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The mammary factor MPBF is a prolactin-induced transcriptional regulator which binds to STAT factor recognition sites

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Abstract

Site-directed mutagenesis of the three binding sites for the mammary factor MPBF in the β-lactoglobulin (BLG) promoter demonstrates that MPBF is a transcriptional activator of the BLG gene in mammary cells. MPBF requires phosphorylation on tyrosine for maximum binding activity and binds to GAS (interferon γ-activation site) elements which are similar to the MPBF binding sites. Prolactin induces MPBF binding activity in CHO cells and is not antigenically related to Stat1 (p91) and Stat2 (p113), suggesting that this transcription factor is likely to be another member of the STAT family of cytokine/growth factor-induced transcription factors.

Key words: Prolactin; Transcription factor; Signal transduction; STAT; Mammary gland

1. Introduction

Prolactin (PRL) is a peptide hormone that regulates the metabolism, proliferation and differentiation of a variety of cells and tissues [1]. In the mammary gland, PRL controls both development of the gland and the expression of milk protein genes [2]. The PRL receptor is closely related to the growth hormone (GH) receptor and is a member of the cytokine/GH superfamily of receptors that lack intrinsic tyrosine kinase activity [3]. Upon GH binding, the GH receptor associates with, and is phosphorylated by JAK2, a non-receptor protein tyrosine kinase (PTK), which is presumed to initiate the signal cascade that results in gene activation [4,5]. The recent demonstration that JAK2 is also essential for both IFN [6] and PRL [7] signal transduction suggests that PRL action may be mediated through a member of the STAT family of transcription factors which are involved in IFN and cytokine signalling pathways (reviewed in [8]).

The putative mammary transcription factor MPBF has three binding sites within the proximal 410 bp promoter of the sheep β-lactoglobulin (BLG) gene [9]. MPBF may be related to the transcription factor MGF which has been shown to be essential for the hormone induced transcription of a transfected β-casein promoter/CAT construct in mammary HC11 cells [10]. The highest affinity MPBF binding site GATTCCGGGAACC is similar to the IFN-γ activation sequence (GAS) ATATTCCTGTAAGTG in the Ly-6E gene [11] and shares an identical 9 bp core with a high affinity acute phase response factor (APRF) binding site [12]. This suggests that MPBF could be related to the IFN-γ activated factor, Stat1, or to other members of the STAT family of transcription factors. STAT factors are latent cytoplasmic transcription factors that are activated by phosphorylation on tyrosine resulting in dimerisation and translocation of the complex into the nucleus where it binds target DNA [13].

We demonstrate here that MPBF is a transcriptional activator of the BLG gene in mammary cells. We also show that MPBF is induced in HC11 cells in response to dexamethasone and PRL and in CHO cells in response to PRL alone. MPBF is not antigenically related to Stat1 or Stat2, but is phosphorylated on tyrosine and binds to GAS elements suggesting that it is probably a STAT factor which mediates the response to prolactin.

2. Materials and methods

2.1. BLG reporter constructs and plasmids

Individual MPBF binding sites in the BLG promoter were mutated as described ([14], T.B. et al., in prep.). The mutated BLG promoters were excised as Sal I/ SphI fragments and cloned 5' of the CAT gene in *SalI/SphI* restricted CAT/pUC19 plasmid. The NFkB site at −172 in the BLG promoter was mutated using the same stategy as described for generating the MPBF mutations. Sequences of the mutant oligonucleotides are: STMm(sense) GGGATTTGGCCAACCGC; STMm(antisense) GCGGTTGGCCAAATCCC; A3m(sense) GTCTACCACCAACCGTC; A3m(antisense) GACGGTTGGTGGTAGAC; A1m(sense) GTGTTCCTCCCACTGGC; A1m(antisense) GCCAGTGGGAGGAACAC; NFkBm(sense) GGCCCAGAGCTCGACTTCCTG; NFkBm(antisense) CAGGAAGTCGAGCTCTGGGCC The bases which have been changed are underlined.

2.2. CAT assays

Before harvesting, cells were washed with phosphate buffered saline, then scraped from the dishes in 10 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1 mM EDTA. Cells were pelleted by centrifugation and resuspended in 200 μl of 0.25 M Tris-HCl. Cytosol extracts were prepared by 3 cycles of freeze thawing [15] followed by centrifugation at 10,000 × *g* for 5 min. The protein concentration of the supernatants was estimated using the BCA protein assay (Pierce). CAT assays containing 1 μg of protein extract were carried out as described previously [15] and assayed using thin layer chromatography.

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Abbreviations: IFN, interferon; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyl transferase.

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2.3. Cell culture and transfection

The growth and hormonal induction of HC11 mammary cells was carried out as previously described [10]. Approximately 5×10^5 cells per 80 cm^2 flask were cotransfected overnight with 1 μg of pSV2neo and 15 μg of BLG plasmid by calcium phosphate precipitation [15]. After 14 days of selection in growth medium containing 200 μg/ml G418, colonies were trypsinised and expanded as a pool of clones. Pools containing greater than 100 colonies were utilised in the hormone induction experiments. The CHO-E3 cell line is a clone derived from CHO-K1 cells stably transfected with the rabbit long-form PRL receptor and was a kind gift from Jean Djiane, INRA, Jouy-en-Josas, France. CHO-E3 cells were induced in serum free medium with PRL as described previously [16].

2.4. Nuclear extracts and EMSA

Nuclear extracts were prepared from tissues and cells using the method of Dignam [17] with minor modifications [9]. Aliquots were flash frozen in liquid nitrogen and stored at −80°C. Daudi cells (human lymphoma derived) were treated with 1000 units IFN-α/ml for 2 h before preparation of nuclear extract. Daudi extracts were generously provided by John Girdlestone, Laboratory of Molecular Biology, Cambridge. EMSAs were carried out as previously described [9] and where indicated, unlabelled oligonucleotide competitors were added simultaneously. Binding sites were created by annealing the following oligonucleotide pairs: STM GATTCCGGGAACCGCT and ACGCGGTTCCCGGAATC; SIE GTCGACATTTCCCGTAAATC and TCGACGATTTACGGGAAATG; GAS CATGTTATGCATATTCCTGTAAGTG and CATGCACTTACAGGAATATGCATAA; ISRE GATCGGGAAAGGGAAACCGAAACTGAAGCC and GATCGGCTTCAGTTTCGGTTTCCCTTTCCC. Supershift analysis was carried out essentially as above with prior incubation of antibody and nuclear extract for 30 min at 4°C plus 10 min at 25°C before the addition of probe. The Stat1 antibody was purchased from Transduction Laboratories and the Stat2 antibody was the gift of Chris Schindler, Columbia University, New York.

2.5. Phosphorylation studies of MPBF

Two protein tyrosine phosphatases were used, T-Cell$_R$ (NEB: a human T-cell tyrosine phosphatase) and LAR (NEB; the human transmembrane leukocyte antigen related tyrosine phosphatase). Sheep nuclear extract (250 ng) was incubated at 30°C for 20–60 min with 5 U of the T-Cell$_R$ (167 ng) or LAR (1.25 μg) phosphatase, after which EMSA was carried out on the extracts. Monoclonal anti-phosphotyrosine, anti-phosphoserine and anti-phosphothreonine antibodies (Sigma) or control mouse ascites fluid (2–5 μg) were incubated on ice for 90 min with approximately 100 ng partially purified MPBF (from sheep lactating mammary gland nuclei) in gel shift buffer and then analysed by EMSA.

2.6. UV cross-linking of MPBF

The double-stranded DNA probe used in the cross-linking experiment was prepared as described in Wakao [18] by annealing the single-strand oligonucleotide that spans the STM binding site (TGGGATTCCGGGAACCGCGTA$(A)_{15}$) with a short primer (TTACGCGG). MPBF was cross-linked to the oligonucleotide probe by irradiation of an EMSA gel on a UV transilluminator for 2 min. To reduce the exposure times required to detect cross-linked proteins by autoradiography, electroelution was eliminated by incorporating the EMSA gel slice into the stacking gel of an SDS-PAGE gel [19]. Labelled polypeptides were detected by autoradiography of dried gels.

2.7. Quantitation

Data were quantitated with a Molecular Dynamics PhosphorImager system.

3. Results

3.1. MPBF is a transcriptional activator of BLG promoter constructs in mammary HC11 cells

Electrophoretic mobility shift assay (EMSA) with nuclear extracts prepared from hormonally induced HC11 cells and the high affinity STM binding site from the BLG promoter revealed a specific MPBF complex in HC11 cells. This MPBF-like activity was present at low levels in uninduced HC11 cells (Fig. 1A, lane 1) and was induced 2- to 3-fold by treating cells with dexamethasone (DEX) and PRL (lane 2).

To determine the role of MPBF in the transcriptional regulation of the BLG gene in mammary cells, the 408 bp BLG proximal promoter sequence was mutated to abrogate MPBF binding at combinations of binding sites within a BLG/CAT reporter gene construct. To abolish MPBF binding two guanine bases, shown to be essential for MGF binding by contact point analysis [10], were changed to cytosine in all three MPBF sites. This dramatically reduced MPBF binding to the A1 and A3S MPBF sites (T.B. et al., in prep.); however, two additional bases, cytosines at positions −100 and −99 had to be changed to T and G, respectively, to completely eliminate MPBF binding to the high affinity STM site.

Three constructs were selected for analysis in HC11 cells: STM, in which the proximal STM site was changed; A1A3, in which the A1 and A3S sites were modified; and SAA in which all thee MPBF sites were mutated. In addition, an NFκB binding site at −172 which does bind an NFκB-like activity in HC11 cells as judged by EMSA (data not shown), was also mutated using the PCR mutagenesis strategy (Fig. 1B). The CAT activity generated by unmodified and mutated BLG promoter-CAT constructs was measured in cytoplasmic extracts from stably transfected HC11 cells treated with either insulin or insulin, dexamethasone and PRL. The constructs were tested in three independently generated transfected pools of cells and the result of a representative experiment is shown in Fig. 1C. Whilst mutation of the NFκB site did not affect expression of the BLG promoter, mutation of MPBF sites significantly reduced CAT activity. Mutation of MPBF sites in the BLG-CAT constructs did not completely abolish the 3- to 4-fold

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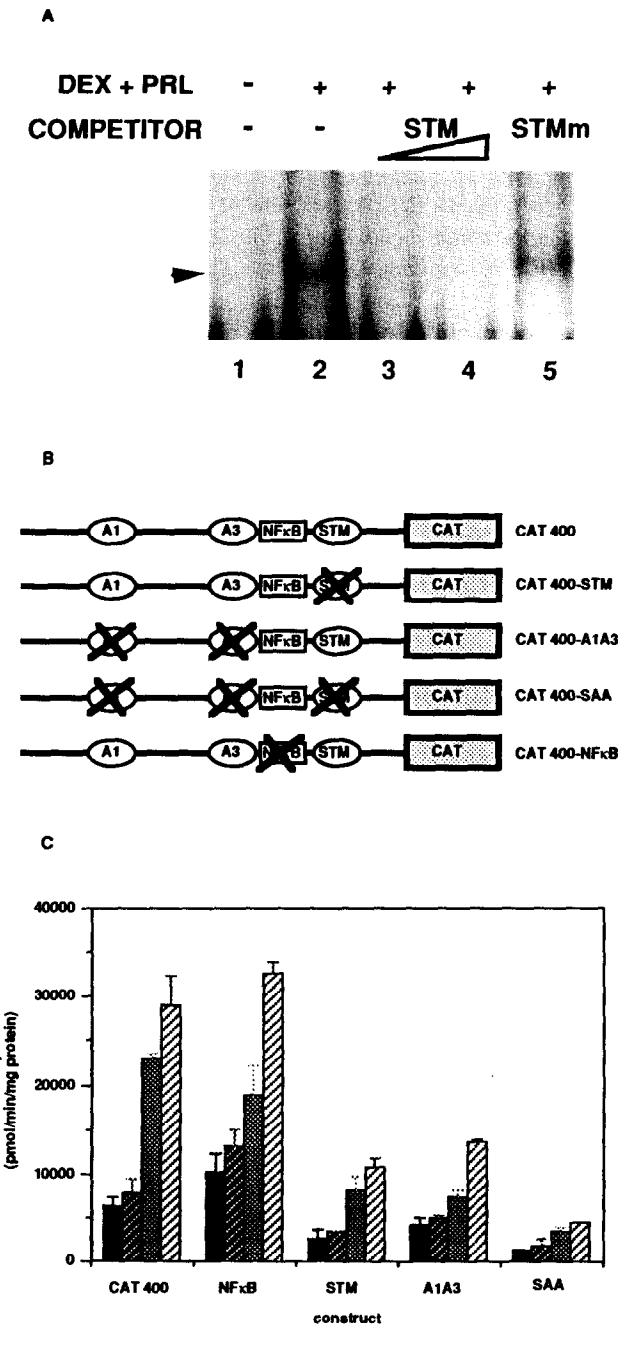
Fig. 1. Generation and analysis of MPBF binding site mutations in the BLG promoter. (A) EMSA with nuclear extract (5 μg) from HC11 cells treated either with insulin alone (−) or with Dex and PRL (+), STM oligonucleotide probe and mutant MPBF binding site oligonucleotide competitors. The molar excess of cold competitor was 4-fold (lane 3) and 40-fold (lanes 4 and 5). (B) Schematic representation of the BLG promoter mutant constructs used in these experiments. Mutant binding site oligonucleotides were used to generate site-specific mutations by PCR. The sequences of these oligonucleotides are given in section 2. Construct STM has the proximal MPBF site mutated, construct A1A3 has the two distal sites mutated and construct SAA has all three sites mutated. (C) Levels of CAT activity for HC11 cells transfected with the unmodified Δ Dp-BLG gene, and the four mutant constructs in response to insulin (solid bars), insulin and PRL (bold crosshatched bars), insulin and Dex (shaded bars), and insulin, Dex and PRL (faint crosshatched bars).The means and standard deviations of triplicate samples for each treatment are shown.

hormonal induction of the BLG promoter. In the context of the normal sheep BLG gene, the SAA promoter is not hormonally induced in HC11 cells (T.B. et al., in prep.), suggesting that the BLG promoter is less tightly regulated when linked to the CAT bacterial reporter gene. These results establish that MPBF is a regulator of BLG gene transcription in mammary cells.

3.2. *The MPBF binding site is related to cytokine/growth factor response elements*

A comparison of the MPBF binding sites in the BLG promoter with a binding site for GAF (IFN-γ induced factor), the GAS motif from the Ly-6E gene [11], a high affinity SIE (sis-inducible element) variant which binds SIF [20], and a high affinity APRE site [12] which binds APRF (Stat3) shows that these sites have a degree of dyad symmetry with the sequence at five positions absolutely conserved (shown in bold in Fig. 2A). Of the three BLG sites, STM is most similar to the GAS-related sequences, matching exactly at seven positions and is almost identical to the APRE variant motif. EMSA analysis showed that a mammary-specific complex of the same mobility as MPBF was observed with SIE and GAS oligonucleotide probes (data not shown). The relative affinity of MPBF for the STM, GAS, and SIE sites was determined by a competition EMSA in which either the STM or SIE elements were used as probe and all three



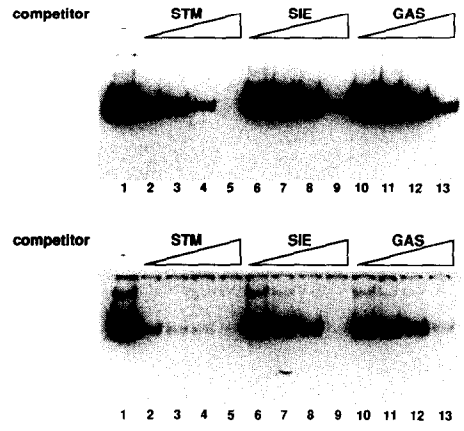


Fig. 2. MPBF recognises GAS elements. (A) Comparison of the three MPBF binding sites in BLG with SIE, GAS, and APRE motifs (a high affinity variant of the c-*fos* SIE element, the GAS site from the Ly-6E gene, and a high affinity variant of the APRF site, respectively). A consensus sequence is indicated – the single bases in the A3S and A1 motifs which are not conserved are underlined. (B) Cross-competition of STM, SIE and GAS sequences. Competition titration of MPBF complex formation with 6 mg of lactating sheep mammary nuclear extract and either STM probe (upper panel) or SIE probe (lower panel) and increasing amounts of STM, SIE and GAS cold competitor oligonucleotides. A 5-fold (lanes 2, 6, 10), 15-fold (lanes 3, 7, 11), 30-fold (lanes 4, 8, 12), or 300-fold (lanes 5, 9, 13) molar excess of the indicated cold oligonucleotide was added to the binding reaction and the amount of complex remaining relative to the uncompeted sample (lanes 1) quantitated with a phosphorimager.

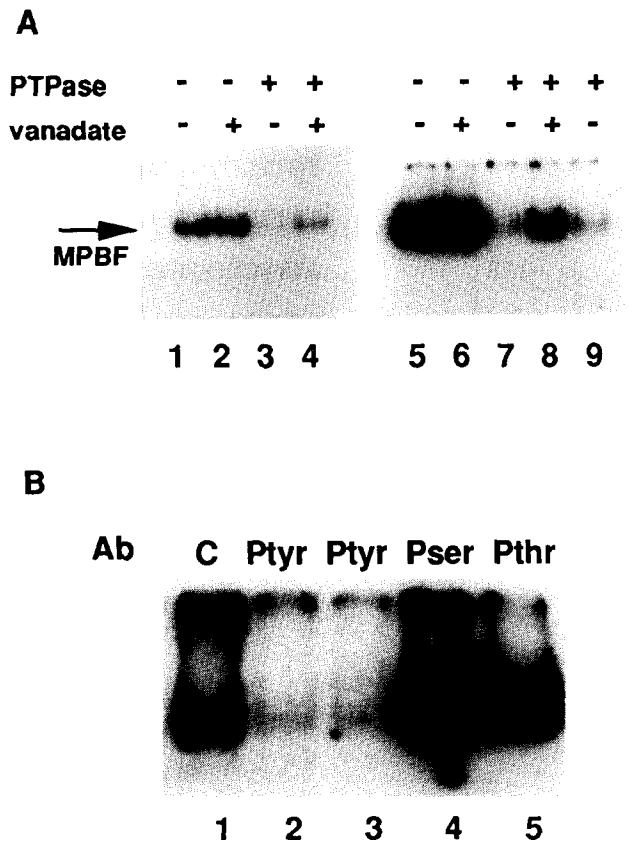


Figure 3. MPBF is phosphorylated on tyrosine. (A) Treatment of MPBF from sheep (lanes 1–4) and mouse (lanes 5–9) lactating mammary glands with LAR (lane 9) and T-cell phosphatases (lanes 3, 4, 7 and 8) and analysis by EMSA using STM probe. Treatment with phophatase, in the presence or absence of sodium vanadate inhibitor, are indicated above each lane. The samples with sheep extract contained 1mg of protein and those with the mouse extract contained 4 μg. (B) Inhibition of MPBF binding activity by antibodies directed against phosphotyrosine. Partially purified sheep MPBF (approximately 100 ng) was incubated with either 5 μg control ascites antibodies (lane 1) or 2.5 μg (lane 2) or 5.0 μg (lane 3) of antiphosphotyrosine antibodies.

binding site oligonucleotides were used as competitors (Fig. 2B). Quantitation of the retarded probe indicated that the affinity of MPBF for both the SIE and GAS sites was approximately five-fold lower than for the STM oligonucloetide. The affinity of MPBF for the GAS and SIE sites was similar to that for the BLG intermediate affinity A3S site (5- to 6-fold lower affinity than STM) but considerably higher than for the BLG A1 motif (44-fold less than STM, [9]). MPBF did not bind an IFN-α response element (ISRE) as judged by EMSA (Fig. 5B) indicating that MPBF recognises only a particular class of cytokine/growth factor response elements and suggests that MPBF may be related to the STAT family of transcription factors that bind GAS/SIE related sequences.

3.3. *MPBF is phosphorylated on tyrosine*

To test whether MPBF requires tyrosine phosphorylation for DNA binding activity, nuclear extracts from sheep and mouse lactating mammary gland were treated with either protein tyrosine phosphatases or with antiphosphotyrosine antibodies and analysed by EMSA. Treatment with either human T-cell or leukocyte antigen related (LAR) protein tyrosine phosphatase prior to EMSA reduced MPBF binding activity by 95% and 86% in mouse (Fig. 3A, lanes 5–9) and sheep (lanes 1–4) extracts respectively, and was inhibited by the specific tyrosine phosphatase inhibitor, sodium vanadate (lanes 2, 4, 6 and 8)). Antibodies directed against phosphotyrosyl residues, also inhibited MPBF activity in partially purified sheep mammary nuclear extract by over 85% (Fig. 3B, lanes 2 and 3). Preimmune serum (lane 1), antiphosphoserine (lane 4) and antiphosphothreonine (lane 5) antibodies did not inhibit MPBF complex formation under these conditions. These results indicate that tyrosine phosphorylation of MPBF or a component of the MPBF complex is required for maximal DNA binding activity.

3.4. *Molecular weight estimation of MPBF from the mammary glands of different species by UV cross-linking*

Fig. 4 (lane 1) shows that for sheep MPBF, two labelled polypeptides of 83 and 85 kDa were cross-linked to the STM oligonucleotide (allowing 19 kDa for the cross-linked oligonucleotide). The molecular weight of the major labelled polypeptide in the mouse extract was

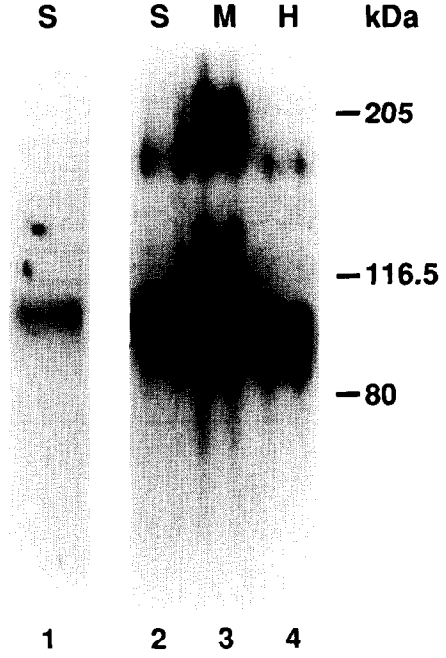


Figure 4. Identification of MPBF DNA-binding components by UV crosslinking. Lane 1 is the cross-linked MPBF polypeptides from 2 μg of lactating sheep nuclear extract, following electroelution, and fractionated on a SDS-PAGE gel. Lanes 2–4 are crosslinked MPBF complexes from lactating mammary nuclear extracts from sheep (0.5 μg), mouse (10 μg) and human (10 μg) respectively, run directly into an SDS-PAGE gel from the UV irradiated EMSA gel.

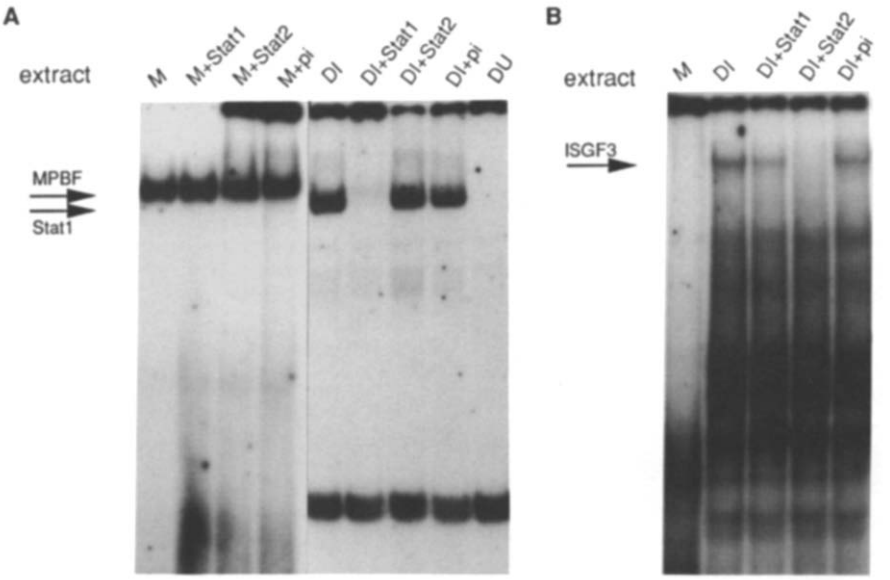


Fig. 5. Analysis of components of MPBF. Nuclear extracts from lactating human mammary gland (4 μg) or IFN-α treated Daudi cells (5 μg) were incubated with p91/84 (Stat1) or p113 (Stat2) specific polyclonal antisera then radiolabelled STM (panel A) or ISRE (panel B) oligonucleotide probes added in a standard gel-shift assay. The source of extract and addition of antibody are indicated above each lane; M is lactating human mammary gland, DI is IFN-α induced and DU is uninduced Daudi cells. Preimmune serum (pi) was used as control. The positions of the MPBF, Stat1 and ISGF3 complexes are indicated.

approximately 90 kDa. A more diffuse cross-linked band was obtained with MPBF from human extracts suggesting the presence of several proteins between 75 and 85 kDa. The presence of multiple bands in human and sheep MPBF suggests that MPBF does not necessarily contain a single polypeptide of 89 kDa as described for rat MGF [18]. The molecular weights of the cross-linked polypeptides in different species correlated with the relative mobilities of MPBF complexes from these species in EMSA (data not shown). Overexposure of the gel (Fig. 4, lanes 2–4) revealed additional high molecular weight complexes of 150–180 kDa, indicating that MPBF binds as a dimer to the palindromic STM site. A minor band of approximately 110 kDa was also detected in all the cross-linked samples.

3.5. *Stat1 and Stat2 are not components of MPBF*

Antibody binding experiments coupled with EMSAs were carried out to determine whether Stat1 and Stat2 are components of the MPBF binding complex. Nuclear extract from lactating human mammary gland was used in these experiments to allow a direct comparison with the Stat1 and ISGF-3 complexes induced by IFN-a treatment of Daudi cells. Nuclear extracts were incubated with antibodies specific for either Stat1 or Stat2 before EMSA with either STM or an ISRE (IFN-a stimulated response element) probe. The Stat1 and Stat2 antibodies inhibited complexes formed on the GAS and ISRE probes respectively in Daudi nuclear extracts but did not affect binding of human MPBF (Fig. 5, lanes 1–9). In addition, the mobilities of the MPBF and Stat1 complexes are clearly different. Western blots of nuclear extracts also indicated that mammary nuclei contain very low levels of Stat1 (data not shown) supporting the conclusion that Stat1 and Stat2 are not components of MPBF which may be a novel member of the STAT family.

3.6. *MPBF-like binding activity is induced directly by prolactin*

To determine whether PRL could induce MPBF binding activity we utilised Chinese hamster ovary (CHO-E3) cells stably transfected with a vector that expresses the long form of the rabbit PRL receptor. This system has been used to demonstrate that the BLG promoter is stimulated by PRL [16, 21] and more recently to define

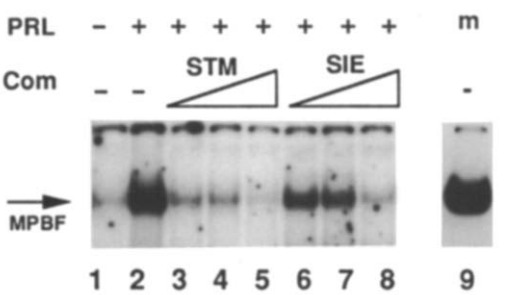


Fig. 6. Prolactin induces MPBF binding activity in CHO-E3 cells. Nuclear extracts were prepared from CHO cells grown in either the absence (lane 1) or presence (lanes 2–8) of PRL and 5 μg extract used in an EMSA with STM probe. Increasing amounts of cold STM or SIE competitor oligonucleotides were added to the binding reactions as indicated : 10-fold molar excess (lanes 3, 6), 20-fold excess (lanes 4, 7) and 100-fold excess (lanes 5, 8). Lane 9 is an MPBF control using 8 μg nuclear extract from lactating mouse mammary gland. This track was exposed for a shorter time than lanes 1–8.

the prolactin response elements in the BLG promoter (Demmer et al., in prep.). A factor of similar mobility to mouse MPBF was induced 5- to 10-fold in the nuclear extracts of CHO-E3 cells treated with PRL. This factor bound the STM oligonucleotide specifically as demonstrated by titration with cold STM competitor binding sites. Competition with the SIE oligonucleotide demonstrated that the induced factor had a reduced affinity for this site compared with the STM oligonucleotide, analagous to the the result obtained with mammary gland derived MPBF (Fig. 2B). These results show that an MPBF-like binding activity can be stimulated by PRL alone and may mediate PRL signalling in tissues and cells other than the mammary gland.

4. Discussion

MPBF has several characteristics of the recently described STAT family of transcription factors. MPBF recognises Stat1 binding sites, requires tyrosine phosphorylation for DNA binding and may be composed of a dimer of an 80–90 kDa polypeptide. Five members of the STAT family of signalling factors have been cloned so far. In addition to Stat1 and Stat2, cDNA clones of mouse and human APRF (Stat3), mouse Stat4 and sheep MGF have been identified [22,23,24]. Although the highest affinity binding site for MPBF, STM, is almost identical to a high affinity variant of the APRF recognition element ([12] and Fig. 1A) preliminary supershift experiments with an antibody to APRF indicate that MPBF does not contain Stat3 (data not shown). Since MGF has been shown to mediate the response to PRL and has binding sites in the promoter of the β-casein milk protein gene, it is likely that MGF and MPBF are similar factors.

The finding that MPBF binds other STAT factor recognition sequences suggests that STAT factors might regulate the BLG promoter through its MPBF sites. EGF is known to inhibit the expression of milk protein genes and can interfere in the induction of MGF in HC11 cells [26] but the mechanism through which this effect is mediated is unknown. Since EGF can induce Stat1 and Stat3, it is possible that these factors could directly antagonise milk protein gene expression by binding to MPBF and MGF sites. The identification of an MPBF-like complex in the virgin and early pregnant sheep mammary gland (CJW and TB, unpublished results), when the BLG gene is inactive, suggests that such a repressor might operate in vivo.

Stat1 is the paradigm for a growing family of transcription factors which can be posttranslationally activated in response to cytokines and growth factors. It will be interesting to determine the role these factors play in the regulation of mammary gland development and milk protein gene expression.

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