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The mouse proline-rich protein MP6 promoter binds isoprenaline-inducible parotid nuclear proteins via a highly conserved NFkB/rel-like site

Stefan G.E.Roberts, Roy Layfield and Charles J.McDonald*
The Krebs Institute for Biomolecular Research, The Department of Molecular Biology, The University of Sheffield, Western Bank, Sheffield, South Yorkshire S10 2TN, UK

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ABSTRACT

Proline-rich protein (PRP) gene MP6 was isolated from a mouse BALB/c genomic DNA library in lambda EMBL3, characterised by hybridisation and restriction mapping and the promoter region, from -162 to +72 around the PRP consensus cap-site, was sequenced. In gel shift assays this region formed complexes C1 and C2 with parotid nuclear proteins which were induced by the β-adrenergic agonist isoprenaline. DNA competition studies and direct binding assays of promoter subfragments showed that it was the sequence from −157 to −91 that was forming the isoprenaline-dependent complexes. All PRP genes conserve a 23bp. sequence, termed PRP Box1, with ets and NFkB / rel binding site-like elements, upstream of their promoters. In the MP6 promoter, PRP Box1 was within the region forming the complexes. Further gel shift assays using PRP Box1 oligonucleotides as competitors and targets indicated that the NFkB / rel binding site-like element was important in formation of the isoprenaline-inducible complexes. HeLa nuclear extracts also formed complexes with PRP Box1 similar to C1 and C2 but nuclear extracts from spleen, submandibular gland and liver did not. These complexes are thus candidate regulators for the isoprenaline-dependent and tissue-specific transcription of PRP genes.

INTRODUCTION

β-Adrenergic stimulation of mice induces expression of multiple closely related proline-rich proteins (PRP) in the parotid glands [1, 2 and literature cited therein]. It does this by elevating transcription rates of the genes for PRPs [3]. PRP gene expression can be induced in cell culture by treatment with dibutyryl cyclic AMP [4, 5] thus cyclic AMP is the probable intracellular messenger, physiologically, for β-adrenergic action upon PRP genes. However, PRP induction is of the slow cyclic AMP-inducible type [3, 5] which does not simply involve the classic type of direct gene activation via AP2 or the CREB family [6, 7].

Induction of growth hormone gene expression in pituitary gland is a well-characterised example of slow cyclic AMP control of transcription [8]. Cyclic AMP, via CREB, first directly induces transcription of the gene for pituitary-specific transcription factor GHF-1. Then, as the GHF-1 protein reaches its critical concentration, GHF-1 activates growth hormone transcription via its own specific binding site in the growth hormone promoter.

Therefore, whereas rapid cyclic AMP transcriptional induction from diverse promoters occurs via a few common regulatory elements active in many cell types, the slow forms of induction are likely mediated via a heterogeneous group of regulators with restricted tissue distribution but which are under direct or even indirect CREB or AP2 control. Slow inductions by cyclic AMP and other messenger systems are therefore akin to many processes of cellular differentiation, growth and development which involve heirarchies of gene control [7].

Cycloheximide has been shown to abolish β-adrenergic-induction of PRP mRNAs [5] indicating that the response requires prior synthesis of one or more protein mediators. This suggests that cyclic AMP may be inducing PRP transcription via an indirect mechanism of the CREB/GHF-1/growth hormone type. The transcription factors which act directly upon PRP genes to mediate their induction by cyclic AMP are unknown. We therefore cloned a mouse PRP gene, MP6, and carried out gel shift assays on the promoter to identify factors which might be involved in PRP gene regulation. We show here that the MP6 promoter formed isoprenaline-inducible complexes with parotid nuclear proteins via an NFkB-like element conserved upstream of all other known PRP genes [9].

MATERIALS AND METHODS

Materials and Chemicals

Restriction enzymes, T4 DNA ligase, DNA polymerase I (Klenow fragment) and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories. Hybond N (nylon membrane), α^{32}P-dATP and γ^{32}P-ATP were from obtained from Amersham International. DNA sequencing reagents were

* To whom correspondence should be addressed

from USB and Poly (dI-dC).(dI-dC) was from Pharmacia. Calf intestinal phosphatase, DNase I and Proteinase K were from Boehringer Mannheim.

Library screening and DNA preparation

A genomic library from mouse strain BALB/c, in the vector EMBL3 was packaged into phage Lambda, transfected into *E. coli* LE392 and plated. The plaques were transferred to nylon membrane and 700,000 were screened with the B-type PRP cDNA, p28 [10], which was ^{32}P-labelled by the random primer method [22]. Hybridisation was performed as described [23] and the membranes were washed to a stringency of 0.1×SSC (20×SSC= 3M NaCl and 0.3M sodium citrate). Positive clones were identified by autoradiography and purified by replating several times. Phage Lambda DNA was prepared by the method of Yamamoto et al [24]. Plasmid DNA was prepared by the alkaline lysis method [25].

DNA analysis and sequencing

DNA, digested with restriction enzymes, was electrophoresed in TAE buffer containing 1μg/ml ethidium bromide. Gels were then transferred to nylon membrane essentially as described [26] and hybridised to ^{32}P-labelled p28 as above. Selected restriction fragments were subcloned into pUC vectors [27] and DNA sequencing was performed using modified T7 polymerase according to the instructions of the supplier, USB. Computer sequence analysis was carried out using the software package of Roger Staden obtained from Amersham International.

Animals

Male BALB/c mice (Sheffield University Field Laboratories colony) of between 8 and 10 weeks of age were given free access to food (CRM diet; Labsure). Mice were injected i.p. with 0.6mg isoprenaline in 0.1ml of 0.14M NaCl daily for three days and on the fourth day were killed by cervical dislocation. Parotid glands, submandibular glands, liver and spleen were removed and used immediately to prepare nuclear protein extracts.

Preparation of nuclear extracts

Nuclear extracts were prepared by the method of Dignam *et al* [28] modified as follows. 1 to 3g of tissue were homogenised in 10ml buffer A (60mM KCl, 15mM NaCl, 0.15mM spermine, 0.5mM spermidine, 14mM β-mercaptoethanol, 0.5mM EGTA, 2mM EDTA and 15mM Hepes pH 7.5) containing 0.3M sucrose at 4°C. This was then layered over 10ml buffer A containing 30% (v/v) sucrose and centrifuged at 2500g for 10 min at 4°C. The nuclear pellet was resuspended in 2ml 20mM Hepes pH 7.5, 20% (v/v) glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF and 0.5mM dithiothreitol. The nuclei were lysed by gentle agitation at 4°C for 1 h and centrifuged at 13000g for 30 min. The supernatant was dialysed against 20mM Hepes pH7.5, 20% (v/v) glycerol, 20mM KCl, 2mM $MgCl_2$, 0.2mM EDTA and 0.5mM dithiothreitol overnight. The protein concentrations of the extracts were then determined [29] and the samples were stored at −70°C.

Gel shift assay

DNA restriction fragments were labelled using α^{32}P-dATP and the large fragment of DNA polymerase I whereas oligonucleotides were labelled using τ^{32}P-ATP and T4 polynucleotide kinase. The ^{32}P-labelled DNA molecules were then purified by polyacrylamide gel electrophoresis, eluted, extracted with phenol and alcohol-precipitated before their use as binding targets. Competitor DNAs were purified in an identical manner except in some experiments where they were generated by PCR in which case they were only subjected to alcohol precipitation before use. DNA yield was determined spectrophotometrically.

The assay for DNA binding activities was a modification of a previously described protocol [30]. Briefly, 1ng of ^{32}P-labelled target DNA was mixed on ice with 1μg poly dI-dC (0.5μg poly dI-dC if an oligonucleotide was used) in 20mM Hepes pH 7.5, 10% (v/v) glycerol, 60mM KCl, 1mM dithiothreitol, 0.2mM PMSF and 4mM EDTA. Nuclear extract (10μg of protein unless otherwise stated) was added then, when appropriate, 1μg of proteinase K or 1 unit of calf intestinal phosphatase. Binding was allowed to proceed for 30 min at 30°C. After adding electrophoresis loading buffer the samples were run on a 5% non-denaturing polyacrylamide gel in 0.5×TBE at 100v. Gels were dried and exposed to X-ray film overnight.

RESULTS

Isolation and characterisation of mouse B-type PRP gene MP6

700,000 plaques from an amplified library of mouse BALB/c DNA in the phage lambda vector EMBL3 were screened for clones harbouring B-type PRP sequences by using the mouse parotid MP5 cDNA insert of p28 [10], labelled with ^{32}P, as the probe at high stringency. Under these conditions only members of the cross-hybridising B-type PRP subfamily are detected whereas A-type PRP sequences are not [10]. The MP5-positive plaque R5 was selected and purified by repeated replating and rescreening with the MP5 probe.

In the mouse there are at least four B-type PRP genes [1] therefore oligonucleotides, specific for mouse PRP sub-classes and for known individual genes [10], were used as hybridisation probes to assess the identity of clone R5. Figure 1A shows that R5 DNA bound oligo. R (detects all mouse PRP genes) and mouse PRP B-type cDNA but did not bind oligo. 2/3 (detects only mouse A-type PRPs). Thus R5 contained a typical B-type PRP sequence.

Probes specific to individual B-type PRPs were then also used (Figure 1A). Oligo. 1 (MP1-specific), oligo. 4.2 (MP4-specific) and oligo. 5.1 (MP5-specific) did not bind to R5 DNA thus R5 contained a novel B-type PRP sequence which we termed MP6. However oligo 4.1 (MP4-specific), oligo. 5.2 (MP5-specific) and oligo. 4/5 (MP4/5 selective) did bind to R5 DNA. Thus the sequence, although distinct, was related closely to both MP4 and MP5. The R5 clone of MP6 was then analysed further by restriction mapping and Southern blotting using MP5 cDNA as the probe. These data are summarised in Figure 1B.

The MP6 promoter region

The whole of the hybridisation-positive region of MP6 was contained on a 5.8Kbp. Eco R1 fragment (data not shown) which was subcloned and this construct (pEcoR5) was mapped further (Figure 1B). The restriction map was not identical to that of MP4 which is the only other mouse B-type PRP gene that is characterised [9]. In particular, the MP4 gene is distinct from MP6 as it is contained on a 4.5Kbp. Eco R1 fragment which contains only a single Hind III site and is not cut by Pst I [9].

However, in all three known mouse genomic PRP sequences [9, 11, 12] there is a Sac I site conserved in Exon 1 which in MP4 is 234bp downstream from an Eco R1 site [9]. It was likely

therefore that the MP6 promoter region would be contained on the 230bp. Eco R1/Sac 1 fragment of pEcoR5 (Figure 1B). This region of MP6 was sequenced and was distinct from the other three known mouse PRP gene sequences [9, R11, 12] although it was highly similar to the MP4 promoter with only 6 nucleotide differences over the 234 bp. region (Figure 2A). There were 4 differences from the MP5 cDNA over the 59bp that were compared (Figure 2A). The PRP Box1 sequence, which is highly conserved in all other PRP genes and implicated in PRP transcriptional regulation [9], was of the exact sequence found in MP4 (Figure 2A).

The PRP promoter binds parotid nuclear factors regulated by isoprenaline

To determine if nuclear proteins bound to the promoter region of B-type PRP genes, extracts from parotid nuclei were prepared from normal mice and mice injected with isoprenaline for 3 days. These samples (10μg) were mixed with 1ng of the ^{32}P-labelled 230bp promoter region of the cloned PRP gene, then subjected to electrophoresis on a nondenaturing polyacrylamide gel. Initially, the addition of R parotid nuclear extracts destroyed the free target DNA used to probe for binding proteins. Parotid glands secrete DNAase into saliva [13] thus it was probable that the nuclei were contaminated with this enzyme. Nuclear extracts were therefore incubated with the target DNA in the presence of different amounts of EDTA, to chelate Mg^{++} ions thus inhibit DNAase, then the mixtures were subjected to non-denaturing gel electrophoresis (data not shown). 4mM EDTA prevented degradation of the target thus was added to all subsequent binding reactions. Under these conditions complexes with parotid nuclear proteins were observed (data not shown and Figures 3–5). The binding reactions were then titrated with different amounts of poly dI/dC to reduce non-specific binding. 1μg and 0.5μg of poly dI/dC were optimal, respectively, for restriction fragments and oligonuclotide target sequences used to probe the ability of nuclear extracts to bind DNA.

Figure 3 shows that in the presence of 4mM EDTA and 1μg poly dI/dC two complexes, C1 and C2, formed between parotid nuclear extracts and the Eco R1/Sac 1 MP6 PRP promoter fragment covering the region from −162 to +72. The complexes were clearly greater in amount when the nuclear extracts came from isoprenaline-treated mice rather than normal mice.

The specificity and localisation of the binding reactions were determined by co-incubations with 100ng of unlabelled DNA competitors. The homologous Eco R1/Sac 1 promoter fragment

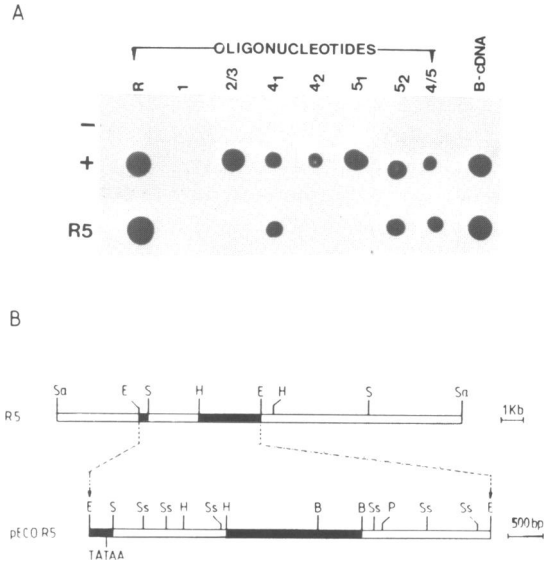

Figure 1. Mouse PRP gene MP6. Panel A: Classification of Lambda clone R5 by oligonucleotide hybridisation. R5 DNA was subjected to dot-blot hybridisation with the probes indicated above each blot. The specificities of the probes to known mouse PRP sequences [10] were: oligo. R (all mouse PRP genes), oligo 1 (MP1), oligo 2/3 (MP2 and MP3), oligos. 4_1 and 4_2 (MP4), oligos. 5_1 and 5_2 (MP5), oligo. 4/5 (MP4 and MP5). The B-type cDNA probe was MP5 [10]. Controls (+) were appropriate cDNAs, (−) was wild type Lambda DNA. No positive control was obtainable for MP1. Panel B: Restriction map of the cloned PRP gene MP6 in Lambda EMBL3 (top) and in subclone pEcoR5 (bottom). The restriction enzymes used were Bst EII (B), Eco RI (E), Hind III (H), Pst I (P), Sac I (S), Sal I (Sa), Ssp I (Ss). The hybridisation-positive region, determined by Southern blotting with ^{32}P-labelled MP5 cDNA as probe, is shown in black. The central Hind III/Pst I fragment contained multiple closely spaced sites for both Bst NI and Dde I, typical of PRP genes, which were not mapped. The promoter region, determined by DNA sequencing, is marked TATA.

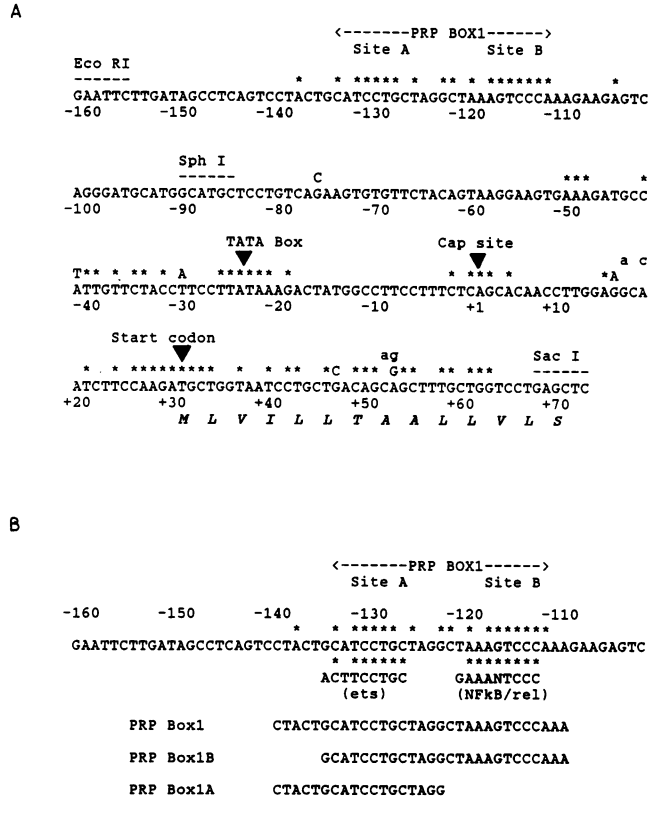

Figure 2. The promoter sequence of PRP gene MP6. Panel A: The left hand end of the pEcoR5 genomic insert (Figure 1B) is shown as far rightwards as the Sac I site conserved in exon 1 of all mouse PRP genomic sequences obtained to date. The sequence is numbered relative to the homology with the MP4 cap site [9] and asterisks indicate identical bases present in all the PRP genes for which sequence data is available (see text). Differences from the other mouse B-type PRP nucleic acids are shown above the MP6 sequence in upper case (MP4) and lower case (MP5). Only the +13 to +72 region of MP6 could be compared with MP5 as the MP5 cDNA sequence extended only as far as +13 [10]. PRP Box1, the cap site position based on the MP4 gene (+1) [9], the −26 TATA motif and the Eco RI, Sph I and Sac I sites used to generate targets in gel shift assays are marked. Panel B: The PRP Box1 region. Similarities of PRP Box1 elements A and B with the binding sites for ets and NFkB, respectively [15], are marked. The PRP Box1, Box1A and Box1B oligonucleotides used in gel shift assays are illustrated in full.

or a 68bp Eco R1/Sph 1 fragment (−157 to −91bp) containing the 23bp. PRP Box1 region (see Figure 2A) with 22bp. of flanking sequence on either side both abolished C1 and C2 formation (Figure 3 and data not shown). However, a 324bp. fragment (+916 to +1240) from the interior of the MP4 gene [9] had no effect on formation of the complexes thus the −157 to −91bp region contained sequence(s) responsible for binding the isoprenaline-inducible nuclear factors in a sequence-specific fashion.

In addition, calf intestinal phosphatase treatment of the nuclear extracts also abolished formation of complexes indicating the importance of phosphorylation to both C1 and C2 formation (Figure 3).

Complexes C1 and C2 are restricted in tissue distribution

C1 and C2 formation occurred with tissue nuclear extracts from parotid glands but not from the submandibular glands, the livers or the spleens of either normal mice or mice treated with isoprenaline (Figure 3). Nuclear extracts from the livers of pigs also did not bind to the PRP promoter fragment. The pig liver extracts, in the absence of EDTA did however form a complex with a consensus Sp1 binding site oligonucleotide (A.Dalton and K.Ford personal communication). However, nuclear extracts from the submandibular glands of isoprenaline-treated mice did have an ability to form a small complex with the PRP promoter fragment and this was absent from normal submandibular samples (Figure 3). DNA molecules containing PRP Box1 did however have the ability to form complexes similar to C1 and C2 with HeLa nuclear extracts (see later text and Figure 5).

Complex formation involves proteins and PRP Box1

As the 68bp Eco R1/Sph 1 subfragment, which contains PRP Box1, abolished formation of complexes between parotid nuclear extracts and the entire Eco RI/Sac I promoter fragment, the subfragment was then used as the target for complex formation in gel shift assays with parotid nuclear extracts. Firstly, parotid nuclear extracts from normal mice and from isoprenaline-treated mice gave results with the subfragment comparable to those obtained using the whole promoter as the target (compare data in Figures 3 and 4A) but only at high concentrations of nuclear extract. At low concentrations of nuclear extract a C2-type complex was the predominant isoprenaline-induced species which formed with the Eco RI/Sph I subfragment (see the next section).

Secondly, the specificities of the binding events which resulted in complex formation between the Eco RI/Sph I subfragment and parotid nuclear extracts were determined by adding competitor DNAs to the gel shift assays. Addition of a hundred fold excess of the unlabelled subfragment inhibited complex formation with the labelled target subfragment but the PRP MP4 gene interior fragment did not affect binding. Thus formation of complexes with the subfragment required specific sequence (Figure 4B) as it did with the whole promoter (Figure 3).

Thirdly, the location within the Eco RI/Sph I subfragment which was the site for complex formation was determined. As PRP Box1 is highly conserved in all PRP promoters this sequence

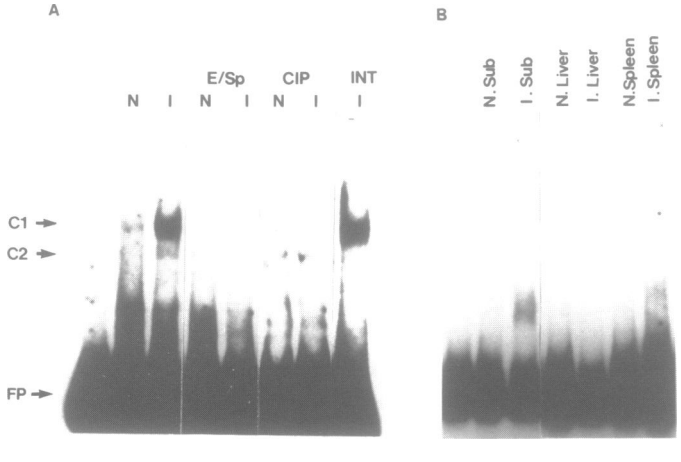

Figure 3. Gel shift assays of the PRP promoter with mouse tissue nuclear extracts. The target was 1ng. of the 226bp. Eco R1/Sac 1 fragment of PRP gene MP6, end-labelled with ^{32}P. It covers −162 to +72 relative to the cap site Figure 2A). The nuclear extracts (10μg protein) were from normal BALB/c mice (N) or the same strain injected daily with 0.6mg. of isoprenaline and killed on day four (I). The left hand panel shows parotid tissue and the right hand panel shows other tissues. Sub = submandibular gland. E/Sp denotes assays which contained 100ng. of the 68bp Eco R1/Sph 1 MP6 promoter subfragment (−157 to −91, see Figure 2A). CIP denotes assays containing 1 unit of calf intestinal phosphatase. INT denotes assays containing 100ng. of a 324bp. fragment from the interior of the MP4 gene (+916 to +1240) [9]. The position of free target (first lane in both panels), complex 1 and complex 2 are marked at left, respectively, FP, C1 and C2.

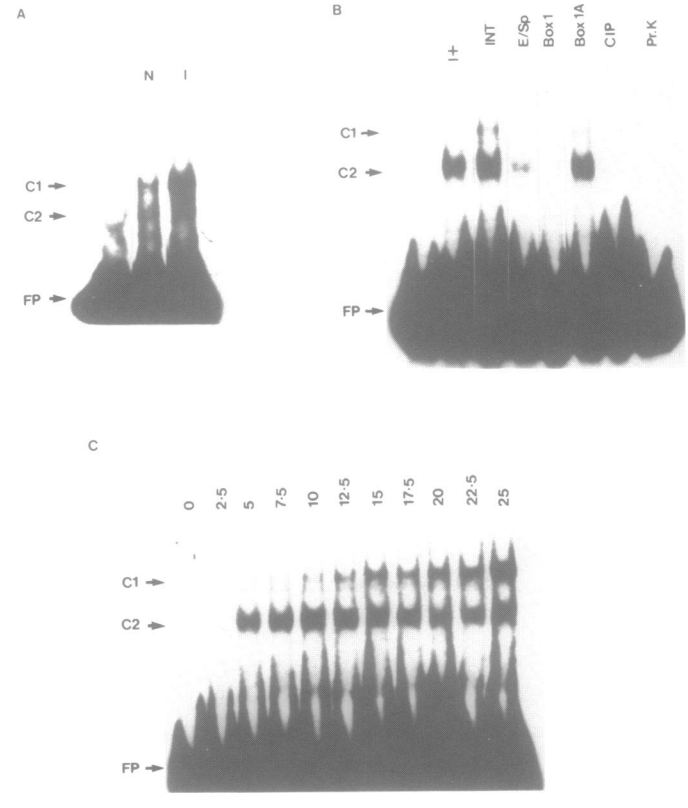

Figure 4. Gel shift assays of the −162 to −91 upstream MP6 PRP promoter Eco RI/Sph I sequence with mouse parotid nuclear extracts. 1ng. of the ^{32}P-labelled 68bp. Eco RI/Sph I target was used in each lane. Abbreviation are as in Figure 3. Panel A: 32μg. of nuclear extract from parotid glands of normal mice (N) and those treated with isoprenaline as in Figure 3 (I). Panel B: Competitions of 100ng unlabelled DNAs (denoted above each lane) with 1ng Eco RI/Sph I target DNA for 10μg of extract as in A:I. I+, No competitor; CIP, +I with 1 unit of calf intestinal phosphatase; PrK, +I with 2μg proteinase K. Panel C: Effect of different concentrations (μg protein/50μl assay) of parotid nuclear extract from isoprenaline-treated mice upon the formation of complexes with the MP6 Eco RI/Sph I fragment. The left lane in each panel is free target. The positions of complexes are denoted C1 and C2.

was a strong candidate for the site of binding thus a 26bp. double-stranded PRP Box1 oligonucleotide (PRP Box1B oligonucleotide, see Figure 2B) was used as a competitor in gel shift assays with the Eco RI/Sph I subfragment as the target. PRP Box1B abolished complex formation establishing this site as crucial for formation of the isoprenaline-inducible complexes (Figure 4B). This is dealt with further in a later section.

Addition of calf intestinal phosphatase to the nuclear extracts abolished their ability to form C1 and C2 with the Eco RI/Sph I fragment as did proteinase K treatment (Figure 4B) thus again showing that phosphorylation was important for complex formation and establishing that protein factors were involved in binding.

The ratio of C1/C2 complex formation is highly dependent on protein concentration

Isoprenaline-treated parotid nuclear extracts always formed a greater amount of both C1 and C2 than did normal parotid extracts, irrespective of the target used (Figures 3, 4 and 5 and data not shown). Results identical to those shown in Figures 3, 4 and 5 were obtained with three different nuclear extracts prepared over a three-month period from both normal animals and isoprenaline-treated animals.

However, in some experiments it was observed that there were different ratios of C1 to C2 in gel shift assays of both isoprenaline-stimulated and normal parotid nuclear extracts when either the whole Eco RI/Sac I promoter fragment or the 68bp Eco RI/Sph I subfragment was used as the target. It was established that this was due to the concentration of nuclear extract that was present in the assay (Figure 4C).

At low concentrations of nuclear extract C2 was the predominant complex which formed on the Eco RI/Sph I subfragment whereas higher concentrations of nuclear extract increased the ratio of C1 to C2 which formed (Figure 4C). Thus C1 formation had a higher apparent binding constant for the DNA than did C2.

A similar ratio of C1 to C2 formation was observed for the whole promoter Eco RI/Sac I target as for the Eco RI/Sph I target when the same molar ratio of nuclear extract to target was used (compare Figure 3 with Figure 4A). At lower molar ratios of extract to target DNA, complex formation shifted in favour of a high C2/C1 ratio. This is apparent in Figure 4B and C for the Eco RI/Sph I target sequence and in Figure 5 for the PRP Box1 oligonucleotide target sequences (see next section).

Thus there was always a greater amount of total complex formation with treated than with non-treated extracts irrespective of the target used to probe for nuclear binding factors. However, the amount of each isoprenaline-inducible complex that formed was dependent on the concentration of nuclear extract in the assay and the molar ratio of extract to target DNA.

Scanning densitometry of the autoradiograph shown in Figure 4C showed that the amount of C1 was linearly related to the amount of nuclear extract in the assay but this was not the case for the amount of C2 which formed, suggesting that cooperative interactions are involved in C2 formation.

The role of PRP Box1 site B in complex formation

As reported above, PRP Box1B, added as a competitor to gel shift assays, abolished complex formation with the Eco RI/Sph I subfragment (Figure 4B) and established PRP Box1 as the target site for complex formation. Figure 5A shows directly that a PRP Box1 double-stranded oligonucleotide (see Figure 2B) formed C1 and C2-type complexes with isoprenaline-inducible nuclear factors from parotid glands and that the PRP Box1B sequence also had the same capability (Figure 5B and data not shown) which was abolished when a hundred fold excess of the same sequence was added as a competitor to the assays.

However, a double-stranded oligonucleotide comprising the 5′ end of PRP Box1 (PRP Box 1A, see Figure 2B), when used as a competitor, did not affect complex formation with the Eco RI/Sph I subfragment (Figure 4B) therefore indicating that the 3′ end of PRP Box1 (Box1B) was the region involved in both C1 and C2 formation.

Therefore PRP Box 1, via its 3′ end, binds isoprenaline-inducible complexes. The striking feature of the conserved

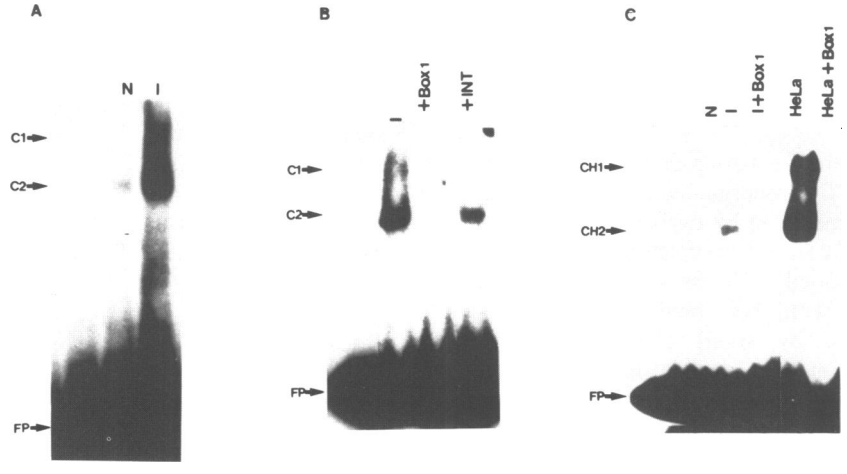

Figure 5. Gel shift assays of MP6 PRP Box1 double-stranded oligonucleotides with parotid and HeLa cell nuclear extracts. 1ng of target DNA and 10μg of nuclear extract was used in each lane unless otherwise stated. Abbreviation are as in Figures 3 and 4 and the left lane in each panel contains free target. Panel A: PRP Box1 oligonucleotide with nuclear extract from parotid glands of normal mice (N) and those treated with isoprenaline as in Figure 3 (I). Panel B: PRP Box1B oligonucleotide with parotid nuclear extracts from isoprenaline-treated mice without competitor DNA (I) or with 100ng of Box1B (+Box1) or 100ng of the MP4 interior fragment (INT; see Figure 3 for details). Panel C: Target DNA was as in Panel B and parotid nuclear extracts N and I were as in Panel A. Hela is 6μg of HeLa nuclear extract. +Box1 marks assays containing 100ng of unlabelled PRP Box 1B oligonucleotide as competitor. The exposure of the autoradiograph was such that HeLa complexes CH1 and CH2 (arrowed) could be distinquished from each other thus parotid C2 is weak and C1 is not apparent.

sequence within PRP Box1B is a strong similarity to an NFkB binding site (Figure 2B), thus NFkB or AGIE-BP1-like factors may be involved in complex formation (see Discussion).

The PRP Box1A competition studies (Figure 4B) and direct binding assays (data not shown) ruled out the 5′ end of PRP Box1 as having direct involvement in the formation of the two isoprenaline-inducible complexes. If the ets-like Box1A site is involved in complex formation it is not apparent from the gel shift assays carried out under the conditions reported here.

C1 and C2-like complexes form between PRP Box1 and HeLa nuclear extracts

Figure 5C shows that an extract of nuclear proteins prepared from HeLa cell paste formed two major complexes with the double-stranded Box1B oligonucleotide. One HeLa complex (CH2) was identical in size to parotid complex C2 and the second HeLa complex (CH1) was slightly larger in size than parotid complex C1. Unlabelled PRP Box1B, as a competitor, in a hundred fold excess over labelled target PRP Box1B abolished formation of both HeLa complexes. Thus HeLa cell nuclei unlike spleen, liver and submandibular gland contain factors, protein in nature (data not shown), that bind to PRP Box1B.

DISCUSSION

We have cloned a mouse gene for a B-type PRP which we term MP6. The promoter region was 97.4% identical to the promoter region in the only other mouse B-type PRP gene sequenced to date, MP4 [9]. The PRP Box1 23bp element, implicated previously in PRP regulation because of its high degree of conservation in all mammalian salivary PRP genes sequenced so far [9], was also present in MP6. The recently published sequences of three rat PRP genes which are close relatives of the mouse A-type PRP genes also contain PRP Box1 conserved to a high degree [14].

We show that the promoter and a promoter-derived subfragment both formed two complexes with proteins from parotid nuclear extracts. These were both clearly under control of the β-adrenergic agonist isoprenaline and the two activities were abolished by dephosphorylation. Both normal and isoprenaline-stimulated parotid nuclear extracts had the ability to form complexes C1 and C2 with the PRP promoter but the total amount of each complex was always greater after stimulation than normally.

These complexes are therefore strong candidates for being factors involved in the slow β-adrenergic induction of PRP gene transcription [3] which is mediated by cyclic AMP [4, 5].

The sensitivity of the C1/C2 ratio to the concentration of parotid nuclear extract used in the gel shift assays suggest that the interaction of the factors with their binding site could be modulated physiologically by small changes in factor concentration thus may point to a mechanism governing the formation of different promoter/protein complexes with different regulatory properties. This interplay could allow a high degree of regulatory flexibility permitting different combinations of positive and/or negative controls over PRP gene transcription during β-adrenergic induction.

Our data indicated that the specific promoter region which undergoes complex formation is PRP Box1. The two regions within PRP Box1 which are conserved to the greatest extent (sites A and B, [9]) are similar, respectively, to binding sites for the oncoprotein transcription factors ets and NFkB / rel [15].

The conserved ets-like PRP Box1 A site was shown not to influence formation of the isoprenaline-inducible complexes with the PRP promoter. However, gel shift assays do not detect all proteins capable of specific interactions with DNA and the presence of EDTA in the assays may compromise the activity of some factors which require divalent cations [16]. Alternatively, site A may be involved in binding events linked to other controls over PRP expression such as developmental regulation, expression in other tissues or glucocorticoid repression [13].

The competition and binding studies with the PRP Box1 oligonucleotides implicated PRP Box1 site B as the binding site for both complexes. The similarity of this site with the binding site for NFkB / rel factors raised the possibility that NFkB itself might constitute the PRP Box1 binding factors. NFkB is a complex regulator of cell function under diverse controls [17, 18] and it can form several different types of complex with its family of DNA binding sites due to protein-protein interactions between its constituent p65 and p50 subunits giving rise to p50 homodimers and p65/p50 heterodimers which both bind to DNA [19,20].

Members of the distinct AGIE-BP1 family of transcription factors also bind to NFkB sites [21]. However, the tissue distributions of NFkB and AGIE-BP1 [17, 18, 21] are different from the binding activities described here suggesting that they may be novel factors. Further studies to assess the binding of purified NFkB and AGIE-BP1 proteins [19–21] and other DNA-binding proteins to PRP promoters and PRP Box1B oligonucleotides will help clarify the relationship of known factors to the HeLa and parotid proteins which gave rise to C1 and C2.

The data presented here will allow the rapid purification of both the HeLa and parotid factors which interact with PRP Box1B but assays of transcription from PRP promoters using transgenic animals or cell lines will determine whether PRP Box1B is indeed necessary and/or sufficient for the well known isoprenaline and dietary regulation of PRP expression in salivary glands.

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