Synthesis of rat muscle carbonic anhydrase III in a cell-free translation system

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Carbonic anhydrase III (CA III) was identified in the products of rat soleus muscle RNA translation in vitro by both a radioimmunoassay and a specific immunoprecipitation technique followed by SDS–polyacrylamide gel electrophoresis analysis of the precipitated antigen. The primary translation product has the same M_r-value as the native isoenzyme. CA III mRNA was found to represent ~0.55% of the total mRNA present in rat soleus muscle.

Carbonic anhydrase *Immunoprecipitation* *mRNA* *Radioimmunoassay* *Muscle* *Translation in vitro*

1. INTRODUCTION

Recently the low-activity, sulphonamide-resistant carbonic anhydrase III (CAIII) isoenzyme has been characterised from skeletal muscle of the rat [1] and shown to be expressed at particularly high concentrations in both male and female soleus muscle [2]. Despite the relative abundance of CAIII in mammalian skeletal muscle [3–8], relatively little is known about its true physiological function, or about the underlying mechanisms which control its gene expression. There is strong evidence that this muscle CAIII and a structurally indistinguishable carbonic anhydrase isoenzyme, which is present at high concentration in the liver of the male rat [2], are products of the same genetic locus. The hormonal regulation of liver CAIII is believed to be complex [9] and it is expected that this isoenzyme will prove to be a valuable marker for studies on the hormonal control of mammalian gene expression. A limited number of reports have described a single-step antibody precipitation of the red-cell carbonic anhydrase I isoenzyme, translated in vitro [10,11], however the immunoreactive polypeptide was not further characterised. The inability to detect and quantitate CAIII mRNA has hampered the investigation of the mechanisms involved in controlling its expression. Here, we report the development of a method for the successful immunoprecipitation of CAIII from the products of rat soleus muscle RNA translated in vitro.

2. MATERIALS AND METHODS

2.1. *RNA isolation and translation in vitro*

Total RNA was isolated from 10 g rat soleus muscle using the guanidinium thiocyanate extraction procedure [12] and tested for biological activity by translation in an mRNA-dependent rabbit reticulocyte lysate (Amersham International) [13]. After denaturation at 68°C for 2 min followed by rapid cooling in a mixture of ice and water the RNA (final conc. 50–250 μg/ml) was incubated in 80% (v/v) lysate for 90 min at 30°C either in the absence or presence of 1 μCi/μl of [^{35}S]methionine (Amersham). Translational activity was determined by liquid scintillation counting of acid-precipitable radioactivity from the ^{35}S-labelled products as described in the lysate batch analysis sheet provided by Amersham.

2.2. *Immunoprecipitation with* Staphylococcus aureus *cells*

Rabbit antisera to rat muscle CAIII and a liver microsomal membrane cytochrome P450 (PB P450) were prepared as in [2,14]. Fixed *Staphylo-*

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coccus aureus cells [15] were obtained from Michelle Ginsberg of the Imperial Cancer Research Fund Laboratories (Lincoln's Inn Fields, London) and prepared for precipitation of the primary antibody immunoprecipitation complex as in [13]. The general immunoprecipitation procedure is described below with any modifications outlined in the legends to fig.2,3.

Translation products ($\sim 6 \times 10^5$ acid-precipitable cpm) were made 1% (v/v) with respect to sodium deoxycholate and diluted with 500 μl 0.5% Triton X-100, 0.5% sodium deoxycholate in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl (pH 7.4), 0.02% NaN_3, 2 mM methionine). The diluted translation products were then adjusted to 1 mM phenylmethylsulphonylfluoride, 100 U aprotinin/ml (Sigma) and 200 μg ovalbumin/ml or bovine serum albumin. The appropriate antiserum or non-immune rabbit serum (5 μl) was then added and the mixture incubated at 30°C for 30 min. Fixed *Staphylococcus aureus* cells (50 μl of a 10% suspension) were added and the samples were agitated by rotation at 4°C overnight. The cells were pelleted, washed and the immunoprecipitated protein was eluted as in [13]. The final supernatants were made 2% (v/v) with respect to mercaptoethanol and heated at 100°C for 3 min.

2.3. *Immunoprecipitation with IgG–Sepharose and protein A–Sepharose*

Goat anti-rabbit IgG (Miles-Yeda) was coupled to cyanogen bromide activated Sepharose-4B (Pharmacia) as in [16]. Protein A–Sepharose was purchased from Pharmacia. Goat anti-rabbit IgG–Sepharose (60 μg IgG in 20 μl) or protein A–Sepharose (40 μg protein A in 20 μl) were substituted for the *S. aureus* cell in the above immunoprecipitation, as indicated in fig.2.

2.4. *SDS gel electrophoresis and fluorography*

Total translation products and immunoprecipitates were analysed by electrophoresis in polyacrylamide gels containing SDS followed by fluorography as in [13].

2.5. *Determination of radioactivity in immunoprecipitates*

Radioactive bands were excised from gels and radioactivity was determined by liquid scintillation spectrometry as in [13].

2.6. *Radioimmunoassay of translation products*

Non-radioactively labelled translation products (20–30 μl) were incubated with 100 μl of a 1/5000 dilution of rat CAIII antiserum for 48 h at 4°C. ^{125}I-Labelled rat CAIII ($\sim$ 15 000 cpm in 100 μl) was added and the incubation was continued for a further 24 h. The antibody-bound phase was precipitated by overnight incubation at 4°C with 100 μl of a 1/20 dilution of donkey-anti-rabbit γ-globulin (Wellcome Reagents) and then collected by centrifugation (1000 × *g* for 30 min) at 4°C. Radioactivity was measured in the precipitates using a programmable γ-counter (LKB 1270 Rackgamma11) and displacement of cpm converted into concentration of CAIII by extrapolation from a standard curve.

3. RESULTS AND DISCUSSION

CAIII was detected in the products of rat soleus muscle total RNA translated in vitro by radioimmunoassay. The amount of CAIII increased with increasing RNA concentration, reaching a maximum at 250 μg RNA/ml (fig.1A). A time course of translation showed that after a short lag, CAIII

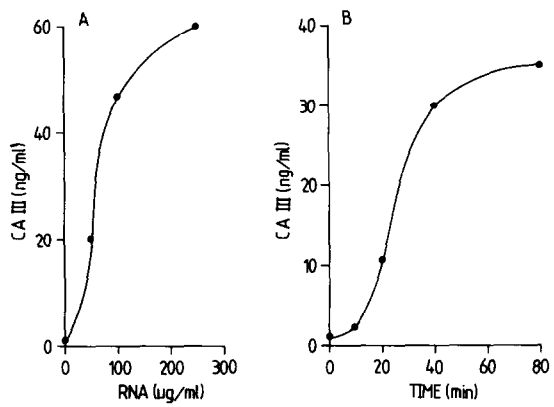

Fig.1. Radioimmunoassay of CAIII in the products of rat soleus muscle total RNA translated in a cell-free protein synthesizing system. RNA was translated at various concentrations of 90 min in the absence of labelled methionine (A) or RNA was translated for various times at 250 μg/ml final conc. in the absence of labelled methionine (B). The amount of CAIII synthesized was determined by radioimmunoassay as described in section 2.6. The sensitivity of the radioimmunoassay was in the region of 250 ng CAIII/litre.

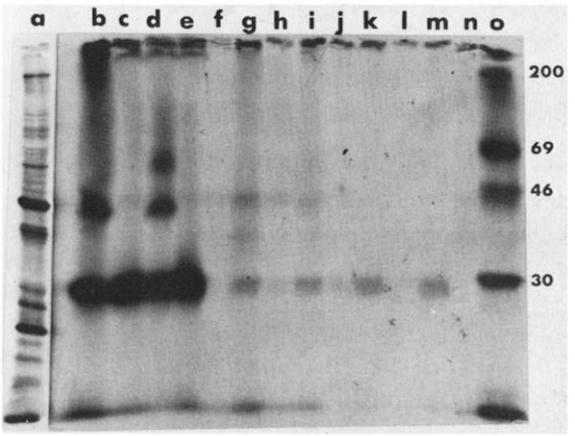

Fig.2. Determination of optimal conditions for the immunoprecipitation of CAIII from the products of rat soleus muscle RNA translation in vitro. SDS–10% polyacrylamide gel electrophoretic analysis of total translation products (a); polypeptides immunoprecipitated by CAIII antiserum with *S. aureus* cells (b–e), goat anti-rabbit IgG–Sepharose (f–i) or protein A–Sepharose (k–m). Translation products were subjected to either a short (f,h,j,l) or long (b–e,g,i,k,m) immunoprecipitation. The short immunoprecipitation involved incubation with CAIII antiserum at 20°C for 15 min, followed by the addition of the second component of the reaction and further incubation at 4°C for 30 min. Long reactions were for 30 min at 30°C in the presence of the first antibody and then at 4°C overnight in the presence of the second component. Immunoprecipitations were done in the presence of either 0.05% NP40 (b,c,f,g,j,k) or 0.5% sodium deoxycholate/0.5% Triton X-100 (d,e,h,i,l,m). Samples in tracks b,d were boiled for 2 min in the presence of 2% SDS before immunoprecipitation. Sample in track n was treated identically to that in track g except that non-immune serum was used instead of CAIII antiserum. Molecular weight markers were run in track o. Numbers give M_r-values of marker proteins ($\times 10^{-3}$). Gel was fluorographed for 11 days.

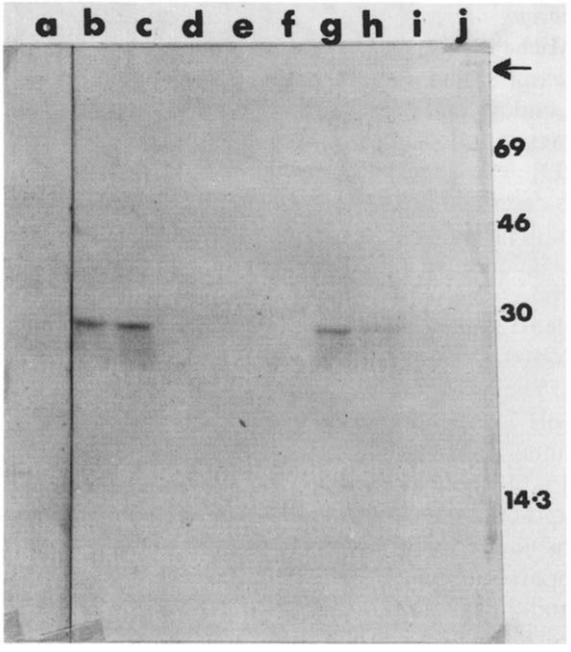

Fig.3. Immunoprecipitational analysis of CAIII in the translation products of rat soleus muscle RNA. Translation products of rat soleus muscle RNA were incubated with 5 μl non-immune serum (a), 5 μl CAIII antiserum (b,g–j), 10 μl CAIII antiserum (c), 5 μl CAI antiserum (d) or 5 μl (30 μg) anti-(PB P450) IgG. Before addition of CAIII antiserum, samples in tracks g–j were mixed with 0.1 (g), 0.5 (h), 2.5 (i) or 5.0 μg (j) of purified CAIII. Track f represents the material immunoprecipitated by 5 μl CAIII antiserum from rabbit reticulocyte lysate incubated in the absence of exogenous RNA. Antigen–antibody complexes were precipitated with *S. aureus* cells and electrophoresed on SDS–13% polyacrylamide gels as in section 2. Gel was fluorographed for 3 days. ← marks the origin of the gel. Numbers give M_r-values of marker proteins ($\times 10^{-3}$).

concentration increased linearly up to about 40 min and then plateaued (fig.1B).

When translated in reticulocyte lysate in the presence of [^{35}S]methionine the translational efficiency of total cellular RNA extracted from rat soleus muscle was 3×10^5 dpm/μg RNA. The RNA directed the synthesis of a wide spectrum of polypeptides up to M_r 200 000 (fig.2a). Two of the major products had M_r-values of about 45 000 and 17 000.

To establish optimum conditions for the immunoprecipitation of CAIII from translation products we investigated the effect of varying the detergent concentration, length of incubation and the reagent used to precipitate the soluble primary antibody–antigen complex. Immunoprecipitation was done as in section 2 with modifications as in fig.2 and immunoprecipitated material was analysed by SDS–polyacrylamide gel electrophoresis (fig.2). Whereas the short-term incubations (fig.2f,h,j,l)

were relatively unsuccessful, all the long-term incubations (fig.2b–e,g,i,k,m) resulted in the immunoprecipitation of a polypeptide of M_r 28 000, the same as that of purified rat CAIII. The use of *S. aureus* cells as the precipitating agent (fig.2b–e) resulted in the immunoprecipitation of twenty times as much of this protein as did either goat anti-rabbit IgG–Sepharose (fig.2g,i) or protein A–Sepharose (fig.2k,m). Varying [detergent] from 0.05% NP40 to 0.5% sodium deoxycholate/0.5% Triton X-100 had no effect on the immunoprecipitation but boiling the translation products in 2% SDS before immunoprecipitation resulted in the precipitation of additional polypeptides (fig.2b,d).

Based on the above results the conditions used in fig.2e (described in section 2.2) were selected for all subsequent immunoprecipitations of CAIII. Using these conditions, CAIII antiserum immunoprecipitated a single polypeptide of M_r 28 000 (fig.3b) from the products of rat soleus muscle mRNA translation in vitro. CAIII antiserum did not precipitate this polypeptide from reticulocyte lysate that had been incubated at 30°C for 90 min in the absence of any exogenous mRNA (fig.3f). Nothing was precipitated from translation products of soleus muscle RNA by non-immune antiserum (fig.3a), rat CAI antiserum (fig.3d), or anti-(PB P450) IgG (fig.3e).

Purified rat CAIII competed with the immunoprecipitated translation product for antigen-binding sites of CAIII antiserum (fig.3g–j), whereas no competition was observed with ovalbumin or bovine serum albumin (not shown). Thus, the immunoprecipitation is specific for CAIII antiserum and the M_r 28 000 protein has antigenic sites common to those of purified CAIII. Doubling the amount of CAIII antiserum used had no effect on the amount of CAIII immunoprecipitated (fig.3c) and it was known that the *S. aureus* cells were in excess [13], indicating that the immunoprecipitation of CAIII was quantitative.

Having established the presence in rat soleus muscle of mRNA coding for CAIII it was possible to use the immunoprecipitation reaction to quantify this mRNA. CAIII mRNA was found to represent about 0.55% of the total mRNA present in that tissue. This figure is dependent on the assumption that, under the conditions used, the efficiency of translation of the CAIII mRNA is equal to the average translational efficiency of the total mRNA population, and that CAIII has an average content of methionine residues. However, methionine represents only ~ 1% of the amino acids in rat CAIII [1] and this is less than the average methionine content found in sequence mammalian proteins or predicted from codon frequencies [17]. Therefore, it is possible that the proportion of CAIII mRNA in rat soleus muscle is of the order of 1–2% and this is fairly close to the proportion of CAIII protein in total rat soleus muscle proteins (about 4%) as determined by immunoassay [2]. The reason that CAIII mRNA is present in a lower proportion than the protein could be due to the CAIII protein and/or mRNA having a longer than average half-life.

We have described a method for the selective immunoprecipitation of CAIII synthesized in a cell-free protein synthesizing system. The primary translation product of rat soleus muscle CAIII mRNA has the same M_r-value as the native enzyme (i.e., 28 000) and apparently does not undergo extensive post-translational modification. The immunoprecipitation technique should prove valuable in the investigation of the developmental and hormonal regulation of CAIII gene expression.

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