RESEARCH COMMUNICATION

A positive regulator of the ribosomal protein gene, β factor, belongs to the ETS oncoprotein family

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The β factor, which interacts with the rpL32 promoter, binds to the sequence 5'-GAGCCGGAAGTG and trans-activates this gene. Comparison of the DNA sequences bound by the β factor with those bound by other known DNA-binding proteins revealed that the ETS proteins interact with similar DNA sequences. Consequently we have examined the relationship of the β factor to the several ETS proteins so far reported. Antibody and oligonucleotide competition experiments, performed by using electrophoretic shift analysis, revealed that the β factor contains ETS epitopes and that it is immunologically related to both of the GA-binding proteins (GABPs), implying that the β factor may consist of two separate protein subunits.

INTRODUCTION

The mouse ribosomal protein L32 gene contains multiple nuclear protein binding sites located upstream and downstream of the transcription start site [1,2]. Both deletion and cell-free transcription analyses have revealed positively acting DNA sequences located at position -79 to -69 (β element) of the promoter region [1,3,4]. In recent studies we have shown that a 55 kDa protein binds to this promoter (β) element and stimulates transcription [3]. Further examination of the DNA sequence bound by the β factor suggests considerable sequence similarity to the ETS DNA-binding site. The ETS element consists of a core purine-rich sequence GGAA, which is also present in other ribosomal gene promoters [5–7; Table 1].

The cellular ETS family of genes has been identified by sequence similarities to the v-ETS oncogene of the E-26 avian leukaemia virus [8–10]. Members of this family include c-ETS 1 and c-ETS 2 [10,11]. Both genes encode for DNA-binding nuclear phosphoproteins and possess characteristics suggesting that they play a role in gene expression [12–14]. The c-ETS 2 gene is expressed in proliferating cells [15]. The c-ETS 1 protein acts as a transcription factor, that binds to the sequence A/CGGAAGT/C, present in many cellular and viral promoters and enhancers [16–19].

Apart from ETS 1 and ETS 2, a number of proteins identified recently also contain amino acid sequences similar to ETS proteins, and the C-terminal regions of these proteins are highly conserved (Table 2). Among these proteins, ETS 2 and GA-binding protein (GABP) are present in all cell types examined [15,20]. In view of these observations we postulate that one of these proteins is identical or possesses qualities equivalent to the β factor.

Recent studies by McKnight and co-workers have revealed that the GABP which activates the herpes simplex virus (HSV) immediate early gene consists of two subunits, and these two distinct proteins form a heteromeric complex which binds to the DNA element [20]. The GABP α -subunit displays characteristics of the ETS family of proteins. On the other hand, the GABP β -

subunit contains the ankyrin repeat [20]. Furthermore, the herpes simplex virus GA DNA element and the rpL32 β element possess significant sequence homology (Fig. 1a).

Table 1. ETS DNA sequence motif in the promoter segment of eukaryotic ribosomal proteins

Gene	Species	Position	Sequence	Reference
L32	Mouse	-69	CCGGAAGTG	[33]
L30	Mouse	-65	ACAAGGCGAA	[34]
S16	Mouse	-211	AGAAGGCTCA	[35]
S14	Human	-69	AGAAGGCCTT	[7]
L14	<i>Xenopus laevis</i>	-55	TGAAGGCCAA	[36]
L14	<i>Xenopus tropicalis</i>	-55	TGAAGGCCAA	[37]
rp59	Yeast	-120	CGAAGGAGTC	[38]
rp51	Yeast	-149	CGAAGGATTC	[39]

Table 2. ETS-related proteins in various species

Protein	Species	Tissue specificity	Reference
c-ETS	Chicken	Induction of erythroblastosis	[8,9]
PU.1/Spi-1	Mouse specific	B-cell and macrophage	[28]
E47	<i>Drosophila</i>	Temporal and spatial pattern expression	[40]
ETS 1	Mouse	T-lymphocytes	[41,42]
ETS 2	Mouse	Proliferating cells	[15]
ETS 2	Human	Most tissues	[12]
erg	Human	?	[43]
elk-1	Human	Lung and testis	[27]
elk-2	Human	Lung and testis	[27]
ETS 2	<i>Drosophila</i>		[45]
PAE3	Mouse	Absent from lymphoid and erythroid	[25]
GABP- α	Rat	Most tissues	[20]
Fli-1	Mouse	Erythroleukaemia	[29]

Abbreviations used: GABP, GA-binding protein; HSV, herpes simplex virus.

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Fig. 1. (a) Comparison of the DNA sequence in the binding sites for ribosomal protein L32 β factor, HSV GABPs and polyoma virus enhancer 3 (PEA3) protein, and (b) comparison of the amino acid sequences of ETS proteins

In (a), the DNA sequences are aligned by the common GGAA motif. In (b), Pan ETS peptide was used to prepare the Pan ETS antibodies.

This investigation is designed to assess the relationship of the β factor to the ETS proteins and to determine specifically whether it is identical with one of the GABPs. Results presented in this paper demonstrate that the β factor is unrelated to ETS 1 or ETS 2, but possesses characteristics similar to those of both GABPs. It is likely that the β factor is the murine homologue of the rat GABPs.

MATERIALS AND METHODS

Cell culture and nuclear extract preparation

Preparation and partial purification of mouse L1210 nuclear extracts were performed as previously described [21,22].

Gel-mobility-shift assay

Electrophoretic-mobility-shift assays were performed as described by Yoganathan & Sells [3]. The labelled fragments used for the gel-shift assays were prepared essentially as described by Yoganathan *et al.* [4]. A specific double-stranded oligonucleotide containing the sequence in the β -element recognition site was prepared by annealing the chemically synthesized oligonucleotides 5'-CCCAGAGCCGGAAGTG-3' and 5'-TGGGCACT-TCCGGCTC-3'. The double-stranded oligonucleotide probes used were β element (5'-CCCAGACCGGAAGTG-3'), PEA3 (5'-GATCTCGAGCAGGAAGTTCTGA-3') and PEA3-Mu (5'-CATCTCGAGCAAGAAGTTCTGA-3'). The influence of antibodies on gel-mobility shift assays was determined by incubating antibody with nuclear extracts at 4 °C for 20 min in a binding reaction, followed by incubation with the labelled DNA probe for a further 30 min at 30 °C.

Preparation of Pan-ETS monoclonal antibody

A Pan-ETS oligopeptide, FKLSDPDEVARRW, corresponding to the amino acid sequence that is conserved in the various known ETS proteins (Fig. 2), was synthesized and used to prepare the monoclonal antibody [23].

Other antisera

The ETS 1- and ETS 2-specific polyclonal antisera were raised against specific oligopeptides; K17C was derived from exon 7 of ETS 1, and L13D was derived from the last 13 amino acids from the C-terminal region of ETS 2. Both were purchased from Cambridge BioResearch Chemicals, DE, U.S.A. Antibodies to GABP α - and β -subunits were kindly provided by Steven McKnight and Catherine Thompson (Carnegie Institute of Washington, Department of Embryology, Baltimore, MD, U.S.A. [20].

RESULTS

β factor is distinct from PEA3 and ETS 1 proteins

In a previous publication we reported that the β element interacts with a 55 kDa protein [3]. This 12-base-pair sequence has considerable homology with the polyoma-virus enhancer element 3 (PEA3) and HSV GABP element (see Fig. 1a). Studies using mobility-shift assays reveal that the oncoprotein c-ETS 1 and the PEA3 binding protein both recognize a common sequence within the PEA3 element [16,24].

The present studies were therefore designed to determine the relationship of the β factor to other identified factors interacting with the ETS element. To perform this investigation, electrophoretic-mobility-shift assays were employed. Partially purified nuclear extracts from L1210 cells were incubated in the presence of a labelled oligonucleotide probe representing the β element. The mobility-shift assays revealed the presence of a specific band (Fig. 2, lane 1). Competition experiments were performed with an unlabelled oligonucleotide representing either the β element or the PEA3 element. The presence of the unlabelled β oligonucleotide dramatically decreased the appearance of a specific band (Fig. 2, lanes 2–4). Although the presence of an equimolar amount of the unlabelled PEA3 element had some effect on the

Fig. 2. Gel shift assay for the β factor

A synthetic double-stranded labelled oligonucleotide containing the β element was incubated with L1210 nuclear extracts; PEA3 wild type and PEA3 mutant oligonucleotides were used as competitors. Lanes: 1, no competitor; 2–4, specific β oligonucleotide 25-, 50- and 100-fold molar excess respectively; 5–7, PEA3 mutant (M) oligonucleotide 25-, 50-, 100-fold molar excess respectively; 8–10, PEA3 wild-type (W) oligonucleotide 25-, 50-, 100-fold molar excess respectively.

Fig. 3. Effect of ETS antibodies on the ability of nuclear extracts to interact with the β element as determined by gel-shift assays

Mouse L1210 nuclear extracts were incubated with individual antibody preparations and tested for their ability to bind to the labelled β oligonucleotide. Lanes: 1, no antibody; 2, Pan ETS antibody; 3, ETS 2 antibody; 4, ETS 1 antibody.

signal as compared with the effect of the specific competitor, this effect was relatively insignificant (Fig. 2, lanes 8–10). Inclusion of a PEA3 mutant oligonucleotide failed to suppress the intensity of the specific band (Fig. 2, lanes 5–7). These results demonstrate that neither the PEA3 protein nor ETS 1 is identical with the β factor.

A parallel mobility-shift experiment was performed with a labelled oligonucleotide probe containing the PEA3 element to determine whether the β element would compete with it for the PEA3 protein(s). Our results revealed that PEA3 proteins were not competed for by the β element (results not shown).

Effect of ETS antibodies on β factor assessed by gel shift assay

To determine the relationship of the β factor to other members of the ETS family, antisera raised against ETS 1, ETS 2 and Pan ETS peptide were employed. The Pan ETS antibody was developed by using the 14-amino-acid sequence which is conserved among the different ETS proteins. Nuclear extracts prepared from L1210 cells were incubated separately with each of these antibodies and then tested for their ability to bind to the β element, as illustrated in the mobility-shift assays shown in Fig. 3. It is evident that only the Pan ETS antibody specifically diminished DNA binding of the β factor (Fig. 3, lane 2). Neither the ETS 1 nor the ETS 2 antibody eliminated specific DNA binding (Fig. 3, lanes 3 and 4). We therefore conclude that the epitopes present in the β factor were not recognized by the antisera produced against ETS 1 and ETS 2. On the other hand, the antisera developed against Pan ETS recognized epitopes in

Fig. 4. Influence of ETS peptides on the interaction between the β element and the β factor in the presence of Pan ETS antibody as determined by gel-shift assays

Lanes: 1, no nuclear extract; 2, plus nuclear extract; 3, nuclear extract and Pan ETS antibody; 4 and 5, nuclear extract, Pan-ETS antibody and 1 and 2 μ g of Pan ETS peptide respectively; 6, nuclear extract, Pan ETS antibody and 2 μ g of ETS 2 peptide. The epitope specificity was tested by absorption of the antibodies by the Pan ETS peptide.

the β factor. These data imply that the β factor contains an ETS recognition site, and this site is important for the binding to the DNA.

The specificity of the Pan ETS antibody was further verified by antigenic competition experiments in which the synthetic Pan ETS peptide was included in the gel-mobility-shift reaction mixture. Inclusion of Pan ETS antibodies abolished the DNA binding (Fig. 4, cf. lanes 2 and 3). Addition of Pan ETS peptide to the reaction mixture before the Pan ETS antibody effectively restored the DNA-binding capacity (Fig. 4, lane 5), whereas addition of the ETS 2 peptide failed to restore DNA binding (Fig. 4, lane 6). Addition of Pan ETS antibodies or peptides Pan ETS and ETS 2 alone to the reaction mixture lacking nuclear extract and containing the labelled β element failed to produce a shift (results not shown). These results suggest that the β factor contains an epitope recognized by Pan ETS antibodies. These observations also eliminate the possibility that the decreased level of the DNA- β -factor complex after treatment of extracts with Pan ETS antibody results from proteolytic activity.

β factor is immunologically related to GABPs

The above experiments support the belief that the β factor contains an ETS DNA-binding domain. Recent studies by McKnight and co-workers indicate that the HSV GA element recognizes two different proteins [20] and that one of the proteins is related to the ETS family of proteins. GABPs contain two different subunits derived from two independent genes. To examine the relationship of the β factor to GABPs, we conducted the following gel-shift experiment to establish whether the β factor is immunologically related to either of the GABPs. As shown in Fig. 5, addition of antiserum specific to GABP α to the

Fig. 5. Effect of GABP antibodies on the formation of the DNA-protein complex with the β element

Lanes: 1, nuclear extract; 2 and 3, nuclear extract with GABP α antibodies; 4 and 5, nuclear extract with GABP β antibodies; 6, nuclear extract with control pre-immune-sera antibodies.

reaction mixture produced a further shift of the specific band (lanes 2 and 3). Furthermore, antiserum specific to GABP β also shifted the specific band (lanes 4 and 5). On the other hand, control pre-immune sera had no effect on the specific band (lane 6). These data suggest that the ribosomal protein β factor contains epitopes related to both the GABP α and GABP β subunits.

DISCUSSION

Previous studies have revealed that the β factor binds to the DNA sequence GAGCCGGAAGTG at position -79 to -69 of the mouse rpl32 gene [1,3]. This sequence functions as a positive *cis*-acting element in this gene [1]. In experiments presented here we further characterize the β factor of the rpl32 gene by using antibodies for the already identified ETS proteins and oligonucleotides in competition gel-shift assays.

We have previously shown that the cloned and purified PEA3 DNA-binding protein binds to the β element and trans-activates the rpl32 gene [4]. A survey of PEA3 mRNA in various cell types have revealed, however, that its distribution is restricted, being absent in cells of lymphoid or erythroid origin [25]. Consequently PEA3 cannot be considered a candidate for the β factor, because the rpl32 gene-transcription factor must be ubiquitous. Furthermore, our gel-shift competition data reveal that the endogenous protein in nuclear extract preparations recognized by the β element is distinct from PEA3 (Fig. 2). Our previous study had shown that cloned and purified PEA3 protein was able to bind to this sequence.

We interpret these results as indicating that the PEA3 protein is able to bind to this sequence; however, the present data demonstrated that the complex formed with the β element has greater specificity for the β element.

The β element possesses a core GGAA sequence (purine-rich) which is also recognized by the ETS family of proteins. We have therefore examined this family of proteins for a member which resembles the β factor. A variety of genes have been described whose protein products have predicted amino acid sequences displaying strong homology to the ETS proto-oncogene ([25-29];

Table 2). Furthermore, several studies suggest that these proteins act as transcriptional regulators [16-19,24-30]. Results in the present paper indicate that the rpl32 transcription factor β belongs to the ETS family of proteins. This has been confirmed by using the Pan ETS antibody, which recognizes the conserved oligopeptide in many identified ETS proteins (Fig. 3).

These results provide additional information concerning the rpl32 β factor. More specifically, our studies reveal that the β factor is immunologically related to both of the GABPs. This finding suggests that the rpl32 β factor consists of two sites identical with GABP α and β or is composed of two separate subunits containing each of these epitopes. McKnight and co-workers have previously shown that GABPs are present in uninfected cells and argue that HSV conveniently utilizes a cellular factor for trans-activating the virus immediate early genes [32]. Recent studies also indicate that mRNAs for GABPs are present in all cell types examined [20]. In this study we have shown that the trans-activating factor, β , for the housekeeping gene rpl32 is immunologically related to GABPs. One criterion for factors such as β , which play a role in the control of ribosomal-protein gene expression, is that they should be ubiquitous in cell distribution. The more broadly distributed members such as the ETS 2 and GABPs may therefore possess characteristics similar to or identical with those of the β factor. Our present results reveal, however, that rpl32 β factor is distinct from ETS 2, but immunologically related to the GABPs.

Whether all ribosomal-protein genes possess sequences which interact with the rpl32 β factor is currently an open question. Several studies have revealed that eight ribosomal-protein promoters so far identified contain ETS binding sites ([5-7]; Table 1). It is tempting to speculate that β or other members of the ETS family of proteins play a role in co-ordinating ribosome biogenesis at the level of ribosomal-protein gene transcription.

Previous studies from our laboratory have shown that the β factor is needed for efficient cell-free transcription and that the PEA3 DNA-binding protein trans-activates the rpl32 gene through the β -element [3,4]. The observation that the β factor responds when cell are physiologically perturbed (T. Yoganathan & B. H. Sells, unpublished work) led us to examine whether this ribosomal-protein promoter sequence is conserved in other species. A search of the literature revealed that *Xenopus* and yeast ribosomal-protein genes also contain the ETS DNA-binding domain in their promoter sequences (Table 1). Reports exist showing that an ETS DNA-binding nuclear protein is present in *Xenopus* [6]; hence it is tempting to speculate that ETS-related proteins co-ordinate ribosomal-protein gene expression in lower and higher organisms.

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