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Regulation of transcription in mammalian cells by yeast Leu3p and externally supplied inducer

Hui Guo, Gunter B. Kohlhaw*

Department of Biochemistry, Purdue University, West Lafayette, IN 47907, USA

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Abstract The Leu3 protein of yeast is a dual-function regulator, stimulating transcription when the inducer α-isopropylmalate (α-IPM) is present and suppressing transcription when the inducer is absent. Here we show that Leu3p retains both its positive and negative regulatory properties when expressed in mammalian cells or when added to a mammalian nuclear extract. α-IPM stimulates reporter gene expression 15–20-fold, both in vivo and in vitro. The concentration of α-IPM required for half-maximal stimulation in vitro is 2.5×10^{-4} M. No yeast-specific factors other than Leu3p itself are required for up- or down-regulation. Since α-IPM is not metabolized in mammalian cells, the Leu3p-α-IPM system might be useful in gene therapy and other studies as a highly specific, externally controlled on/off switch of gene expression.

Key words: Eukaryotic transcriptional regulation; Transactivator Leu3p; Gene therapy; Cross-species modulation; Yeast (*Saccharomyces cerevisiae*); Mouse fibroblast

1. Introduction

Leu3p belongs to a family of lower eukaryotic transcriptional activators that have several features in common. Their DNA binding domains conform to a $Zn(II)_2$-Cys_6 cluster located near the N-terminus [1,2], they have acidic-hydrophobic transcriptional activation domains, usually located near the C-terminus [3–5], and some of them are modulated, i.e. changed from an inactive to an active form by a small effector molecule. For example, Gal4p responds to an unidentified intermediate of galactose metabolism that alters the interaction between Gal4p and the negative regulator GAL80 [4]; Put3p, an activator in the proline utilization pathway of yeast, responds to proline [6]; Hap1p responds to heme which apparently affects both the binding of Hap1p to DNA and the unmasking of its activation domain [7]; and Leu3p responds to α-IPM, an early intermediate in leucine biosynthesis [8]. Leu3p binds to UAS_{LEU} motifs in the promoters of genes involved in branched-chain amino acid biosynthesis and nitrogen assimilation [9–11]. It does so irrespective of the presence or absence of α-IPM [10,12]. When α-IPM is absent, Leu3p acts as a repressor [8,10,13]. When α-IPM is present, the activation domain of Leu3p becomes available for interaction with factor(s) of the transcription initiation machinery and elicits a strong activating signal [8].

The activation domains of Gal4p and Leu3p have been shown to stimulate transcription not only in yeast, but also in mammalian cells, consistent with the idea that the apparatus involved in gene activation has been conserved among eukaryotes [14,15]. By contrast, modulation of yeast (or other lower eukaryotic) transactivators apparently has not been demonstrated outside of their native environment. In some instances, such as in the case of Gal4p, modulation across species would be difficult to achieve since additional protein factors are required for masking and unmasking of the activation domain, and possibly other functions [16,17]. Experiments with LAC9, a Gal4p-related transactivator from *Kluyveromyces lactis*, support this notion: to achieve masking of LAC9 in mammalian cells, it was necessary to co-express GAL80 [18]. Moreover, masking could not be reversed by galactose, presumably because factors required for the unmasking process are absent from mammalian cells.

We report here that Leu3p adopts a masked conformation in mammalian cells on its own and that unmasking can be achieved simply by including the inducer α-IPM in the culture medium. Masked Leu3p causes repression, unmasked Leu3p causes activation of gene expression.

2. Materials and methods

2.1. Plasmid construction

Plasmid pC3-LEU3 was constructed to express full-length Leu3p from the human cytomegalovirus (CMV) major intermediate early promoter in mammalian cells. A 3.1 kb fragment containing the *LEU3* sequence from −45 to +3063 [19] was excised from plasmid pZRL [20] by complete digestion with *Eco*RI and partial digestion with *Hin*dIII. The fragment was separated on a 0.7% agarose gel and extracted with the QIAEX II gel extraction kit (QIAGEN Inc.). It was then ligated into the polylinker region of the expression vector pcDNA3 (Invitrogen) that had also been digested with *Eco*RI and *Hin*dIII. The plasmid carrying the luciferase (LUC) reporter gene was pGL3-LUC (Promega). To construct pGL3-UAS_L-LUC, four consecutive UAS_{LEU} 30-mers [10] were inserted at the *Sac*I site of the polylinker region of pGL3-LUC.

2.2. Transfection of mammalian cells and luciferase assays

Mouse 30A5 preadipocytes [21], a cell line derived from 10T1/2 fibroblasts and kindly provided by K.-H. Kim, were cultured to about 70% confluence, then transfected, incubated, and lysed according to published procedures [22]. Luciferase assays were performed by luminometry following the supplier's protocol (Promega, Publication TM033). Transfection efficiency was monitored by cotransfecting the cells with pRSV-lacZ [22] and measuring β-galactosidase activities.

2.3. Preparation of nuclear extracts and in vitro transcription assays

30A5 cells were harvested by centrifugation in a table-top centrifuge at $1000\times g$ for 10 min. Nuclear extracts were prepared by established procedures [23]. In vitro transcription was carried out by the method of Sierra et al. [24]. Plasmid pML(C_2AT)Δ-53sh, which carries the −53 to +10 nucleotide sequence of the adenovirus type 2 major late (Ad2-ML) promoter followed by a G-free cassette [25], was modified by inserting five consecutive UAS_{LEU} elements at the unique *Sac*I site of the Ad2-ML promoter. Modified or unmodified plasmid was

*Corresponding author. Fax: (1) (317) 494-7897.
E-mail: kohlhaw@biochem.purdue.edu

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digested with *Pvu*II, and the shorter of the two fragments obtained in each case served as template. The total volume of each transcription assay was 30 μl. Transcripts were electrophoresed on a 4% polyacrylamide-urea gel, subjected to autoradiography, and quantitated by densitometry using an UltroScanXL laser densitometer.

2.4. SDS-polyacrylamide gel electrophoresis and immunoblotting

After cell lysis, performed according to established procedures (Promega Publication TM033), aliquots of whole cell extract corresponding to 20 μg of total protein were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting. The antibodies used were prepared against a chimeric protein that contained the 28 C-terminal amino acid residues of Leu3p fused to key limpet hemocyanin [26]. Immunoblotting was performed following existing protocols (Amersham International Life Science).

2.5. Special chemicals

DL-α-Isopropylmalic acid was from Aldrich; β-isopropylmalic acid was a gift from H.E. Umbarger. Highly purified yeast Leu3 protein was kindly provided by F. Zheng.

3. Results

3.1. Leu3p expressed in mammalian cells can be modulated by α-IPM

To express full-length Leu3p in mammalian cells, we transiently transfected 30A5 mouse preadipocytes (a fibroblast-derived cell line) with a plasmid (pC3-LEU3) that carried the *LEU3* gene behind a CMV promoter (see Section 2). A plasmid containing the luciferase gene controlled by an SV40 promoter served as the reporter. The reporter plasmid was used either as such or with four UAS_{LEU} elements inserted upstream of the SV40 promoter. The luciferase activity observed with reporter plasmid devoid of UAS_{LEU} elements (Fig. 1, column 1) was taken to reflect basal level expression. This level of expression did not change when either Leu3p alone or Leu3p and α-IPM were present (columns 2,3), indicating that there was no 'squelching', i.e. sequestration of transcription factors by unbound Leu3p. Inserting UAS_{LEU} elements upstream of the SV40 promoter resulted in a slight (< 2-fold) increase in luciferase activity (columns 4,5). This increase was probably caused by a weak interaction of factors present in 30A5 cells with the UAS_{LEU} flanking regions (data not shown). The increase was α-IPM-independent. In cells that had been cotransfected with UAS_{LEU}-containing reporter plasmid and pC3-LEU3, luciferase gene expression dropped about 4-fold relative to cells that carried only the reporter plasmid (compare columns 4,6). This negative effect probably has two components: competition with the weakly interacting factors, and suppression of basal level transcription to about 40% of normal (compare columns 1,6). Adding α-IPM to cells that had been cotransfected with UAS_{LEU}-containing reporter plasmid and pC3-LEU3 led to strong activation of the reporter gene. At what appeared to be saturating levels of α-IPM in the culture medium (10 mM), an overall increase in expression of about 17-fold was observed (compare columns 6,7).

To determine if full-length Leu3p was expressed in the mouse cell line in both the presence and absence of α-IPM, Western blots were performed using whole-cell lysates of 30A5 cells (Fig. 2). The results show that Leu3p molecules of the expected mass (approx. 100 kDa [19]) were produced in transfected mammalian cells irrespective of the presence or absence of inducer (compare columns 2,3). No crossreacting material could be detected in non-transfected cells (column 4).

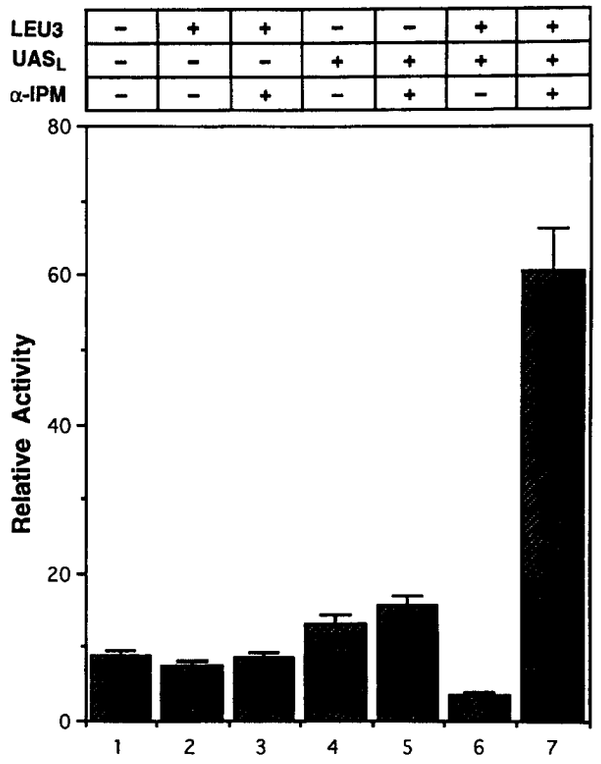

Fig. 1. Regulation of reporter gene expression by yeast Leu3p and α-IPM in mouse cells. Preadipocytes (30A5)were transfected as indicated and incubated for 48 h in the absence (−) or presence (+) of α-IPM (10 mM). The cells were then lysed and assayed for transfection efficiency and luciferase activity. Boxes labeled LEU3: cells were transfected with pC3-LEU3 where indicated (+). Boxes labeled UAS_L: cells were transfected either with pGL3-LUC (−) or with pGL3-UAS_L-LUC (+). See Section 2 for further details. Columns: 1, cells were transfected with pGL3-LUC reporter plasmid only (basal level expression; no UAS_{LEU} elements present); 2, cells were cotransfected with pGL3-LUC and the *LEU3*-containing plasmid pC3-LEU3; 3, same as column 2 except that cells were incubated in the presence of α-IPM; 4, cells were transfected with pGL3-UAS_L-LUC only; 5, same as column 4 except that cells were incubated in the presence of α-IPM; 6, cells were cotransfected with pGL3-UAS_L-LUC and pC3-LEU3; 7, same as column 6 except that cells were incubated in the presence of α-IPM. Increasing the α-IPM concentration from 10 to 20 mM did not change the -fold activation; decreasing the α-IPM concentration to 5 mM resulted in a smaller activation amplitude (not shown). No special effort was made to facilitate the uptake of α-IPM into the cells. The α-IPM concentrations given are those of the biologically active isomer. The error bars indicate S.D. of the mean calculated from three experimental samples assayed in duplicate.

3.2. Negative and positive effects of Leu3p in a mammalian cell nuclear extract

To substantiate further the idea that Leu3p is modulated in a mammalian environment in a fashion similar to what had been seen in yeast, we performed in vitro transcription assays using nuclear extract from untransfected 30A5 cells and Leu3p that had been purified to near homogeneity from yeast cells overproducing the protein [26]. Extract was incubated with a template that contained the Ad2-ML promoter followed by a G-free cassette (Fig. 3). To allow efficient binding of Leu3p, five copies of the UAS_{LEU} element were inserted upstream of the promoter. The amount of transcript produced in the absence of Leu3p and α-IPM is shown in Fig. 3A (lane 1). Adding highly purified Leu3p but no α-IPM resulted in a

5-fold drop in the rate of transcription (lane 2). Adding both Leu3p and α-IPM caused strong activation of transcription (lanes 3–9), with a maximal amplitude between non-induced and induced levels of about 18-fold. Supplying the in vitro system with increasing concentrations of α-IPM had a saturation effect (Fig. 3B). The α-IPM concentration necessary to cause half-maximal stimulation of transcription was 0.25 mM. To again address the question of whether the negative effect of Leu3p was due to squelching, Leu3p was added to an incubation mixture containing template without the UAS_{LEU} elements. There was essentially no effect on transcription, whether α-IPM was present or not (Fig. 3C, compare lanes 1,2 with lanes 3,4). Fig. 3C further demonstrates that, like in the in vivo experiment, the mere presence of the UAS_{LEU} elements caused a slight (2.5-fold), α-IPM- and Leu3p-independent activation (lanes 5,6). Again, this effect was abolished and the basal level expression (as seen in lanes 1–4) was reduced (by 3-fold) when Leu3p was added (lane 7). Leu3p-dependent transcriptional activation (lane 9) specifically required α-IPM since no activation was observed when β-IPM was used instead (lane 8). In fact, when β-IPM replaced α-IPM, Leu3p returned to its repressive mode. The amplitude between the repressed and activated levels in this experiment was 19-fold.

4. Discussion

The results reported here are consistent with a scenario according to which Leu3p synthesized in 30A5 cells enters the nucleus and binds to the UAS_{LEU} elements of the reporter gene in a masked conformation that represses gene expression below basal level. Externally supplied α-IPM also enters the nucleus and causes a conformational change in Leu3p that allows the transcriptional activation domain to establish contact with the general transcription factors, thereby stimulating gene expression. A synopsis of these events is given in Fig. 4.

Qualitatively and quantitatively, the behavior of Leu3p in mammalian cells and in mammalian cell nuclear extracts very much resembles that seen in its native yeast environment. For example, the presence of Leu3p causes a 2.5–5-fold drop in basal level expression of reporter genes in the mammalian

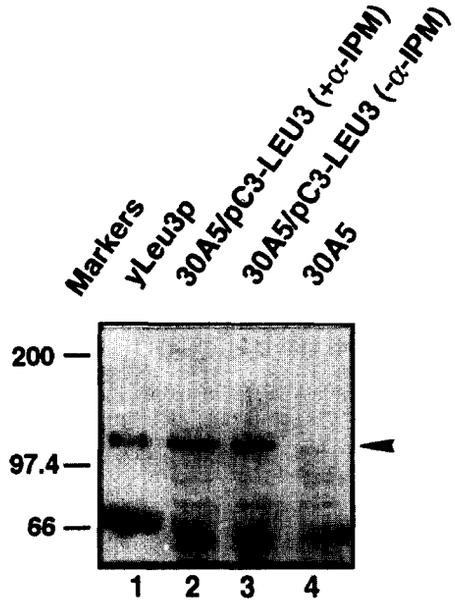

Fig. 2. Immunoblot analysis of Leu3p synthesized in 30A5 cells. Lanes: 1, control, highly purified Leu3 protein from yeast; 2, whole-cell lysate of 30A5 cells transfected with pC3-LEU3 and incubated in the presence of α-IPM (10 mM); 3, same as lane 2 except that cells were incubated in the absence of α-IPM; 4, whole-cell lysate of untransfected 30A5 cells. The position of three molecular weight markers is indicated (kDa). The arrowhead points to the position of Leu3p (approx. 100 kDa). See Section 2 for further details.

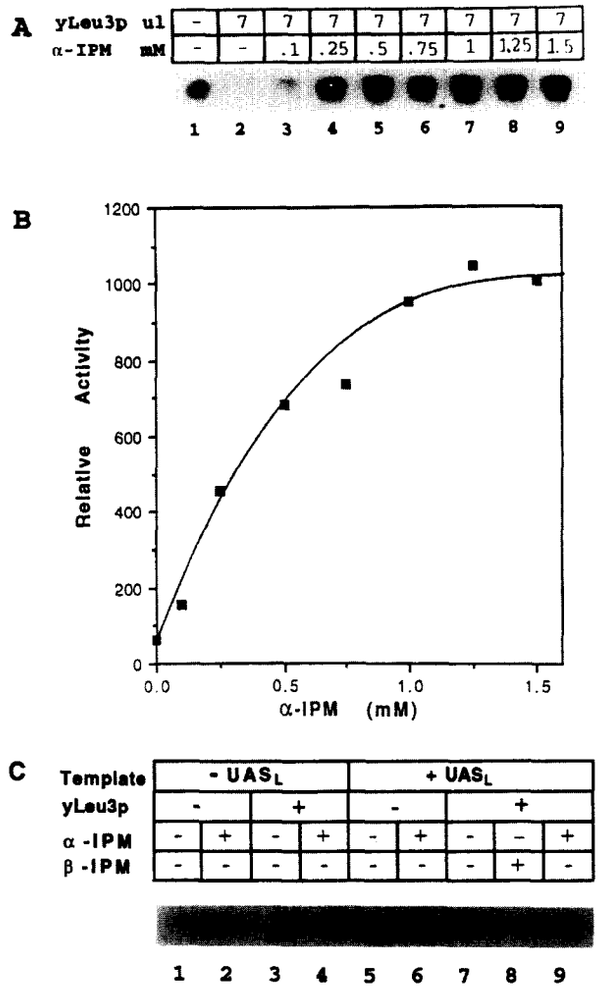

Fig. 3. Regulation of gene expression by Leu3p and α-IPM in vitro in a 30A5 cell nuclear extract. See Section 2 for preparation of nuclear extracts and other details. (A) Autoradiograph of transcripts generated from a promoter containing UAS_{LEU}. Lanes: 1, control, neither Leu3p nor α-IPM was present; 2–9, transcription in the presence of a constant concentration of highly purified Leu3p (175 ng per assay) and increasing concentrations of α-IPM, as indicated. The concentrations given are those of the biologically active isomer. (B) Dependence of transcriptional activation (in arbitrary units) on α-IPM concentration. The data points correspond to lanes 2–9 of A. (C) Comparison of templates lacking (lanes 1–4) or containing (lanes 5–9) UAS_{LEU} elements. Lanes: 1, incubation mixture contained neither Leu3p nor α-IPM; 2, same as lane 1 except that α-IPM (1 mM) was present; 3, same as lane 1 except that purified Leu3p (175 ng) was present; 4, same as lane 1 except that both α-IPM and Leu3p were present; 5–7,9, same as lanes 1–4 except that UAS_{LEU} elements were present in template; 8, same as lane 9 except that α-IPM was replaced with β-IPM (2 mM).

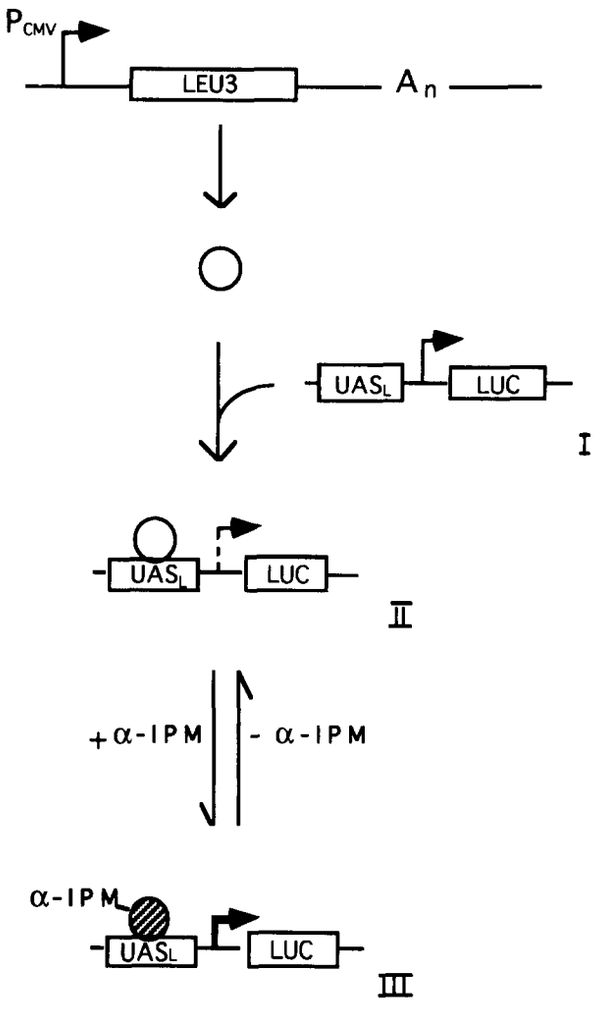

Fig. 4. Synopsis of LEU3- and α-IPM-dependent regulation of a reporter gene in mouse adipocytes. The top of the figure shows part of plasmid pC3-LEU3 that expresses the yeast *LEU3* gene from a CMV promoter. A second plasmid, pGL3-UAS_L-LUC, carries the luciferase gene under the control of an SV40 promoter containing four copies of UAS_{LEU} elements. The expression of the LUC gene in the absence of *LEU3* is identified as state I (corresponding to column 4 of Fig. 1). In the presence of *LEU3*, but in the absence of α-IPM, LUC gene expression is suppressed (state II, corresponding to column 6 of Fig. 1). When both *LEU3* and α-IPM are present, LUC gene expression is induced (state III, corresponding to column 7 of Fig. 1). The open circle represents Leu3p in the absence of α-IPM (repressive mode), the shaded circle represents Leu3p in the presence of α-IPM (active mode).

system, compared with a 4–5-fold negative effect on specific gene expression in yeast [8,10,13]. The concentration of α-IPM needed for half-maximal activation by Leu3p is 0.25 mM in mammalian nuclear extract and 0.2 mM in yeast whole-cell extract [8]. The difference between repressed and fully activated levels of expression of target genes (15–20-fold) is also very similar in both organisms. It is evident from these observations that no yeast-specific factors other than Leu3p itself are required for reporter gene activation or repression. In this sense, Leu3p is a self-contained regulator. In particular, there appears to be no need for a specific activation domain masking factor (corresponding to, for example, GAL80 in the Gal4p induction system). It is highly unlikely that such a masking factor would be elaborated in mammalian cells that do not normally contain a Leu3p-type regulator. Masking (and unmasking) of the transcriptional activation domain are therefore likely to be Leu3p intramolecular processes.

The functioning of Leu3p in mammalian cells could be most easily explained if it were assumed that it interacted with analogous components of the yeast and mammalian transcription machineries, for both its positive and negative effects. However, the possibility that the detailed mechanism of action of Leu3p in mammalian cells is different from that in yeast (while still leading to similar results) cannot be excluded at this point. One reason for this caveat is the recent observation that repression by Leu3p in yeast appears to depend on the negative factor Mot1p, also known as $yTAF_{II}180$ [27]; so far, a Mot1p homolog has apparently not been identified in mammalian cells.

The Leu3p-α-IPM system is highly specific. In yeast, the only proteins other than Leu3p itself that would be expected to interact with α-IPM are IPM isomerase (which interconverts α- and β-IPM), α-IPM synthase (which produces α-IPM), and a hypothetical α-IPM transporter in the inner mitochondrial membrane. All three are part of the leucine biosynthetic pathway in yeast and are expected to be absent from mammalian cells which are incapable of synthesizing leucine. In agreement with this notion, we could not detect any cross-reacting material in 30A5 cells using polyclonal antibodies against β-IPM dehydrogenase, the enzyme that catalyzes the third step in the leucine pathway (data not shown). The specificity of the α-IPM-Leu3p interaction, i.e. the absence of pleiotropic effects of α-IPM, should be useful when triggering gene expression in developmental studies or in gene therapy experiments. Since the absence of the leucine pathway from mammalian cells eliminates the only known metabolic route for α-IPM, delivery of α-IPM should, and apparently does, result in a strong, persistent 'on' signal. Subsequent withholding of α-IPM (the 'off' signal) should lead to a gradual return of gene silencing, governed by the rate of diffusion of α-IPM out of the cells. While the kinetics of these processes remain to be established, it is evident that the Leu3p-α-IPM system has the potential to become a powerful alternative to regulatory systems depending on hormones [28] or antibiotics [29].

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