ plus $\text{FeOTF}/2\text{N}$ to chick-embryo reticulocyte receptors. As shown in Table 1, the antibody does not inhibit binding to chick-embryo reticulocyte receptors of either the combined half-molecules or of holo-OTF, at the antibody/antigen molar ratios of 2:1 and 10:1. The presence of the anti-peptide antibody leads to a slight increase in binding over control values. Preincubation of

Fig. 3. Elution profiles of radioiodinated $\text{FeOTF}/2\text{C}$ in the presence and in the absence of antibody $\alpha(\text{OTF})_{570-584}$ on a Superose 6 column

Portions of ^{125}I -labelled $\text{FeOTF}/2\text{C}$ (50 pmol, 30000 c.p.m.) alone (○) and in the presence of antibody $\alpha(\text{OTF})_{570-584}$ at antibody/antigen molar ratios of 2:1 (●), 10:1 (△) and 20:1 (▲) were chromatographed on a Superose 6 column. Fractions (0.5 ml) were collected and assayed for radioactivity.

the antigens with anti-(human TF) antibody, which does not recognize OTF, leads to some decrease in binding of the combined half-molecules and of holo-OTF at a molar ratio of 10:1. Further controls include two domain-specific monoclonal antibodies ($\alpha\text{OT}+\text{C}_1$ and $\alpha\text{OT}+\text{C}_2$), one of which blocks binding to receptor whereas the other enhances binding of OTF and of the combined half-molecules to receptor, as shown previously (Mason *et al.*, 1987a; Church *et al.*, 1988b). The results show that antibody $\alpha(\text{OTF})_{570-584}$ does not appear to effect the binding of OTF or of the combined half-molecules to chick receptors.

To demonstrate that antibody $\alpha(\text{OTF})_{570-584}$ is bound to the OTF-receptor complex, cells saturated with ligand at 0 °C were incubated with this antibody or with anti-OTF antibody (positive control) or anti-(human TF) antibody (negative control). After incubation and washing, the antibody-OTF-chick-embryo reticulocyte complexes were probed with ^{125}I -labelled goat anti-(rabbit IgG) antibody. As shown in Fig. 4, there is significant binding of the radioiodinated antibody to cells incubated with both anti-OTF antibody and antibody $\alpha(\text{OTF})_{570-584}$. There is no significant binding to cells incubated with anti-(human TF) antibody. Thus antibody $\alpha(\text{OTF})_{570-584}$ is able to bind to OTF that is bound to its specific receptor, suggesting that the OTF molecule when bound to receptor is oriented with disulphide bond 6 away from the OTF receptor-binding site.

The highly conserved length of the loop composed of residues 570–584 led to speculation (Bailey *et al.*, 1988) that it might play an important role in the function of TF. Binding of the 150000- M_r antibody to the loop in the 40000- M_r C-terminal domain has no effect on binding of OTF to the receptor. Neither specific inhibition nor steric hindrance is observed. This indicates that the loop lying on the surface opposite from the iron-binding cleft is at some distance from the receptor-binding region of the C-terminal domain and remains accessible to antibody.

Table 1. Effect of various antibodies on the binding of the combined half-molecule domains and holo-OTF to chick-embryo reticulocytes

Cells treated with 20 mM-NH₄Cl were incubated with 75 pmol of radioiodinated Fe₂OTF or with 150 pmol of radiolabelled FeOTF/2C and 300 pmol of FeOTF/2N with or without antibody for 30 min at 37 °C and processed as described in the Experimental section.

Antibody	Antigen/antibody molar ratio	Binding (% of control) ($\pm$ S.D., $n = 2$)	
		FeOTF/2C* + FeOTF/2N	¹²⁵ I-labelled Fe ₂ OTF
α (OTF) _{570–584}	1:2	124	92
	1:10	126 (± 26)	92 (± 33)
Anti-(human TF)	1:2	112	77
	1:10	76 (± 6)	75 (± 18)
α OT + C ₁	1:1	32 (± 9)	1 (± 0)
	1:2	9	1
α OT + C ₂	1:2	340	–
	1:10	113 (± 0.4)	84

* The number of binding sites/cell for ¹²⁵I-labelled FeOTF/2C alone (no FeOTF/2N or antibody present) was 8.8 % of the number of binding sites/cell found for the control.

Fig. 4. Binding of ¹²⁵I-labelled anti-(rabbit IgG) antibody to chick-embryo reticulocytes in the presence of Fe₂OTF and purified antibodies

Chick-embryo reticulocytes were saturated with unlabelled Fe₂OTF at 0 °C, washed, incubated with anti-(chicken OTF) antibody (●), antibody α (OTF)_{570–584} (■) and anti-(human TF) antibody (▲), washed and incubated with increasing amounts of ¹²⁵I-labelled anti-(rabbit IgG) antibody as indicated on the abscissa. After the final incubation, portions (3 × 50 μl) of cell suspension were washed as described in the Experimental section and assayed for radioactivity. Results were normalized to c.p.m./7 × 10⁷ cells and the average for the triplicate determinations is shown. A 50 μl portion of ¹²⁵I-labelled anti-(rabbit IgG) antibody contained 1 × 10⁶ c.p.m.

Delivery of iron to cells by TF is critical to cell proliferation. A thorough delineation of the first step of this process, the mutual recognition and binding of TF to its receptor, is essential and might provide insights into the mechanism of iron transport and delivery. The present study illustrates one approach to this ultimate goal.

This investigation was funded by U.S. Public Health Service Grant DK/ML-31729. We thank Dr. Robert C. Woodworth for helpful discussions and continuing support. W.R.C. is supported in part by U.S. Public Health Service Grant HL-35058.

REFERENCES

- Aisen, P. & Listowsky, I. (1980) *Annu. Rev. Biochem.* **49**, 357–393
- Bailey, S., Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnain, S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R. & Watson, J. L. (1988) *Biochemistry* **27**, 5804–5812
- Bailes, E. M., Richardson, P. J. & Luzio, J. P. (1987) in *Biological Membranes: A Practical Approach* (Findlay, J. B. C. & Evans, W. H., eds.), pp. 73–101, IRL Press, Oxford
- Baldwin, G. S. & Weinstock, J. (1988) *Nucleic Acids Res.* **16**, 8720–8721
- Bartek, J., Viklicky, V. & Stratil, A. (1985) *Br. J. Haematol.* **59**, 435–441
- Brock, J. H. (1985) in *Metalloproteins, Part II: Metal Proteins with Non-Redox Roles* (Harrison, P., ed.), pp. 183–262, Macmillan Press, London
- Brown-Mason, A. & Woodworth, R. C. (1984) *J. Biol. Chem.* **259**, 1866–1873
- Church, W. R., Walker, L. E., Houghten, R. A. & Reisfield, R. A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 255–258
- Church, W. R., Messier, T., Howard, P. R., Amiral, J., Meyer, D. & Mann, K. G. (1988a) *J. Biol. Chem.* **263**, 6259–6267
- Church, W. R., Brown, S. A. & Mason, A. B. (1988b) *Hybridoma* **7**, 471–484
- Greene, F. C. & Feeney, R. E. (1968) *Biochemistry* **7**, 1366–1371
- Huebers, H. A. & Finch, C. A. (1987) *Physiol. Rev.* **67**, 520–582
- Jeltsch, J.-M. & Chambon, P. (1982) *Eur. J. Biochem.* **122**, 291–295
- MacGillivray, R. T. A., Mendez, E., Shewale, J. G., Sinha, S. K., Lineback-Zins, J. & Brew, K. (1983) *J. Biol. Chem.* **258**, 3543–3553
- Mason, A. B. & Brown, S. A. (1987) *Biochem. J.* **247**, 417–425
- Mason, A. B., Brown, S. A. & Church, W. R. (1987a) *J. Biol. Chem.* **262**, 9011–9015

- Mason, A. B., Brown, S. A., Butcher, N. D. & Woodworth, R. C. (1987*b*) *Biochem. J.* **245**, 103–109
- Mason, A. B., Brown, S. A. & Church, W. R. (1988) *Comp. Biochem. Physiol. B* **91**, 541–549
- Metz-Boutigue, M.-H., Jollès, J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G., Montreuil, J. & Jollès, P. (1984) *Eur. J. Biochem.* **145**, 659–676
- Newman, R., Schneider, C., Sutherland, R., Vodinelich, L. & Greaves, M. (1982) *Trends Biochem. Sci.* **7**, 397–400
- Oratore, A., D'Andrea, G., Moreton, K. & Williams, J. (1989) *Biochem. J.* **257**, 301–304
- Park, I., Schaeffer, E., Sidoli, A., Baralle, F. E., Cohen, G. N. & Zakin, M. M. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3149–3153
- Reichlin, M. (1980) *Methods Enzymol.* **70A**, 159–165
- Rose, T. M., Plowman, G. D., Teplow, D. P., Dreyer, W. J., Hellstrom, K. E. & Brown, J. P. (1986) *Proc. Natl. Sci. U.S.A.* **83**, 1261–1265
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Trowbridge, I. S., Newman, R. A., Domingo, D. L. & Sauvage, C. (1984) *Biochem. Pharmacol.* **33**, 925–932
- Turkewitz, A. P., Amatruda, J. F., Borhan, D., Harrison, S. C. & Schwartz, A. L. (1988*a*) *J. Biol. Chem.* **263**, 8318–8325
- Turkewitz, A. P., Schwartz, A. L. & Harrison, S. C. (1988*b*) *J. Biol. Chem.* **263**, 16309–16315
- Williams, J. (1982) *Trends Biochem. Sci.* **7**, 394–397
- Williams, J., Elleman, T. C., Kingston, B., Wilkins, A. G. & Kuhn, K. A. (1982) *Eur. J. Biochem.* **122**, 297–303
- Williams, S. C. & Woodworth, R. C. (1973) *J. Biol. Chem.* **248**, 5848–5853

Received 15 February 1989/18 September 1989; accepted 1 November 1989