

D-site of albumin promoter binding protein (DBP) may regulate hematopoiesis

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Abstract

It is hypothesized that the various cyclic phenomena in hematopoiesis [circadian (24hrs) and cyclic hematopoiesis (7days in mice and 21days in human)] reflect the involvement of circadian and/or metabolic regulatory mechanisms in hematopoietic processes. It is further hypothesized that D-site albumin promoter binding protein (DBP), a (circadian) clock controlled gene (CCG) may play a role in coordinating such phenomena. It is found that DBP can both activate and inhibit the activity of transcriptional regulators such as HIF-1, NF- κ B and AP-1 families. DBP itself appears to be a target for the signalling molecules' proline-rich tyrosine kinase 2 (PYK2), and dual-specificity Yak1-related tyrosine kinase 3 (DYRK3). Further evidence indicates possible roles for serum-glucocorticoid regulated kinase-1 (SGK1), protein kinase C (PKC) and glycogen synthase kinase (GSK3) in the regulation of DBP activity. These observations would indeed be consistent with a potential role for DBP in the regulation of survival, proliferation and differentiation of hematopoietic progenitors.

Keywords: DBP; cyclic hematopoiesis; HIF-1; circadian; phosphorylation; metabolism.

Introduction

Hematopoietic stem cells (HSCs) have been used therapeutically in the form of either bone marrow transplantation (BMT) for over 40 years or more recently cord blood/stem cell transplantation. However, there are problems associated with graft-rejection in cases of allogenic BMT whereas small quantities of cord blood is a major problem in transplanting an adult recipient. Essentially, the success of both these procedures depends on there being sufficient stem cells in the graft to effect long term reconstitution of the patient. For effective therapy, it is highly advantageous to have plentiful of HSCs rather than a miniscule amount of HSCs. However, the amplification of HSCs in the absence of differentiation occurs *in vivo*, while it has been difficult to reproduce *in vitro*. One of the major research objectives in this field is to understand the mechanisms that control self-renewal and lineage commitment at the molecular level and thus pave the ways to identify potential targets and strategies through which stem cells may be manipulated *in vitro* such that they amplify by self-renewal without undergoing lineage commitment.

The complexity of such cellular processes and their intracellular dynamics are associated with highly dynamic behaviour of living cells. Theoretical and mathematical arguments suggest that such dynamic state of living cells can only be explained with periodicity (Gilbert and Lloyd, 2000). Although

periodic or rhythmic behaviour in biological systems have been recognized for a long time, the recent findings that even immortalized cells manifest circadian phenomena (Balsalobre et al., 1998) and the dissection of genetic components of circadian clocks have brought the different types of oscillations into the focus of research and now it is widely accepted that oscillations are prime attribute of life. The different types of oscillations are classified essentially based on the periodic length or time and they are summarized in the Table 1.

Here, based on the involvement of D-site albumin promoter binding protein (DBP), a circadian transcription factor known to be expressed at the mRNA level in fetal liver CD34⁺ lin⁻ HSCs (Phillips et al., 2000), in the regulation of several metabolic and non-metabolic genes (Table 2), and on the evidences both of metabolic features (Table 3) and of cyclic phenomena (Table 4) in hematopoietic cells, it is hypothesized that DBP may regulate both the circadian and cyclic aspects of hematopoiesis.

In order to test this hypothesis, the functions of DBP in hematopoietic progenitor cells have been investigated by using the multipotential FDCP-Mix cells. In liquid culture in the presence of low concentrations of interleukin-3 (IL-3) and appropriate concentrations of different growth factors these cells differentiate into erythroid and myeloid lineage cells but not lymphoid cells. For example, in the presence of erythropoietin

(EPO) and hemin along with low levels of IL-3, FDCP-Mix give rise to erythroid cells over a 7-10 days period. Likewise, in the presence of granulocyte/macrophage colony stimulating factors (GM-CSF, G-CSF, or M-CSF) and low levels of IL-3, the FDCP-Mix generate macrophages, and neutrophilic granulocytes (Heyworth et al., 1991; Heyworth et al., 1995; Mclvor et al., 2000). However, at high concentrations of IL-3, the cells undergo self-renewal and demonstrate little or no ability to undergo differentiation even when other growth factors are present. Further, the signalling mechanisms of DBP function have been investigated by using the human embryonic kidney cells (293 HEK cells) which complements the work on FDCP-mix cells as we found that 293 cells do not express the DBP at the protein level whereas it is present in the FDCP-mix cells. It has been found that DBP has a strong potential to regulate the hematopoietic processes by regulating the expression of activator protein-1 (AP-1), nuclear factor kappa factor B (NF- κ B) and hypoxia inducible factor-1 (HIF-1) family of transcription factors.

Materials and Methods

Cell culture

FDCP-Mix cells were cultured and maintained in self-renewal culture medium consisting of Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% pretested horse serum and mouse IL-3-conditioned medium at a concentration that stimulated optimal cell growth (corresponding to 100 U recombinant (r)IL-3 per milliliter) as described previously (Mclvor et al., 2000). Human embryonic kidney 293 cells were grown in DMEM medium supplemented with 10% (v/v) FCS under standard cell culture conditions in either 6 well plates or in 10cm animal cell culture petri dishes, and transfected by using the calcium phosphate method (Jordan et al., 1996) to an efficiency of 60-90%. Cell counting was done by using the Trypan Blue dye exclusion method and colony forming units (CFU) assay for FDCP-Mix cells was carried out as previously described (Mclvor et al., 2000). Tetrazolium salt reduction (MTT) assay was done by measuring the absorbance on an ELISA reader at 570nm.

Vector constructions

The DBP coding sequence was excised from plasmid pET-mDBP vector was cut with XbaI and NheI and inserted into CMVeGFP (GFP) cut similarly with XbaI and NheI to generate the plasmid AntiDBPeGFP (AntiDBP), while

DBPeGFP (DBP) was obtained by blunt-end ligation of the vector and the insert after repairing the DNA termini. Insert orientation was determined by cutting with Scal. The PYK2/hrGFP coexpression vectors PYK2hrGFP and AntiPYK2hrGFP were made by inserting an EcoR I fragment carrying the human PYK2 coding region from plasmid pRK5-hPYK2 into the EcoRI site of the vector IREShrGFP (hrGFP or hr in graphs, Stratagene, Amsterdam), and determining insert orientation by BclI digest. The expression plasmid DYRK3hrGFP was obtained by inserting a BamHI-EcoRI fragment carrying the hDYRK3 coding sequence from plasmid pGex-hDYRK3 into BamHI-EcoRI digested IREShrGFP. The presence of the insert was verified by cutting with NotI and Sall. The IRES2eGFP control vector (IRES2 or IR) was generated by deleting the hSGK1 sequence from hSGK1-IRES2eGFP by digesting with BglII and BamHI. Table 5 acknowledges the generous gifts given by scientific community to enable this work.

Reporter gene assays

A total of 2 μ g of the luciferase reporter plasmid was transfected with 10 μ g of pCMVExp3eGFP or equimolar amounts of other vectors. For 293 cells, 5 times less DNA were used as the transfection efficiency is very high. After overnight incubation, the cells from each electroporation/transfection were washed twice in phosphate-buffered saline, resuspended in 100 μ L luciferase assay lysis buffer (Promega). After removal of debris by centrifugation, 80 μ L of supernatant was assayed using a Berthold 9501 Luminometer and the readings are recorded as relative light units (RLUs). The assay tanks and tubes were flushed thoroughly with sterile distilled water before and after use to keep the background as low as possible. In the graphs, the X-axis represents the different conditions and the Y-axis represents the relative light units.

FACS sorting

Viable cells were recovered 12 to 18 hours after electroporation, washed, and resuspended at 2 to 5×10^5 cells/mL in ice-cold phosphate-buffered saline containing 0.05% heat-inactivated bovine serum albumin and 2 U/mL IL-3. FACS was performed using a FACSVantage SE (Becton-Dickinson) as described previously (Mclvor et al., 2000).

Western blotting

Cells were cultured under self-renewal conditions. Harvesting, electrophoresis, and Western blotting of protein extracts were

performed as described previously. Blocked membranes were incubated for 1hr at room temperature with mouse anti-phosphotyrosine, anti-phosphoserine (Calbiochem, US), or rabbit anti-DBP antibody all diluted 1:1000 in the blocking buffer (TTBS buffer: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). After washing the membranes once for 5 min and then three times for 10 min in the blocking buffer, the membranes were incubated with goat anti-mouse IgG or anti-rabbit IgG coupled to horseradish peroxidase (Calbiochem, US) at 1:2000 for 1hr at room temperature. Membranes were washed stringently in four changes of TTBS as described above. SuperSignal West Chemi-luminescent Substrate (Pierce, USA) was used to obtain the signals which was documented with Fuji CCD camera system.

Results and Discussion

The role of circadian clock in the control of vital life activities in mammals is well documented. It is now known that the central oscillator functions through several clock output genes also known as clock controlled genes (CCG). The gene for D-site albumin promoter binding protein is a CCG that is expressed in several different cell types and tissues including fetal liver CD34⁺ lin⁻ HSCs. It is known that DBP is involved in the transcriptional control of several key genes encoding rate-limiting enzymes of metabolism and other processes (Table 2). The role of DBP in the self-renewal, commitment and differentiation of HSCs is not known. However, there are several lines of evidences both from circadian, cyclic hematopoiesis and also from other cell systems that have all pointed to an important role for DBP in controlling several aspects of hematopoiesis. The hypothesis of this work can be summarized as follows:

DBP function is regulated by DYRK3, PYK2, GSK3beta, one or 2 isoforms of PKC and SGK-1. Differential phosphorylation of DBP leads to different functions such as activation and inhibition of NF-κB, AP-1 and HIF-1 family of transcription factors. Differential degradation of phosphorylated DBP by elastase (known to be involved in the etiology of cyclic neutropenia) may play a role in the orchestration of circadian and cyclic aspects of hematopoiesis by regulating survival, differentiation and apoptosis of hematopoietic progenitors. DBP may be involved in the circadian regulation of several genes such as erythropoietin and PYK2.

This work represents a preliminary effort to test this hypothesis which evolved as the work progressed. Several lines of new evidences are presented in support of an important role for DBP in hematopoiesis using FDCP-Mix cells as a model system and 293 HEK cells complements some of the conclusions drawn in favour of a role for DBP.

Expression and Phosphorylation of DBP in 293 and FDCP-Mix Cells

Post-translational modifications of proteins play an important role in the regulation of vital processes such as cell proliferation and differentiation. Since DBP is involved in such processes, it is likely that DBP is regulated by phosphorylation, although other modifications may also play a role. A simple estimation for the number of phosphorylation sites in mouse DBP protein using Netphos version 2 (Blom et al., 1999) has been done. The number of serine, threonine and tyrosine residues in mouse and human DBP which have high probability (more than 0.5) of being targets for phosphorylation are 16, 5 and 3; and 12, 8 and 3 respectively. The results show that DBP protein is expressed in FDCP-Mix cells (Figure 1b) but not in 293 cells (Figure 1a). When FDCP-Mix cells were exposed either to hypoxia for 30mins and 6hrs or to 1mM ATP for 6hrs, there was a 2 fold reduction in the concentration of DBP. It is likely that when cells are exposed to hypoxia, there is an increase in the intracellular concentration of calcium which may activate PYK2 (Beitner-Johnson et al., 2002). The phosphorylation of DBP by PYK2 may in turn protect it from proteolytic degradation, while the rise in calcium may have perturbed the serpin-elastase complexes (Calugaru et al., 2001) resulting in the degradation of a substantial portion of DBP. The finding that there is no reduction in HIF-1 activity mediated by DBP/PYK2 transfected 293 cells (Figure 7e) when exposed to hypoxia at an earlier or later time point may suggest that PYK2 phosphorylated DBP is likely resistant to proteolytic degradation, although it does not specifically prove that DBP phosphorylated by PYK2 is resistant to elastase. However, there also appeared to be a rise in the serine phosphorylation of DBP when the cells were exposed to hypoxia or ATP since there was no reduction in the phosphoserine content in comparison to the reduction in the DBP concentration seen with the blot probed with AntiDBP antibody. This suggests that DBP may be phosphorylated by yet another serine kinase during hypoxia and upon treatment

with ATP. The results obtained here also show that 1mM ATP, the optimum concentration of ATP that is known to sustain the survival of FDCP-Mix cells in the absence of growth factor such as IL-3 (Whetton et al., 1984) causes an increase in the tyrosine phosphorylation of DBP together with a 2 fold reduction in the concentration of DBP. It is already known that an aberrant purine metabolism is linked to cyclic neutropenia (CN), and there is an increase in the concentration of ATP and other nucleosides in erythrocytes and plasma of the cyclic neutropenic patients but not of chemical-induced neutropenic patients (Osborne et al., 1983, 1985). Since DBP is involved in erythroid differentiation, it is now conceivable that the increased ATP concentration may result in the reduction of DBP at the protein level, which may in turn causatively be involved in CN. Alternatively, the increase in ATP may be an adaptive response to protect the hematopoietic progenitors against the apoptosis typical of CN (Aprikyan and Dale, 2001), possibly resulting from an excessive degradation of DBP by mutant elastase. This high level of ATP accumulated in the progenitors may be carried over to subsequent stages of differentiation. However, it needs to be addressed how this excess ATP is generated. In this context, it may be relevant that the plus strand of mouse DBP (GenBank Accession: NM_016974) has 99% homology with the negative strand of mouse sphingosine kinase 2 (Sphk2, GenBank Accession: AK016616, Figure 1c). In both human and mouse, Sphk2 and DBP are adjacent to each other at their chromosomal locations. (In human, Sphk2 and DBP has been mapped to chromosome 19q13.2 and 19q13.3 whereas in mouse it has been mapped to chromosome 7 B2 and 7, 23.0cM respectively; LocusLink at NCBI). Although this possible regulatory region in opposite strands between 3' end of *DBP* and 5' promoter region of *Sphk2* by itself at the level of DNA may not be sufficient to suggest a functional relation between DBP and Sphk2, when one integrates what is known about sphingosine kinases and DBP, it is possible to understand that DBP and Sphk2 may not only be linked to each other evolutionarily but also functionally. Sphingosine kinase is an enzyme that catalyses the formation of sphingosine-1-phosphate (S1P) from sphingosine and ATP. Sphingosine is produced from the enzymatic activity of ceramidase upon ceramide (that is synthesized *de novo*) or as part of the sphingomyelin cycle in animal cells but it has also been found in insects, yeasts and plants.

The reverse reaction of sphingosine kinase is mediated by sphingosine phosphatase, and these two enzymes act in concert to control the cellular concentrations of S1P. It is known that S1P is an important cellular metabolite with several functions such as survival, proliferation, vasculogenesis, migration and differentiation (Pyne and Pyne, 2000). The current opinion seems to suggest that the balance between S1P and ceramide and/or sphingosine levels in cells is critical for their viability (Maceyka et al., 2002) and it is also known that S1P inhibits the expulsion of cytochrome c from mitochondria to cytosol, a principal event to trigger apoptosis (Cuvillier and Levade, 2001). It is also known that S1P is stored at high concentrations in platelets which in turn plays an important role not only in blood clotting but also in hemostasis (Igarashi and Yatomi, 1998). It is also known that medium change has profound effects on numerous bioactive lipids such as sphingosine, diacylglycerol (DAG), and ceramide (Smith et al., 1997). Since serum shock induces the expression of several clock genes and CCGs (Balsalobre et al., 1998), it is possible that either sphingosine and its derivative S1P play an important role in the expression and regulation of the activities of DBP or DBP plays a role in the regulation of the activities of sphingosine kinase and thereby influence the concentrations of S1P to regulate vital functions such as survival, proliferation and hemostasis. This connection between Sphk and DBP needs to be addressed to understand the mechanism by which DBP may act to regulate hematopoiesis. There are a number of results presented here which support a link between DBP and Sphk activity and these results are considered as they come across.

Getting back to the question on the source of excess ATP, it is possible that this results from underutilization of ATP by Sphk, while the increase in other nucleotides and nucleosides may be the result of accumulation of ATP and defective proliferation. The Sphk may have underperformed owing to its own degradation by mutant elastase or DBP by mutant elastase, resulting in the accumulation of ATP. Since sphingosine/S1P/ceramide balance would be disturbed due to changes in Sphk/DBP function, the hematopoietic progenitors may apoptose and have a defective proliferation. The involvement of sphingosine and its derivatives in the neutrophil priming (MacKinnon et al., 2002) further lends support to the link between DBP and Sphk owing to the fact that priming is impaired in the neutrophils from several cyclic

diseases (Iki et al., 1997). However, treatment of CN patients with hG-CSF is known to result in shortening of the period length of the cyclic hematopoiesis from 21 days to 14 days (Hammond et al., 1989) owing to increased proliferation suggesting that the defects in survival and proliferation are cured. In this context, it must be said that DBP did occasionally advance the formation of CFUs to day 4 but this effect was not found to be reproducible under the conditions of the assay. This may be because once the cells have passed through certain stages of development, they may not return to the same stage at least under *in vitro* conditions. In other words, the circadian and cyclic features of hematopoiesis will most likely follow regular patterns *in vivo*, while circadian and cyclic features may not follow such regular features strictly in a cyclic manner *in vitro*. There are lots of reasons which may introduce variations to the culture *in vitro*. For example, the irregular intervals in changing the media on the cells and the absence of proper *in vivo* signals from SCN which is known to coordinate circadian functions in peripheral tissues, may have profound effects and they may have altered the behaviour of cells resulting in loss of several cyclic features or they may show features which appear at irregular intervals.

The results also show that DBP is phosphorylated by DYRK3 in 293 cells. DYRK3 is a dual-specificity (yeast) Yak1-related tyrosine kinase expressed in response to EPO and known to play an important role in the early stages of erythroid differentiation (Geiger et al., 2001). An unusual enzymatic property of DYRK-related kinases is their ability to catalyze tyrosine-directed autophosphorylation as well as phosphorylation of serine/threonine residues in exogenous substrates. In yeast, Yak1 and its related kinases are known to be involved in the regulation of cell cycle and cell symmetry (Bahler and Nurse, 2001). It has been recently shown that DYRK-related kinases are involved in similar functions in neural progenitors and other cells (Hammerle et al., 2002; Ewton et al., 2003). It is possible that DBP phosphorylated by DYRK3 is involved in the regulation of cell cycle and symmetric cell divisions associated with erythroid differentiation.

PYK2 is a calcium-dependent proline-rich tyrosine kinase (Girault et al., 1999), also known as calcium dependent tyrosine kinase (CADTK), related adhesion focal tyrosine kinase (RAFTK), focal adhesion kinase 2 (FAK2) and cellular adhesion kinase beta

(CAKbeta). It is a member of the focal adhesion kinase (FAK) subfamily expressed in CD34⁺ bone marrow cells, myeloid, lymphoid, megakaryocytic lineages and brain cells (Avraham et al., 1995; Salgia et al., 1996). PYK2-H is an isoform of PYK2 mainly expressed in hematopoietic cells including T-cells, B-cells, and natural killer cells and it may have different signaling functions (Dikic and Schlessinger, 1998). The results show that DBP is phosphorylated by PYK2 in 293 cells. Although PYK2 is known to phosphorylate tyrosine residues, the residual serine phosphorylation seen may be due to basal phosphorylation at serine residues by endogenous kinases in 293 cells. A limitation of these results on the phosphorylation of DBP by both DYRK3 and PYK2 is that the autophosphorylation of both DYRK3 and PYK2 in 293 cells has not been checked. However, the functional luciferase assays performed with the above kinases and DBP suggest that DBP is indeed regulated by both DYRK3 and PYK2.

It is also clear from Figure 1b that lithium chloride at the concentration of 10mM does not have any detectable effect on the concentration of DBP but it does increase the tyrosine phosphorylation of DBP. Lithium chloride was initially used as an inhibitor of GSK3 (Stambolic et al., 1996) to study its effects on DBP and to test whether GSK3 is also involved in the regulation of the activities of DBP. However, LiCl does not reduce the serine phosphorylation of DBP by inhibiting GSK3. Since there are multiple serine phosphorylation sites on DBP, inhibiting GSK3 alone may not be sufficient to cause a significant reduction in serine phosphorylation. The increase in tyrosine phosphorylation of DBP by lithium may further suggest its involvement in cyclic neutropenia. It is known that lithium (carbonate) is an effective therapy to treat cyclic neutropenia in grey collies dogs but not in human (Hammond et al., 1983; Williams and Jones, 1984). It has been found that lithium carbonate ameliorates neutropenia associated with cancer chemotherapy in humans (Richman et al., 1984). It is possible that lithium also affects the phosphorylation state of DBP. However, since lithium at different concentrations have multiple effects in different cell systems (Gurvich and Klein, 2002), further work would be needed to clarify this issue and to assess whether GSK3 is involved in the regulation of DBP.

Effect of DBP on CFU formation in FDCP-Mix Cells

It is well known that cell size and the concentration of DNA polymerase alpha, an enzyme crucial for DNA replication vary in inverse proportion. Further, an inverse relation between cell size and clonogenic potential has been observed in human diploid fibroblast-like cells (Pendergrass et al., 1989). Since DBP protein level has been correlated to cell size (Schmidt and Schibler, 1995), it is possible that DBP may influence the proliferative potential and therefore the colony forming potential of FDCP-Mix cells. The CFU efficiency (the number of colonies per 100 cells plated), varied between 3-8% for untransfected cells and between 0.1-0.5% with transfected cells probably indicative of procedure related damage. There was found to be a 2 fold increase in the number of colonies when DBP was overexpressed in FDCP-Mix cells compared to the empty GFP (Figure 2). This nominal increase was found to be significant with the P value of 0.006. Although colony numbers were routinely counted after 7 days of incubation, colonies were occasionally detected from DBP transfected cells just 4 days after plating. It is also possible that the low level increase in CFU potential observed here is due to an increase in the serpin2a, a serine protease inhibitor whose overexpression in FDCP-Mix cells has already been found to result in a modest increase in CFU formation (Hampson et al., 1997). Similarly, it is possible that upregulation of alpha-2,6-sialyl transferase, a transcriptional target of DBP, known to be essential for the cells to invade agar and matrix (Le Marer and Stehelin, 1995; Zhu et al., 2001), may play a role in conferring clonogenic potential to the cells. However, the low level increase in the number of colonies suggest that DBP is likely connected to the mechanisms of homeostasis.

Effect of DBP on Survival and Proliferation in FDCP-Mix Cells

It has been found that DBP increases the number of viable FDCP-Mix cells by 2-3 fold compared to empty GFP vector (Figure 3a). The role of DBP in the control of cell cycle and proliferation is not well known. However, it is well known that there is a circadian rhythmicity in proliferation in several tissues including bone marrow (Smaaland, 1996) and also in the expression of several cell cycle proteins (Bjarnason et al., 1999). The results presented here suggest that DBP is indeed involved in the regulation of survival and proliferation in FDCP-Mix cells. A potential positive effect of DBP on survival and proliferation may encompass activation of thymidine kinase,

NF- κ B and AP-1 family of transcription factors. It is known that retinoblastoma (pRb) control element binding proteins bind to the DBP promoter during differentiation (Leggett and Mueller, 1994). Hence, a balancing negative effect on survival and proliferation may also be expected from the tumor suppressor activity of DBP. It is therefore possible that the moderate increase in viable cell number is the sum of both positive and negative influences. The negative effect of DBP is likely to be reminiscent of tumor suppressors such as retinoblastoma (pRb, Knudsen et al., 1999). This pathway may involve two different mechanisms like pRb namely inhibition of cell cycle progression and induction of cell death. More work would be needed to further understand the exact mechanisms by which DBP may be involved in both tumor promotion and suppression.

It is known that DBP is involved in the catabolism of cholesterol which plays an important role in cell proliferation (Suarez et al., 2002). DBP binding sites were found in the thymidine kinase (TK) gene promoter (using MatInspector version 2.2) which is upregulated during cell proliferation (Sherley and Kelly, 1988). Hence, the effect of DBP on the regulation of the thymidine kinase promoter using the TK109 Luc construct was tested. The results (Figure 3b) show that DBP transactivates TK109 by nearly 10 fold. Repeat experiments confirmed a 5-10 fold activation of TK109 by DBP. Evidences in the literature clearly suggest a role for DBP in proliferation. DBP has been known to be involved in the control of proliferation of hepatocytes since DBP expression becomes down regulated during chemical induced liver regeneration (Mueller et al., 1990). However, the results presented here suggest that DBP is likely to play an important role in the proliferation not only when it is downregulated but also when its expression is upregulated.

Transactivation Experiments with Serpin2a Promoter

Since DBP is known to be involved in the transcriptional control of the expression of serine protease inhibitors (serpin) namely spi2.1 in liver (Rossi et al., 1992) and angiotensinogen (Narayanan et al., 1998), the potential involvement of DBP in the control of serpin2a (S2a) was assessed. S2a is specifically expressed in FDCP-Mix cells during self-renewal and downregulated during differentiation (Hampson et al., 1997). It was found that DBP can activate the S2a promoter by 100 fold. However, the extent of activation seen in repeat experiments performed on

separate occasions varied from 2 to 3 fold. (Figure 4a, b and c). This may be because of the variations in the cell state, passage numbers, endogenous level of DBP, serpins, elastase and other cycling kinases that may control the activity of DBP. However, more than 2 fold activation with respect to S2a promoter by DBP has been consistently seen in all the experiments irrespective of these factors which could affect the level of transactivation by DBP. Interestingly, the 100 fold activation of S2a by DBP was observed only once at an early stage following thawing and it was not found to recur in any of the subsequent experiments. As mentioned before, once the cells have crossed certain stages of development, they may not return back to the same stage. It is possible that there is a dampening of the culture that is dysregulation of the synthetic and degradative pathways of DBP *in vitro* which may lead to accumulation of DBP protein in the cells. The accumulation of DBP protein in the cells after few passages (within 3 weeks from thawing) may result in severe reduction in the fold transactivation of S2a upon overexpression of DBP. The fact that both DBP and S2a are expressed at the fetal liver stage (Hampson et al., 1997) may further suggest that DBP may indeed regulate the expression of S2a developmentally.

Transactivation experiments were carried out with GATA.1 (GATA binding factor 1, an erythroid transcription factor) and DBP to see whether there is any increase in activity of these proteins on S2a promoter luciferase expression. Although footprinting or gel shift assays would have to be done to measure the increase in DNA binding activity directly, the luciferase assays should reveal any functional synergism between GATA.1 and DBP. The results (Figure 4a) show that there is a 3 fold increase in the Luc activity when DBP is cotransfected with a small amount of GATA1 or vice versa. This clearly shows that there is a synergistic interaction between GATA1 and DBP, but it does not prove the physical interaction between GATA1 and DBP. It is already known that GATA proteins inhibit EPO gene expression (Imagawa et al., 1997), and that DNA binding activity of GATA proteins are needed for this inhibition, but the mechanisms are not known completely. It remains to be seen whether GATA1 and DBP interaction leads to the expression of appropriate target genes during erythroid differentiation, including shutting off EPO expression. The fact that DBP mediated transactivation requires the histone acetyltransferase p300 (Lamprecht and

Mueller, 1999) but not CREB binding protein (CBP), and that GATA1 is activated by acetylation (Boyes et al., 1998) raises the possibility that DBP acts here to accelerate GATA1 activation. It is not known whether DBP is acetylated or not and it is possible that DBP may simply act as a recruiter of the acetyltransferases to the particular domain without becoming itself acetylated.

PU.1 has been found to transactivate the S2a promoter considerably despite the lack of a PU.1 binding site on S2a promoter (Cross and McIvor, unpublished observation). Based on this result and also host of other reasons like promiscuous expression of genes of multiple lineages at low level in the progenitors, it is hypothesized that there is a threshold level of general transcription factors that act as a lid on several hematopoietic transcription factors (HTFs) maintaining the stem cell compartment. This lid gets lifted off once a particular HTF is overexpressed leading to the expression of several HTFs in the progenitors and these HTFs decide the lineage choice in a stochastic manner leading to the differentiation towards a particular lineage. This hypothesis explains the effect of PU.1 on FDCP-Mix cells like reduction in CFU efficiency and cell death of PU.1 overexpressing cells in self-renewal media since self-renewal media can not support growth of committed progenitors (McIvor et al., 2003). However, it is possible that reduction in CFU efficiency may be because PU.1 overexpressing cells have simply lost their ability to penetrate the agar and grow as colonies due to loss of sialylation and not really committed to any lineage. This is indeed supported by the finding that transfection of antisense beta-galactoside alpha 2,6-sialyltransferase (ST6Gal I), (a transcriptional target of DBP), in human colon cancer cells reduces the formation of colonies in soft agar and invasion of extracellular matrix material (Matrigel) *in vitro* by approx. 98% and more than 3-fold respectively compared to parental cells (Zhu et al., 2001). It is also possible that cell death caused by PU.1 may also be because of the interference of overexpressed PU.1 with endogenous survival pathway of self-renewing cells connected to DBP and not because of the commitment. It is also known that growth factors do play an important role in the commitment and it is not possible to simply rule that growth factors are mere factors for survival and proliferation. Hence, an alternative hypothesis is proposed wherein transactivation of S2a promoter by PU.1 is an adaptive response of the cells to prevent the cell death since PU.1 upregulates the elastase

gene which could cause cell death by degrading the survival factors like DBP. In order to test whether PU.1 has a negative effect on DBP activity through elastase, (a target gene of PU.1, Srikanth and Rado, 1998), DBP and PU.1 were cotransfected to see how the transactivation of the S2a promoter is affected. The results in Figure 4b confirmed the previous finding that PU.1 is capable of upregulating S2a gene but also showed that the level of transactivation of S2a by PU.1 is more than 3 fold compared to GFP whereas the Luc activity seen with DBP is 2 fold. When DBP is cotransfected with PU.1, the Luc activity was still higher than the Luc activity seen with the transfection of DBP but it is about the same level of Luc activity seen with PU.1 alone. This could mean either the initial hypothesis that PU.1 will have a negative effect on DBP through elastase pathway is wrong or that the level of S2a activation is much higher essentially as an adaptive response to prevent cell death. However, it is also possible to have another alternative view. It has been previously shown that the activity of liver activator protein (LAP), a transcriptional activator is inhibited by liver inhibitory protein (LIP), a transcriptional repressor. Owing to its higher affinity for its cognate DNA sequences, LIP can attenuate the transcriptional stimulation by LAP in substoichiometric amounts. But a moderate increase in the LAP/LIP ratio results in a significantly higher transcriptional activation of appropriate target genes (Descombes and Schibler, 1991). Since PU.1 does not have any binding site on S2a promoter and it also directly interacts with GATA.1 (that has binding site on S2a) to prevent its DNA binding activity through protein-protein interactions (Zhang et al., 1999; Nerlov et al., 2000), it is possible that this increased S2a Luc activity is largely mediated by DBP due to the increase in the ratio of activator (DBP) and inhibitor (PU.1). But the difference between LAP/LIP and DBP/PU.1 is that the inhibitory effect of PU.1 is not immediate and direct but it is rather acting at a distance through the elastase. Variations in the proteolytic activity of elastase among different phosphorylated and unphosphorylated DBP species together with the transcription factor like PU.1 whose concentration is essential in deciding the lineage fates (DeKoter and Singh, 2000) could generate different patterns needed for the differentiation. These explanations fit well with the pioneering pattern theory previously put forward to explain pattern formation during development and also proved (Meinhardt and Gierer, 1972; Gierer and

Meinhardt, 2000). However, the results obtained with this experiment does not disprove but makes the proposition that PU.1 has a negative effect on DBP through elastase not only untenable but also contradicts it which is based on multitudes of previous observations. In order to further understand the mechanisms of S2a activation, the kinetics of S2a activation by DBP and PU.1 together with control GFP vector was studied.

The results (Figure 4c) show that DBP and PU.1 direct different levels of S2a Luc activity at different time points and the kinetics of S2a activation by these transcription factors are different. Interestingly, at early stages following transfection the level of Luc activity seen with PU.1 is much lower than the Luc activity seen with DBP and empty GFP vector. This clearly shows that after PU.1 overexpression, it has had a negative effect on the endogenous transcription factors that are involved in the S2a transactivation considerably and there has been an increase in the Luc activity only after the initial dip. This initial dip can not be brushed aside simply as an effect of electroporation or an artifact of the luciferase done at a very early time point after electroporation. It is known that luciferase does not need any post-translational modification for its activity. Since FDCP-Mix cells are heterogenous population having a subpopulation of cells that are always going through cell divisions, the cells immediately take up DNA after electroporation and respond to the external perturbations. This is also supported by the previous finding that IL3-dependent cell lines possess an extremely rapid transcription mechanism that respond within 2hrs for introduced DNA (Garland et al., 1992). Even 6hrs after electroporation, the Luc activity seen with PU.1 is nearly two time less than the Luc activity seen with DBP, though after the initial dip, the PU.1 mediated Luc activity picks up much faster than GFP by 4.30hrs. It is very interesting to note that the Luc activity seen with DBP and GFP follow similar pattern of S2a activation but the level of S2a Luc activity seen with DBP is much higher than GFP. The stress effect of electroporation reaches a maximum at 6hrs and exactly at the same time point the Luc activity seen with DBP also reaches the maximum. By 18hrs after electroporation, the Luc activity seen with DBP and GFP goes down, though it is still higher in DBP than GFP. This again strengthens the initial hypothesis that DBP is a survival factor and vindicate the similar kinetics seen with GFP and DBP. The difference in the level of Luc activity seen with GFP and DBP may easily be explained by the difference in the

level of DBP protein. The reduction in the Luc activity seen with DBP is not surprising since it is known that DBP is a circadian factor and the level of transactivation by DBP is bound to change over time. It is also previously known that DBP at the mRNA and protein level is controlled by the cell size (Schmidt and Schibler, 1995) and it is likely that either the concentration of DBP regulates the cell size or cell size regulates the level of DBP protein. However, little is known about this aspect of DBP. It is also interesting to note that S2a promoter activity seen with PU.1 is stable even after 18hrs and it is higher than the Luc activity seen with DBP and GFP. This result has been discussed in the previous section. It is also possible that an interaction between a circadian transcription factor and a non-oscillating factor may be able to generate a different pattern in the gene expression which may well explain the higher level of S2a activity in PU.1, a non-oscillating transcription factor transfected cells compared to DBP transfected cells. The overall results obtained from this experiment does not prove that PU.1 has a negative effect on DBP through elastase pathway but it certainly makes this hypothesis highly tenable.

Comparisons of fold activation of S2a promoter mediated by DBP, GATA.1 and PU.1 (in Figure 4d) suggest that the protein complex between PU.1 and GATA.1 is unlikely to be in an equilibrium with the individual concentrations of PU.1 and GATA.1 within multipotential cells. Since disturbance of such an equilibrium by overexpressing PU.1 should result in two fold more activation of S2a promoter by PU.1 compared to GATA1 but PU.1 results in only two fold more activation of S2a than DBP. This may suggest that DBP may be connected to an equilibrium between S2a and elastase. Briefly, the transactivation experiments with S2a promoter not only suggests that DBP is involved in the regulation of S2a but also suggests that DBP may be a central transcription factor involved in the upregulation of several transcription factors such as NF- κ B and AP-1 family of transcription factors.

Transactivation Experiments with IgK Promoter

Nuclear factor kappa B (NF- κ B) factors have been found to promote cell survival in a number of cells and growth conditions (Sonenshein, 1997). Classical NF- κ B/Rel factors are composed of p50 and p65 (RelA) subunits. In most cells other than mature B lymphocytes, NF- κ B/Rel proteins are expressed ubiquitously, but sequestered in

inactive forms in the cytoplasm by association with inhibitory proteins, termed I κ Bs for which I κ B α represents a paradigm. Multiple stimuli, as varied as exposure to proinflammatory cytokines like tumor necrosis factor alpha (TNF α) or IL-1 or other cytokines, viral infection, or chemical agents such as phorbol esters, UV irradiation, and phosphatase inhibitors, converge via distinct signaling pathways to activate NF- κ B/Rel factors (Ghosh, 1999). This activation pathway involves I κ B phosphorylation on serine residues, followed by ubiquitination and rapid degradation of the inhibitory protein by the proteasome, which allows for nuclear translocation of the NF- κ B/Rel factor (Verma et al., 1995; Karin and Ben-Neriah, 2000). Constitutively active NF- κ B/Rel factors have been found in mature B cells and in pro-B cells, including the Ba/F3 cell line (Miyamoto et al., 1994; Rice and Ernst, 1993; Besancon et al., 1998). Further, constitutive NF- κ B activity is known to protect Ba/F3 cells from apoptosis induced by IL-3 deprivation (Besancon et al., 1998). It has been previously shown that NF- κ B is involved in the proliferation of several cells such as bovine vascular smooth muscle cells and hepatic epithelial cell line (Bellas et al., 1995; Kirillova et al., 1999). It is also interesting to note that there is an accumulation of evidence to support an I κ B-independent level of NF- κ B regulation (Beyaert et al., 1996; Schmitz et al., 2001). These findings suggest that NF- κ B in an I κ B-dependent and independent manner is involved in the regulation of a number of physiological processes. It has been reported that DNA binding activity of NF- κ B follows a circadian pattern and it is also regulated by melatonin (Chuang et al., 1996). No regulation of NF- κ B activity by DBP has so far been described. It has been recently shown that hypoosmolarity induced a sustained NF- κ B binding activity whereas the hyperosmotic NF- κ B response was only minor in hepatoma cells (Michalke et al., 2000). DBP mRNA and protein levels are correlated with cell size in several tissues, although it is not known whether DBP controls cell size or cell size controls DBP. Based on these data, it was hypothesized that DBP is connected to the survival pathway via NF- κ B mediated pathway.

The results in Figure 5a show that DBP activates the NF- κ B activity by 6 fold compared to the basal IgK promoter activity seen with GFP. There is no NF- κ B activation when AntiDBP is expressed. This shows that expression of AntiDBP alone is not sufficient

to activate NF- κ B. Since AntiDBP does activate the TRE promoter (Figure 6), it is likely that it can indeed interfere with endogenous DBP expression in FDCP-Mix cells. It has been known that NF- κ B is minimally activated by hyperosmotic condition in hepatoma cells (Michalke et al., 2000). It is possible that AntiDBP expression may not be able to bring about hyperosmotic stress sufficient to activate NF- κ B. However, the overexpression of PYK2, (a kinase activated under hyperosmolar conditions) does activate the NF- κ B minimally in FDCP-Mix cells (Figure 5c).

It is interesting to note that nuclear factor regulated by IL-3 (NFIL3), also known as adenovirus E4 promoter binding protein (E4BP4), closely related to PAR family of transcription factors, initially cloned and characterized as a transcriptional activator of the IL-3 promoter (Zhang et al., 1995) is regulated by IL-3 in mouse pro-B cell lines (Baf-3 and FL5.12). But in the absence of IL-3, enforced expression of the human *NFIL3/E4BP4* cDNA promoted the survival but not the growth of IL-3 dependent pro-B cells (Ikushima et al., 1997). It has been shown that NFIL3 can function either as a repressor or an activator depending on the cell type (Cowell et al., 1992; Cowell and Hurst, 1994; Zhang et al., 1995). Consistent with this observation, it has been recently shown that enforced expression of *NFIL3* failed to protect M1p53ts_{val} myeloid leukemia cells from p53-dependent apoptosis and actively antagonized the ability of IL-6 to rescue cells from that fate (Altura et al., 1998). E2A-HLF, a fusion gene with the DNA sequence specificity of PAR family of transcription factors prevents apoptosis due to growth factor deprivation or gamma-irradiation in interleukin-3 (IL-3)-dependent murine pro-B cells. When E2A-HLF is introduced into M1p53ts_{val} cells, they were resistant to p53-mediated apoptosis and that E2A-HLF effectively substituted for the survival functions of IL-6 (Altura et al., 1998). It is previously known that HLF binds to the site IV of the DBP promoter and DBP promoter is highly active in the UOC-B1 cells which bears a 17:19 translocation resulting in the creation of an E2A:HLF fusion protein (Newcombe et al., 1998). Hence, it is possible that E2A-HLF mediates survival via DBP which in turn could activate NF- κ B function. However, there is no direct evidence to show any role for DBP in the survival of any IL-3 dependent hematopoietic cell lines or other cells. The results obtained here suggest that DBP may play an important role in NF- κ B mediated survival and proliferation pathways. It was

found that PU.1 transactivates NF- κ B 2 fold more than does DBP (Data not shown). The fact that PU.1 causes apoptosis of FDCP-Mix cells in media supporting either self-renewal or erythroid differentiation (Mclvor et al., 2003), suggests that PU.1 does not play any role in the survival of FDCP-Mix cells under these conditions. Since the luciferase assays were performed with the cells from self-renewing media, the activation of NF- κ B by PU.1 is merely an adaptive response. It is likely that PU.1 activates NF- κ B via DBP which may become upregulated when the serpin2a-elastase balance is disturbed.

The NF- κ B activation is either I κ B dependent or independent. In order to further define the pathways leading to NF- κ B activation by DBP, the effect of aspirin, an inhibitor of I κ B kinases (Kopp and Ghosh, 1994) is studied. The results in Figure 5b clearly show that 5mM aspirin inhibits DBP mediated NF- κ B activation only partially. It is known that NF- κ B, an activator of angiotensinogen (AGT) is activated by angiotensin II (Ang II) through a positive regulatory loop (Brasier et al., 2000). Although NF- κ B is ubiquitously expressed, surprisingly the mechanism for Ang II-inducible NF- κ B regulation differs between aortic smooth muscle cells (VSMCs) and hepatocytes. In VSMC, Ang II induces nuclear translocation of cytoplasmic NF- κ B proteins through proteolysis of its inhibitor, I κ B. By contrast, in hepatocytes, Ang II induces large nuclear isoforms of NF- κ B to bind DNA through a mechanism independent of changes in I κ B turnover (Brasier et al., 2000).

The results obtained here suggest that the activation of NF- κ B by DBP is largely independent of I κ B kinases. It may be possible that Ang II induces the NF- κ B activation via DBP in hepatocytes since DBP is also involved in the expression of AGT (Narayanan et al., 1998). The partial inhibition of DBP mediated NF- κ B activity by aspirin suggests that DBP is also involved in I κ B-dependent NF- κ B activation. This I κ B-dependent NF- κ B activation may be mediated by nonreceptor tyrosine kinase, proline-rich tyrosine kinase 2 (PYK2) which is involved in the osmoregulation and variety of other processes (Tokiwa et al., 1996; Avraham et al., 2000). The presence of binding sites of DBP in the PYK2 promoter (found out using Matinspector), and the role of PYK2 in osmoregulation (Tokiwa et al., 1996) support a hypothesis wherein DBP upregulates PYK2 which becomes activated when the level of DBP proteins crosses a threshold. PYK2 has

recently been shown to link signaling via a variety of G-protein-coupled receptors leading to the activation of NF- κ B in an I κ B-dependent manner in HeLa cells (Shi and Kehrl, 2001).

The results presented in Figure 5c show that PYK2 activates NF- κ B by 1.5 fold in FDCP-Mix cells. Since the level of activation is low, equimolar concentrations of PYK2 is also used to see the NF- κ B activation. Equimolar concentration of PYK2 gives nearly 2 fold more Luc activity compared to the basal activation by hrGFP construct. However, this level of activation by PYK2 is nearly 2 fold less than NF- κ B activation by DBP. The results also show that PYK2 mediated NF- κ B activity is completely inhibited by 5mM aspirin whereas the NF- κ B activity by DBP is only partially inhibited by the same concentration of aspirin. It has been previously shown that 20mM salicylate is needed to inhibit PYK2 phosphorylation (Wang and Brecher, 2001). However, the present experiment suggest that 5mM aspirin, (acetylsalicylate, a derivative of salicylate) may be sufficient to inhibit a biological effect of PYK2 such as NF- κ B activation. Further experiments are needed to further define the mechanisms of NF- κ B activation by DBP and also to prove the apparent connection between DBP and PYK2.

The connection between cell size and NF- κ B activity (Michalke et al., 2000), and the connection between DBP and cell size (Schmidt and Schibler, 1995) suggest a link between DBP and NF- κ B activity via cell size. It was hypothesized that the phosphorylation of DBP by SGK1 which is involved in the regulation of cell volume (Lang and Cohen, 2001), may confer on DBP the ability to activate NF- κ B in an I κ B independent manner. However, the results presented in Figure 5d suggest that DBP inhibits the NF- κ B activity mediated by SD-SGK1, a constitutive form of SGK1 by nearly 2 fold in 293 cells. The SD-SGK1 was chosen in preference to SGK1 for testing the effects on IgK and TRE promoters in 293 cells since it was found that SGK1 and SD-SGK1 had the same effect on IgK or TRE promoter in 293 cells, but the fold activation was much higher in case of SD-SGK1 which may be because SGK1 needs to be activated by other kinases (Kobayashi et al., 1999). The mechanism through which DBP may inhibit the SD-SGK1 mediated NF- κ B activation is not clear. It is possible that a combination of phosphorylation of DBP by a PKC member and SGK1 may confer on DBP the capability to activate NF κ B in an I κ B-independent pathway.

The results presented in Figure 5e show that SD-SGK1 inhibits the NF- κ B activity 1.5 fold in FDCP-Mix cells. However, this inhibition of NF- κ B activity by SD-SGK1 was found to be cyclic and at the crest, there was a 1.5 fold inhibition, while there was no effect on the NF- κ B activity during the trough phase of the cycle. Since DBP inhibits SD-SGK1 mediated NF- κ B activity in 293 cells (which does not express DBP protein), these results may suggest that DBP may be oscillating in a cyclic manner in FDCP-Mix cells which may inhibit NF- κ B activation at the crest phase of the cycle, but not at the trough phase of the cycle. However, there is a 1.5 fold activation of IgK promoter when the cells are transfected with SGK1 irrespective of cell state. It has been previously shown that cytokine-independent survival kinase (CISK or SGK3), a SGK-related kinase acts downstream of the phosphatidylinositide 3-kinase (PI3 kinase) cascade (Liu et al., 2000) and it is also known that SGK1 activity is regulated by 3-phosphoinositide-dependent kinase 1 and 2 (PKD1 and PKD2; Shelly and Herrera, 2002). It is also known that PI3 kinase pathway is linked to the activation of NF- κ B via I κ B pathway (Vermeulen et al., 2002). It is therefore possible that SGK1 may activate the NF- κ B via PI3 kinase pathway.

Briefly, the experiments performed with IgK promoter suggest that there are 2 different mechanisms of NF- κ B activation in FDCP-Mix cells: They are I κ B-independent and dependent pathways. DBP may activate the NF- κ B partially via I κ B-independent pathway and partially via I κ B-dependent pathway through PYK2. However, SGK1 may activate NF- κ B via the I κ B-dependent pathway, while SD-SGK1 either inhibits or has no effect on NF- κ B activity. It is possible that a combination of phosphorylation of DBP by a PKC member and SGK1 may confer DBP the capability to activate NF κ B in an I κ B-independent pathway.

Transactivation Experiments with TRE Promoter

The AP-1 family of transcription factors is thought to play key roles in oncogenic transformation, cell proliferation, differentiation and apoptosis, as well as in the cellular response to various stimuli such as physical and chemical stress, and cellular damage. AP-1 is not a single protein but dimeric basic leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra2), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2,

LRF1/ATF3, B-ATF, JDP1, JDP2) sub-families, which recognize either 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE; 5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGACGTCA-3'). The combination of all existing AP-1 subunits theoretically allows 50 different homo- or heterodimeric proteins which may serve nonreplacable specific functions or redundant functions. In this work, the AP-1 activity has been studied with the TRE reporter construct, to which the Jun and Fos homo- or heterodimers and heterodimers containing Jun and any other protein partner are known to bind. The regulation of AP-1 activity is complex and may occur through changes in *jun* and *fos* gene transcription, mRNA turnover, protein turnover, post-translational modifications of Jun and Fos proteins and interactions with other transcription factors that can either synergize or interfere with AP-1 activity (Papavassiliou, 1997; Liebermann et al., 1998; Shaulian and Karin, 2002).

The Figure 6a shows that DBP and AntiDBP activate AP-1 family of transcription factors nearly 4 and 2 fold respectively. It is known that EPO induces the expression of early response genes such as *c-jun*, *junD* and *c-fos* mRNA, and also stimulates Jun protein synthesis and induces activation of AP-1 in human erythroleukemia K562 cells (Adunyah et al., 1996). It is also known that *c-fos* and *junB* are induced during the latent period preceding commitment of Friend erythroleukemia cells to differentiation (Francastel et al., 1992). Since DBP is likely involved in the erythroid differentiation, it is likely that DBP is involved in the regulation of *c-fos* which is expressed in the erythroblasts from fetal liver and from adult blood BFU-E-derived colonies but not in granulocytic or monocytic precursors (Caubet et al., 1989). However, the mechanism through which DBP may regulate *c-fos* is not clear but may involve transcriptional and post-translational mechanisms or protein-protein interaction. It has been shown that the AP-1 family of transcription factors are activated during hypoxia (Bandyopadhyay et al., 1995). Since there is a reduction in the concentration of DBP in FDCP-Mix cells when exposed to hypoxia, it is likely that AntiDBP expression also leads to downregulation of DBP at the mRNA and protein level to a certain extent and AntiDBP expression may mimic the cells exposed to hypoxia. It was initially thought that AntiDBP expression may lead to *c-Jun* activation in a *c-Jun* N-terminal kinase (JNK)-dependent manner similar to that observed in

HepG2 cells (Minet et al., 2001). However, subsequent results obtained with both PYK2 and SGK1 in FDCP-Mix cells show that *c-Jun* is not activated detectably either by JNK activation (mediated by PYK2; Figure 6b) or by SGK3 inactivation (mediated by SGK1; Figure 6c). Hence, it is likely that AP-1 activation mediated by AntiDBP also involves *c-fos* via either transcriptional or post-translational modifications. Since JNK and *c-Jun* activation are involved in apoptosis (Shaulian and Karin, 2002), it is possible that they are under tight control in FDCP-Mix cells. It was found that PU.1 transactivates the AP-1 family of transcription factors 2 fold more than does DBP (Data not shown) suggesting that it is involved in the activation of TRE promoter indirectly. Figure 6b shows that PYK2 does not activate TRE promoter even when equimolar concentration of PYK2 is used, suggesting that it does not activate AP-1 family of transcription factors in FDCP-Mix cells.

Figure 6e shows that DBP inhibits the AP-1 activation mediated by PYK2 in 293 cells. Since 293 cells do not express DBP, it is clearly DBP which inhibits the JNK activation mediated by PYK2 in FDCP-Mix cells. It has been shown that elevated levels of cAMP inhibit JNK activation in liver epithelial cells (Li et al., 1997). Since the cotransfection of DBP and PYK2 leads to activation of HIF-1 (Figure 7a), it is likely that there is an increase in the cAMP concentration which would be needed to activate the protein kinase A (PKA) and to upregulate the HIF-1 activity (Kvietikova et al., 1995). It is possible that this increase in cAMP also has the effect of inhibiting the JNK activation mediated by PYK2. However, the AP-1 activity mediated by PYK2 is also inhibited under high or low cell concentrations when the DBP/PYK2 combination does not activate the HIF-1 suggesting that the cAMP dependent pathway alone may not be sufficient to inhibit the JNK activation. It is likely that DBP controls the kinase activity of PYK2 such that PYK2 does not phosphorylate DBP until a critical concentration of DBP protein builds up within cells. Once the threshold cell size beyond which the cell size can not grow any further is reached, PYK2 may become activated to protect a substantial portion of DBP and to degrade another substantial portion of DBP. Frank et al. (2001) have recently concluded based on their studies with PYK2 kinase-inactive mutant K457A and a tyrosine phosphorylation site mutant Y402F that PYK2 kinase activity negatively regulates JNK activation, whereas Tyr⁴⁰² phosphorylation of PYK2 positively regulates it in AngII-stimulated

vascular smooth muscle cells, suggesting a unique mechanism of PYK2. However, it has been shown that both PYK2 kinase activity and Tyr⁴⁰² phosphorylation have a positive effect on JNK activation in neonatal cardiac fibroblasts stimulated by AngII (Murasawa et al., 2000) indicating a cell-type specific role of the PYK2 kinase activity in JNK regulation. Such variations can be explained based on the differences in the PYK2 substrates which may control the JNK activation positively or negatively. It is likely that DBP is one of PYK2 substrates which is involved in the negative regulation of JNK. The mechanisms of this negative regulation needs to be further explored.

There are at least 12 isoforms of PKC enzymes and individual PKC isoforms have been implicated in many cellular responses such as contraction, migration, secretion, hypertrophy, proliferation, apoptosis and differentiation (Dempsey et al., 2000) of different cell systems. They show varying patterns of tissue- and cell-type-specific expression. They also differ in subcellular localization and regulatory and enzymatic properties. The PKC isotypes can be classified into three major groups: the "conventional" isozymes (cPKC), activated by calcium and diacylglycerol; the "novel" isozymes (nPKC), which require only diacylglycerol for activation; and the atypical isozymes (aPKC), which require none of these cofactors. The conventional (α , β I, β II and γ) and novel (δ , ϵ , η , and θ) PKCs can be activated by PMA. Since it is hypothesized that DBP is a tumor suppressor, it is likely that DBP inhibits the protein kinase C (PKC) activation mediated by PMA. It has been known that PMA at low concentration potentiates superoxide generation in neutrophils (Tyagi et al., 1988). Thus, it is hypothesized that phosphorylation of DBP by PYK2 is essential to activation of PKC, while the activation of PKC by PMA (Kikkawa et al., 1983) at high concentrations may interfere with the functions of DBP such as neutrophil priming of cyclic disorders (Iki et al., 1997). Since PKC activation leads to AP-1 activation, the TRE promoter has been used as a readout for the PKC activation. The Figure 6d shows that the expression of AntiPYK2 inhibits the TRE activity and it also shows that AntiPYK2 inhibits TRE activation mediated by PMA in FDCP-Mix cells. These results suggest that PYK2 is essential for the activation of TRE promoter but overexpression of PYK2 is not sufficient to activate the TRE promoter via JNK activation. The results also show that PMA transactivates TRE promoter by 2 fold but when the AntiPYK2 expressing

cells are treated with PMA, the fold activation goes down to less than 1.5 fold in FDCP-Mix cells. However, this reduction is very low owing to the severe reduction in the baseline TRE activity by AntiPYK2. The results presented in Figure 6g shows that DBP inhibits the TRE activation mediated by PMA in 293 cells. Since 293 cells do not express DBP at the protein level, these results suggest that DBP itself is involved in the inhibition of TRE activation seen in FDCP-Mix cells. The inhibition of AP-1 activity seen with DBP overexpressed cells compared to GFP transfected cells even in the absence of PMA may suggest that DBP modulates the endogenous PKC activity. It has been known that sphingosine inhibits the PKC activation (Smith et al., 1997), while the S1P is known to activate PKC (Lampasso et al., 2001). It is possible that (basal phosphorylated) DBP prevents or reduces the conversion of sphingosine into S1P and thereby inhibits the PKC activation mediated by PMA considerably. However, the DBP phosphorylated by PYK2 is unlikely to inhibit the PKC activation mediated by PMA. An experiment was specifically performed to test whether DBP phosphorylated by PYK2 inhibits the TRE activation mediated by PMA revealed that the DBP (and hrGFP) inhibited TRE activity minimally which may suggest that the concentration of DBP is essential to inhibition of TRE activation mediated by PMA. When three different constructs (GFP/DBP, hrGFP/PYK2hrGFP and TRE reporter) are simultaneously transfected into 293 cells, it is likely that the level of protein expressed from each construct will be severely reduced. However, the fact that the HIF-1 activity mediated by DBP/PYK2 combination is inhibited by PMA (Figure 7d) supports the above conclusion that DBP phosphorylated by PYK2 does not inhibit PKC activation mediated by PMA. It is also known that PMA mediated down-regulation of PKC is inhibited by sphingosine (Grove and Mastro, 1988). Briefly, DBP may modulate the activity of PMA and PMA in turn may modulate the activity of DBP possibly via regulating the concentration of sphingosine, S1P and the activity of Sphk. As mentioned earlier, it will be necessary to understand this plausible link between Sphk and DBP to further understand the mechanism by which DBP inhibits the TRE activation mediated by PMA.

Figure 6f shows that SD-SGK1 activates the TRE promoter 2 fold compared to control GFP/IRES2 cotransfected cells. The cotransfection of SD-SGK1 with DBP does not inhibit the TRE activation mediated by SD-

SGK1. It is known that SGK1 inhibits the GSK3 (Kobayashi and Cohen, 1999) while the GSK3 inhibition is known to activate the c-Jun (Boyle et al., 1991). These results together may therefore suggest that DBP does not inhibit the c-Jun activation mediated by SD-SGK1. As discussed above, SGK1, SD-SGK1 and PYK2 expression do not activate the TRE promoter detectably in FDCP-Mix cells (Figure 6c and d). It is known that c-Jun is also involved in apoptosis (Shaulian and Karin, 2002). This may be one reason why c-Jun activity is under stringent control in FDCP-Mix cells during self-renewal under high concentrations of IL-3.

The discussion of TRE promoter activation can be summarized as follows: DBP and AntiDBP may transactivate AP-1 family of transcription factors and this may likely be c-fos. Since both PYK2 and SD-SGK1 fail to activate the TRE promoter detectably which are known to activate c-Jun, it is likely that c-Jun activation is under stringent control in self-renewing FDCP-Mix cells. DBP may inhibit the PKC activation mediated by PMA possibly via regulating Sphk activity. PYK2 is essential for the endogenous TRE promoter activity in FDCP-Mix cells but overexpression of PYK2 is not sufficient to activate the TRE promoter.

Role of DBP in the Regulation of HIF-1 Activity

Hypoxia is normally encountered by humans under varying conditions. During development, the human fetus encounters hypoxic conditions *in utero* until birth. Humans living at high altitudes are adapted to chronic hypoxia owing to lower oxygen in high altitude conditions. Hypoxia also manifests itself under clinical conditions such as wound healing, anaemia, myocardial infarction, ischemic heart disease, retinopathy, chronic pulmonary disease, and cancer. The hypoxia inducible factor-1 (HIF-1) is a principal factor involved in the regulation of hypoxia. It is a heterodimeric protein composed of HIF-1 α and HIF-1 β . Under normoxia, the HIF-1 α is unstable while under hypoxic or anoxic conditions, the HIF-1 α protein gets stabilized. Upon stabilization, the HIF-1 α enters nucleus to form the HIF-1 complex which is involved in the modulation and coordination of the cellular responses to hypoxia. It is known that HIF-1 regulates the expression of a battery of genes involved in increasing the blood flow and oxygen supply to tissues such as EPO, inducible nitric oxide synthase (iNOS) and vascular endothelial growth factor (VEGF), and another battery of genes involved in adapting the cells to anaerobic metabolic conditions such as aldolase, lactate dehydrogenase,

phosphofructokinase, phosphoglycerate kinase and pyruvate kinase (Wenger, 2002). The work on the regulation of HIF-1 has been analysed by using two HIF-1 reporters: One has the HIF-1 binding sites from the 3' end of the EPO gene and another has the HIF-1 binding sites from the transferrin gene promoter. The EPO-HIF-1 reporter was used to study the HIF-1 activity in 293 cells which are known to express EPO (Withy et al., 1992) while the Tf-HIF-1 reporter was used in the studies involving FDCP-Mix cells which do not express EPO under self-renewing conditions.

The results indicate that cotransfection of DBP and PYK2 leads to 5 fold activation of HIF-1 while the control cotransfection of GFP and PYK2 inhibits the basal HIF-1 activity more than 3 fold (Figure 7a). It is known that HIF-1 activation involves the PKA pathway and it is likely that DBP phosphorylated by PYK2 leads to generation of cAMP which may in turn activate the PKA and HIF-1. It is known that calcium ionophore inhibits the expression of EPO (Faquin et al., 1993) and it is possible that this inhibition is mediated by PYK2. This inhibition of HIF-1 activity by PYK2 also occurs under hypoxia. It has been shown that GSK3 β is activated by PYK2 (Hartigan et al., 2001). It has also been shown that activation of protein kinase B (Akt, a human homolog of viral Akt) by hypoxia resulted in the phosphorylation of GSK-3 α and GSK-3 β at Ser-9 and Ser-21, two well characterised Akt phosphorylation sites, respectively, that are inactivating modifications of each GSK-3 isoform (Chen et al., 2001). It is likely that PYK2 mediated HIF-1 inhibition occurs via GSK3 β . However, the exact details of this GSK3 mediated HIF-1 inactivation are not known.

The Figure 7b shows that cotransfection of DBP and DYRK3 results in a 6 fold activation of HIF-1 compared to the basal HIF-1 activity seen with GFP and hrGFP. The cotransfection of GFP and DYRK3 increases the HIF-1 activity nearly 1.5 fold. However, the combination of DBP and DYRK3 is needed to transactivate the HIF-1 reporter strongly. It is likely that this activation of HIF-1 also involves the PKA pathway given the recent finding that DYRK3 stimulates the cAMP response elements (CRE) in 293 and HeLa cells (Li et al., 2002). The Figure 7c shows that DBP/hrGFP mediated HIF-1 activity is not affected by PMA which is not surprising given the previous result that DBP inhibits the TRE promoter activation mediated by PMA (Figure 6g). The Figure 7d shows that DBP/PYK2 mediated HIF-1 activation is more susceptible to inhibition by PMA than the

DBP/DYRK3 mediated HIF-1 activity. However, whether DBP resists the inhibitory effect of PMA or not (with respect to HIF-1) is dependent on cell concentration. The results presented in Figure 7e show that the kinetics of HIF-1 activation mediated by DBP/PYK2 and DBP/DYRK3 are different. When the DBP/DYRK3 transfected cells are exposed to hypoxia at an earlier time point (27hrs post-transfection), there is a reduction in the HIF-1 activity to nearly 1.5-2 fold HIF-1 activation compared to basal level. Since the DBP/DYRK3 mediated HIF-1 activation is relatively resistant to PMA (PKC activation), these results may reflect a different mechanism. This pathway may involve the degradation of DBP protein phosphorylated by DYRK3 via elastase followed by the onset of high level of endogenous PKC activation by the hypoxia. However, when DBP/DYRK3 transfected cells are exposed to hypoxia at a later time point, the activation of HIF-1 is not reduced. This may reflect the downregulation of elastase expression at the transcriptional level by DBP through binding to the introns of the elastase gene (see below). However, the DBP/PYK2 transfected cells do not show this reduction of HIF-1 activity when exposed to hypoxia at either time point. The results also show that HIF-1 activity in these 293 cells is constitutive. Interestingly, it was found that this kinetic phenomenon was abolished when the experiments were performed with the cells that were kept for a day after confluency (prior to transfection). However, it is not known whether this phenomenon returns if the cells are grown under normal conditions for sufficient time.

The Figure 7f shows that DBP inhibits the HIF-1 activity nearly 6 fold while DBP/PYK2 and DBP/DYRK3 have no effect on HIF-1 activity under high cell density in 293 cells. Although a potential role of DBP in the suppression of metastasis was considered in the early stages of this work based on the hypothesis that DBP is involved in the pathogenesis of polycythemia vera (PV, a benign myeloproliferative disorder), a role for DBP in the inhibition of HIF-1 activity was recognized only after obtaining this result. It lends further support to the hypothesis that DBP may be involved in the pathogenesis of PV, since upregulation of HIF-1 activity is a hallmark of metastatic tumours where HIF-1 is known to be involved in the vasculogenesis (Dachs and Tozer, 2000). It has been recently shown that the PKC isoforms PKCdelta and PKCepsilon are involved in contact-mediated inhibition of proliferation and inhibition of proliferation at low cell densities respectively (Heit et al., 2001; Petrovics et al., 2002). The

results presented here may suggest that one or more PKC isoforms interferes with the functions of DBP phosphorylated by both PYK2 and DYRK3. Since this PKC activation is endogenous, it would affect the DBP phosphorylated by both PYK2 and DYRK3 equally, although DBP phosphorylated by DYRK3 appears to be relatively less susceptible to PKC (PMA) interference (Figure 7d). However, the basal phosphorylated DBP (that is DBP not phosphorylated by PYK2 and DYRK3 but phosphorylated by an endogenous kinase present in 293 cells) inhibits the HIF-1 activity. It is known that loss of tumor suppressor function of PTEN facilitates the HIF-1 activation (Zundel et al., 2000). Since DBP is a likely tumor suppressor, the inhibition of Akt by DBP may lead to activation of GSK3 which may in turn inactivate the HIF-1. However, further work would be needed to understand the mechanistic details of HIF-1 inhibition by DBP. Similar results are also observed if the concentration of cells were low at the time of cell harvest. However, the DBP/PYK2 combination inhibited the HIF-1 activity nearly 1.5-2 fold compared to the basal HIF-1 activity seen with GFP and hrGFP under low cell density. This inhibition of HIF-1 activity by DBP/PYK2 was also observed if the cells reached the high cell density a few hours prior to harvest. In order to further define the mechanisms of HIF-1 activation or inhibition by DBP/hrGFP and DBP/PYK2, the effect of fresh media on HIF-1 activation was studied. The results presented in Figure 7g show that DBP/hrGFP activates HIF-1 nearly 3 fold while DBP/PYK2 inhibits the HIF-1 activity nearly 5 fold. It has been known that fresh media change may lead to an increase in the bioactive lipids including sphingosine (Smith et al., 1997). It is likely that sphingosine has a stimulating effect on the basal phosphorylated DBP, while this increase in sphingosine has a negative effect on DBP phosphorylated by PYK2. But the small change in the sphingosine concentration caused by nearly 2.5 fold dilution of conditioned media is unlikely to have a strong effect on DBP phosphorylated by DYRK3. It has been known that sphingosine stimulates the synthesis of cAMP while S1P is known to reduce the cAMP concentration (Pyne et al., 1996; Pyne and Pyne, 1996). Thus, it may be possible to exert an influence on HIF-1 activity via regulating sphingosine/S1P or ceramide balance. It is likely that different phosphorylated forms of DBP have different effects on the sphingosine/S1P or ceramide concentration. It

is not clear how DBP is involved in this process.

The Figure 7h shows that LiCl at the concentration of 10mM inhibits the HIF-1 activity in DBP+ DYRK3 transfected 293 cells nearly 2 fold under hypoxia. It was found that there was no detectable inhibition when the experiment was performed under normoxia. Since DYRK-related kinases are known to prime protein substrates for subsequent phosphorylation by GSK3 (Woods et al., 2001), it is possible that GSK3 phosphorylates DBP primed by DYRK3. The results discussed above show that a substantial portion of DBP is degraded when FDCP-Mix cells are exposed to hypoxia. It has been recently shown that GSK3 is activated by PYK2 (Hartigan et al., 2001). These data taken together suggest that a substantial portion of DBP protein phosphorylated by PYK2 may become resistant to degradation by elastase, while the rest of the DBP protein phosphorylated by GSK3 may be degraded by elastase. It is possible that SGK1 or a member of PKC primes the DBP protein in normal self-renewing FDCP-Mix cell population, while during erythroid differentiation DYRK3 may prime DBP for subsequent phosphorylation by GSK3. This priming by SGK1 or a PKC member may have caused an increase in the serine phosphorylation of DBP in FDCP-Mix cells exposed to hypoxia. The results presented in Figure 7i show that neutrophil elastase inhibitor at the concentration of 0.02mM reduces the HIF-1 activity mediated by DBP nearly 2 fold under normoxia in 293 cells. It is found that VBP binding sites are in the intron of the elastase gene using MatInspector. These data suggest that DBP may be involved in the downregulation of elastase at the transcriptional level by binding to the introns of the elastase gene. This suggests a possible antagonistic interaction between DBP and elastase which is perturbed when elastase inhibitor is added. This perturbation may have caused a reduction in HIF-1 activity mediated by DBP.

The Figure 7j shows that there is a nearly 1.5 fold activation of transferrin HIF-1 reporter by PYK2 under normoxia in FDCP-Mix cells. When the PYK2 expressing cells are exposed to hypoxia, there is no further increase in HIF-1 compared to the HIF-1 activity seen with hrGFP transfected cells. It is known that IL-3 activates the PKC in FDCP-Mix cells (Shearman et al., 1993) and it is also known that PMA can partially substitute for the IL-3 (Whetton et al., 1988). It is possible that this high level of endogenous PKC activity mediated by IL-3 inhibits the HIF-

1 activation mediated by PYK2. However, there is a low level of activation of HIF-1 by PYK2. The results may also suggest that HIF-1 is largely constitutive in FDCP-Mix cells.

Briefly, the summary of the discussions on the experiments performed with HIF-1 reporters is as follows: DBP activates HIF-1 nearly 2-3fold under optimal cell concentrations, while it inhibits the HIF-1 activation at both low and high cell concentration. The DBP/PYK2 combination inhibits HIF-1 activity nearly 3-4 fold at low cell concentration or upon addition of fresh media, followed by activation of HIF-1 by 8-14 fold at optimal cell concentration, but has no effect on HIF-1 activity at high cell density. However, the DBP/DYRK3 combination had no effect on HIF-1 activity at either low or high cell density, while at optimal cell density it activated HIF-1 by 8-15 fold. The HIF-1 activity mediated by DBP/DYRK3 was reduced by exposure of the cells to hypoxia at an earlier stage, but activated by exposure at a later stage suggesting that DYRK3 is a developmental kinase while DBP/PYK2 mediated HIF-1 activity is not influenced by early or late stages of hypoxia. The DBP/PYK2 mediated HIF-1 activity is more susceptible to PMA inhibition compared to DBP or DBP phosphorylated by DYRK3. Since elastase inhibition leads to nearly 2 fold inhibition of HIF-1 activity, it is possible that DBP is involved in the transcriptional down regulation of elastase.

Transactivation Experiments with c-fms Promoter

The c-fms gene codes for the receptor for colony stimulating factor 1 (CSF1, also known as M-CSF), a cytokine which controls the production, differentiation, and function of macrophages. It is a tyrosine kinase transmembrane receptor and member of the CSF1/platelet-derived growth factor (PDGF) receptor family of tyrosine-protein kinases. The M-CSF binding activates the receptor kinase through a process of oligomerization and transphosphorylation. Mutations in c-fms were most prevalent (20%) in chronic myelomonocytic leukemia and acute myelogenous leukemia (AML) type M4 (23%), both of which are characterized by monocytic differentiation (Ridge et al., 1990). Kondo et al. (2000) showed that the receptors for GM-CSF and M-CSF are expressed at low to moderate levels on the primitive hematopoietic stem cells, are absent on common lymphoid progenitors, and are upregulated after myeloid lineage induction by IL2. It is also known that PU.1, a transcription factor essential for

monocytic differentiation, upregulates c-fms (Inaba et al., 1996).

The results presented in Figure 8a show that cotransfection of PU.1 with PYK2 leads to nearly 1.5 fold activation of the c-fms promoter compared to PU.1 and hrGFP transfected FDCP-Mix cells. Since PYK2 may lead to reduction in the concentration of DBP, it is possible that this reduction orients the multipotential cells towards myeloid lineage. However, it was found that transfection of PYK2 alone was not sufficient to transactivate the c-fms promoter. It is known that c-fms mediates survival via the Akt pathway (Lee and States, 2000). Since FDCP-Mix cells express high level of DBP, a candidate tumor suppressor, it is highly unlikely that DBP permits the higher level expression of c-fms (under self-renewing conditions). Hence, PYK2 was cotransfected with a known c-fms transactivator PU.1. The results showed that PYK2 can indeed potentiate PU.1 mediated c-fms transactivation 1.5 fold. However, it was found that this coactivation by PYK2 is cyclic and at the trough phase, there was no activation (Data not shown), while at the crest, there was 1.5 fold potentiation of PU.1 mediated c-fms activation. PYK2 has been previously shown to activate the NF- κ B by 1.5 fold in FDCP-Mix cells (Figure 5c). However, this 1.5 fold activation of NF- κ B by PYK2 was not found to be cyclic suggesting that the activation of c-fms is likely more susceptible to endogenous cycling factors unlike the activation of NF- κ B by PYK2.

The Figure 8b shows that cotransfection of PU.1 with SGK1 or SD-SGK1 leads to nearly 1.5 fold potentiation of the c-fms promoter compared to PU.1 and control IRES2 cotransfection. However, this coactivation by SGK1 was found to be cyclic. At the crest, there was 1.5 fold coactivation of c-fms promoter while there was no effect at the trough phase of the cycle (Data not shown). It is likely that there are several other proteins and kinases which are also oscillating in a cyclic manner apart from DBP. When the cells are orienting themselves towards erythroid lineage, it is possible that exogenous expression of these kinases may not be effective in redirecting the cells to a different lineage at least under self-renewing conditions. The results may also suggest that cells may be cycling to and fro between erythroid and myeloid orientations.

Conclusion

The work presented here on DBP is too preliminary and it may be used as a launch pad to address a large number of issues that

are summarized here. In particular, it will be interesting to search for defects in hematopoiesis in DBP knock out mice. Although there are defects in knock out mice such as low locomoter activity (Lopez-Molina et al., 1997) which could conceivably be due to either neurological defects or hematological defects such as reduced erythrocytes, it is not known what role DBP plays in hematopoiesis *in vivo*. Does GATA1 upregulate the expression of DBP at the mRNA level? Does DBP upregulate PYK2 transcriptionally? Do DBP and GATA1 interact with each other physically to accomplish an erythroid specific gene expression pattern? How many phosphorylation sites are there in DBP *in vivo*? What is the site of phosphorylation in DBP by DYRK3 and PYK2? What are the sites of phosphorylation in DBP by SGK1, PKC and GSK3? What is the PKC isoform involved in the regulation of the activities of DBP? What is the relation between sphingosine kinase and DBP and how does DBP regulate (both activation and inhibition of) Sphk2 activity? Is DBP involved in the pathogenesis of polycythemia vera and cyclic neutropenia? What is the kinetics of degradation of DBP phosphorylated by different kinases both *in vitro* and *in vivo*? Does elastase involved in this degradative pathway? Does DBP downregulate the elastase expression transcriptionally? What is the exact mechanism of activation and inhibition of NF- κ B and AP-1 family of transcription factors? What is the exact mechanism of activation and inhibition of HIF-1 activity by DBP? Is DBP involved in the expression of EPO? What other role does DBP play in the differentiation of HSCs? How does other hematopoietic transcription factors such as *HOXB4* and *c-myb* interact with DBP? What is their relation with DBP? What is the role of DBP in apoptosis? Is E4BP4 involved in the lymphoid commitment and differentiation? What are all the protein partners of DBP? What are all phosphatases involved in the dephosphorylation of DBP?

There are several parameters of bone marrow cells which show large circadian variations in addition to well-known variations in DNA synthesis (Smaaland et al., 1991). Further studies of the mechanisms of this circadian control through detailed analysis of DBP may therefore indicate potential routes to the effective chronotherapy of hematopoietic disorders.

Bauer (1995) suggests that there are two kinds of knowledge: They are maps and stories. The maps are reliable knowledge (facts) while the stories are meaningful. In

short, the emerging story of this work on DBP suggests that DBP may be involved in the regulation of survival, proliferation, apoptosis, commitment and differentiation of HSCs. It also suggests that DBP may regulate circadian and cyclic hematopoiesis. However, the data (maps) presented in spite of the fact that it is incomplete still suggest that this story may be true. But the future may hold the keys to work out all the issues raised above which may provide us the complete map of how DBP regulates hematopoiesis.

Acknowledgement

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Figure 1a: Expression of DBP in 293 cells. 50µg of whole cell extracts from untransfected 293 cells and DBP/hrGFP transfected cells were used for the Western blotting with anti-DBP antibody. It shows that DBP is not expressed in 293 cells.



Figure 1b: Expression and phosphorylation of DBP in 293 and FDCP-Mix cells. 25µg of whole cell extracts from 293 and FDCP-Mix cells were run on 15% SDS-PAGE and stained with Coomassie blue (a) to show the equal loading of proteins. A blot probed with anti-DBP antibody (b) shows that DBP is expressed in self-renewing FDCP-Mix cells day 3 cells (SRd3) and there is a 2 fold reduction in the concentration of DBP when FDCP-Mix cells are treated with 1mM ATP or exposed to hypoxia for 30mins and 6hrs but not when cells are exposed to 10mM LiCl. Similar blot probed with anti-phosphoserine antibody (c) shows that DYRK3 phosphorylates DBP in 293 cells. The blot probed with anti-phosphotyrosine antibody (d) shows that PYK2 phosphorylates DBP in 293 cells and it also shows that there is an increase in the tyrosine phosphorylation of DBP when FDCP-Mix cells are treated with 10mM LiCl and 1mM ATP or when cells are exposed to hypoxia for 30mins and 6hrs.

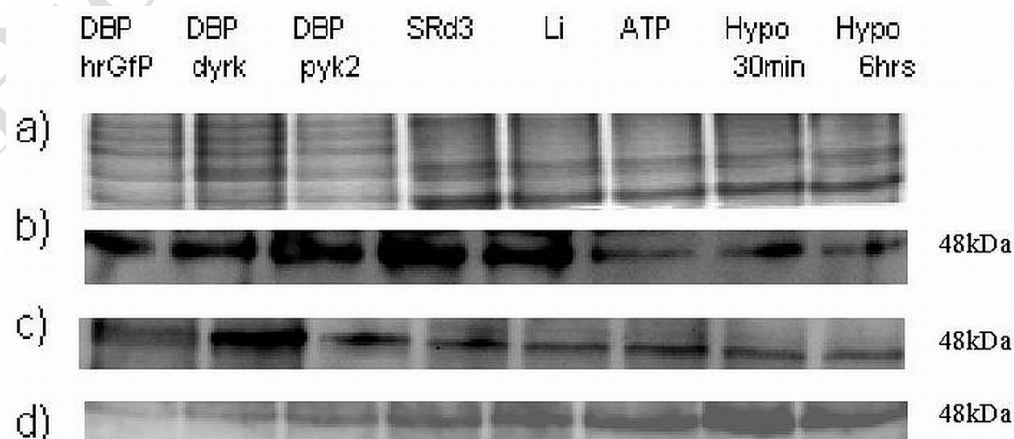


Figure 1c: Pairwise alignment of mouse DBP and Sphingosine kinase 2 by using BLAST. The plus strand of mDBP is aligned to the minus strand of Sphk2. Identities = 486/489 (99%), Gaps = 1/489 (0%), E value = 0.

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mDEP: 1151 aggatgagaagtactggagccggaggtacaagaacaatgaagcagccaagaggtcgagag 1210
      |||
Sphk: 2370 aggatgagaagtactggagccggaggtacaagaacaatgaagcagccaagaggtcgagag 2311

mDEP: 1211 atgcaagaagactcaaggagaaccagat atctgtgagggtgcctcctggagaaggaaa 1270
      |||
Sphk: 2310 atgcaagaagactcaaggagaaccagat atctgtgagggtgcctcctggagaaggaaa 2251

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Sphk: 2250 acgccctgttggcgcaggaggtggtggtgtgcccagcagagctgtcccactaccgtgtg 2191

mDEP: 1331 tgctttcacgctaccagccccagcatgggacactgtgagggc-accctccaccctgccaggy 1389
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Sphk: 2190 tgctttcacgctaccagccccagcatgggacactgtgagggcaccctccaccctgccaggy 2131

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Sphk: 2130 cagagtcctggtccttgcctcagacttacaactgacttcctcctgtcccatggccagtg 2071

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Sphk: 2070 tctggccagctaggtgccggaagaacgtcatgatgcagacaat acatttatat tttt aa 2011

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      |||
Sphk: 2010 gaaaaagctagcctccccacctcccttggggggtggggagggctcctgtgtgtgctct 1951

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      |||
Sphk: 1950 tagcatgttggggaaccatccatccaaccgctccatcaacacaatcctgaataaatct 1891

mDEP: 1630 tgagaacc 1638
      |||
Sphk: 1890 tgagaacc 1882
    
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Figure 2: Effect of DBP on CFU formation. It shows that there is a 2 fold increase in the number of colonies when FDCP-Mix cells are transfected with DBP compared to empty GFP transfected cells. Blank (untransfected cells) have 30-40 times more colonies than the transfected cells. The results represent a single CFU assay experiment performed in sextuplicate.

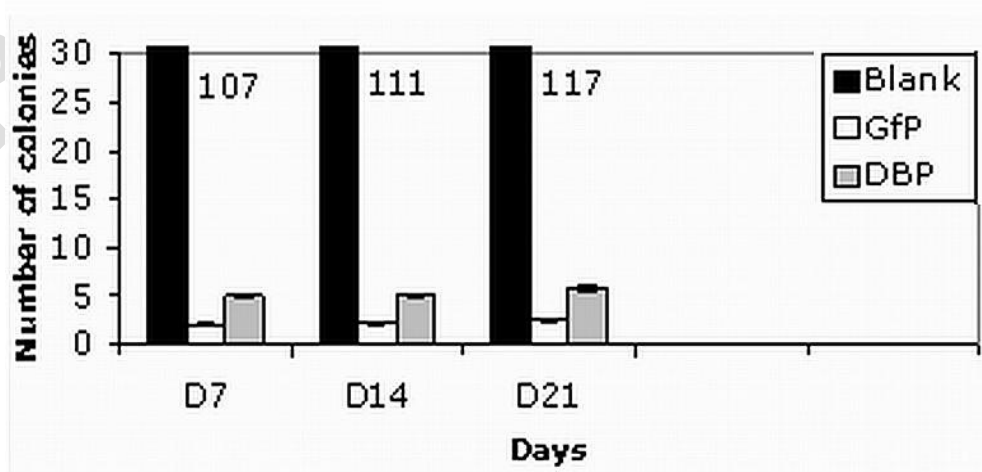


Figure 3a: Effect of DBP on cell survival and proliferation. It shows that when FDCP-Mix cells are transfected with DBP there is more than 2 fold increase in the number of live cells compared to GFP transfected control cells. The survival and proliferation of untransfected cells (blank) is beyond the range (40-50 times more than the transfected cells). The results represent the mean and standard deviation of a single experiment performed in triplicate.

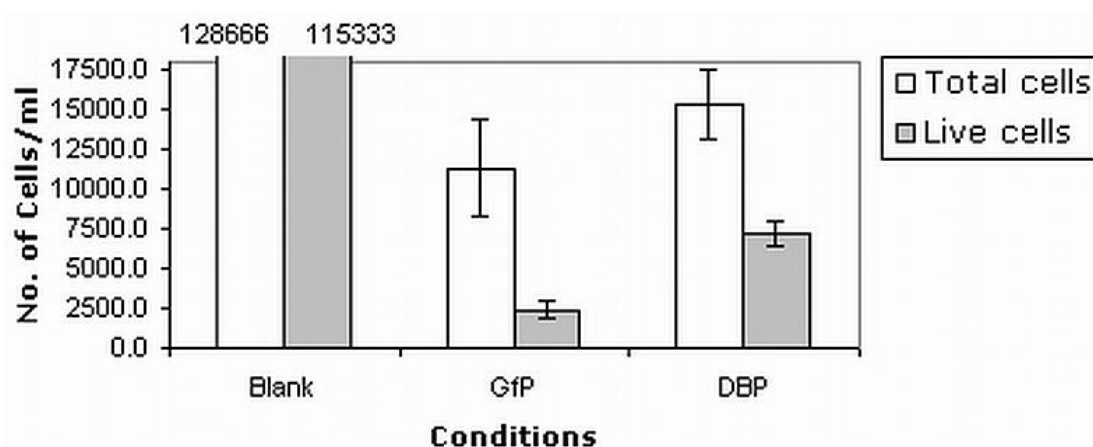


Figure 3b: Effect of DBP on the thymidine kinase promoter. DBP transactivated TK109 promoter 10 fold compared to GFP vector. The graph shows the mean +/-SD of a single experiment performed in triplicate.

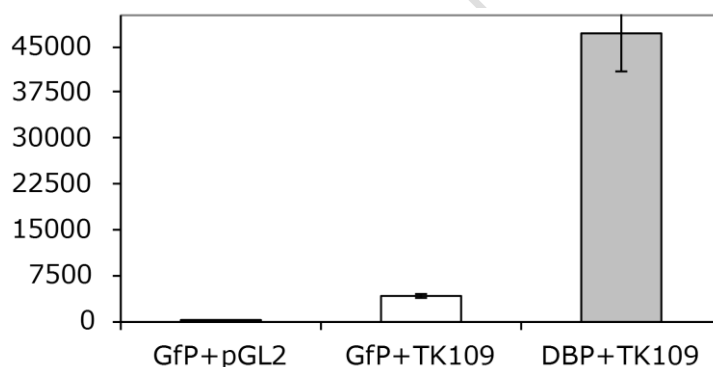


Figure 4a: Effect of cotransfection of DBP and GATA1 on S2a promoter. It shows that when DBP is cotransfected with small amounts of GATA1 or vice versa, there is a 3 fold activation of S2a promoter compared to the Luc activity seen with DBP/GFP or GATA1/GFP. The Luc activity of GATA1/DBP is beyond the range and it is given therein. The data represent mean +/- SD of a single experiment performed in triplicate.

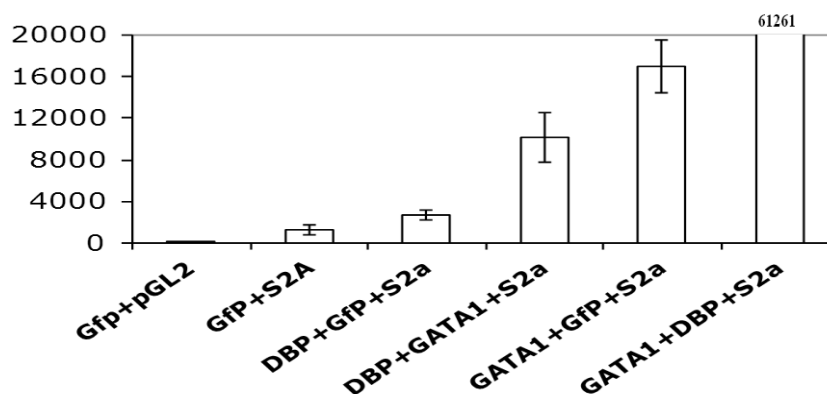


Figure 4b: Effect of cotransfection of DBP and PU.1 on S2a promoter. It shows that DBP transactivates S2a promoter 2 fold more than GFP vector, while PU.1 transactivates S2a nearly 2 fold more than DBP (and GfP). Cotransfection of DBP with small amounts of PU.1 gives the same level Luc activity as that of PU.1 transfection. The data represent mean with standard deviation of a single experiment performed in triplicate.

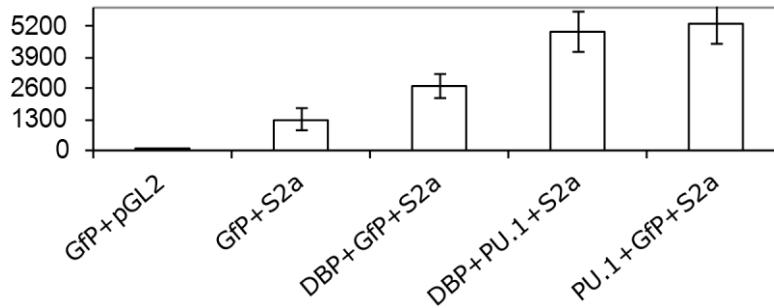


Figure 4c: It shows that the kinetics of Serpin2a activation by GFP and DBP follows the same pattern except the Luc activities which is 2-3 fold more in DBP. However, PU.1 causes an initial dip in endogenous S2a level at 1.30hrs before upregulating S2a activity nearly 2 fold more than the Luc activity seen with DBP at 18hrs. The data represent mean +/- SD of a single experiment performed in triplicate.

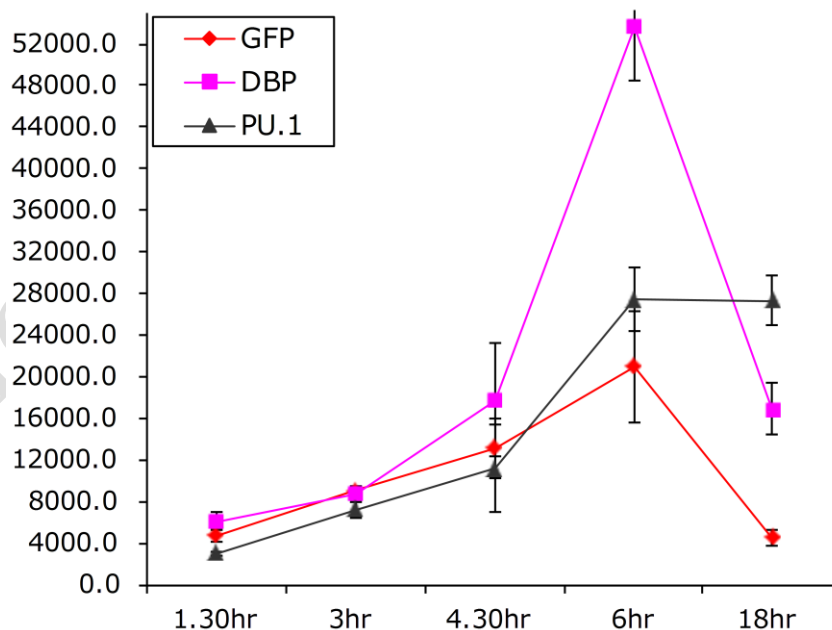


Figure 4d: Comparison of S2a activation by different transcription factors. It shows that PU.1 transactivates S2a 2 fold more than DBP, while GATA1 transactivates S2a 6 fold more than DBP. DBP transactivates S2a 2 fold more than empty GFP vector.

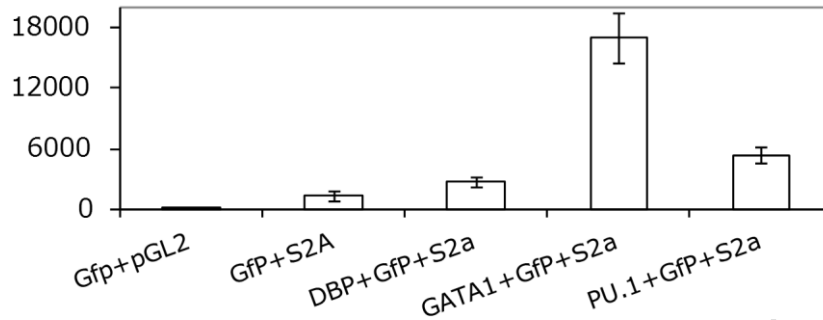


Figure 5a: Effect of DBP and AntiDBP on IgK promoter in FDCP-Mix cells. It shows that DBP transactivates IgK promoter 6 fold compared to empty GFP vector, while AntiDBP activates IgK promoter at a low level. The graph shows the mean +/-SD of a single experiment performed in triplicate.

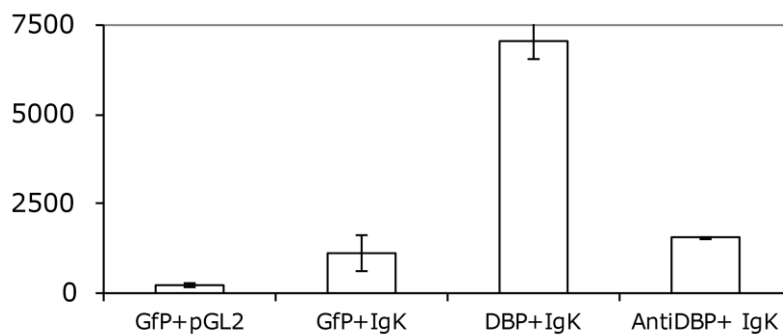


Figure 5b: Effect of 5mM aspirin on NF-κB activation mediated by DBP in FDCP-Mix cells. It shows that 5mM aspirin can partially inhibit the NF-κB activation mediated by DBP.

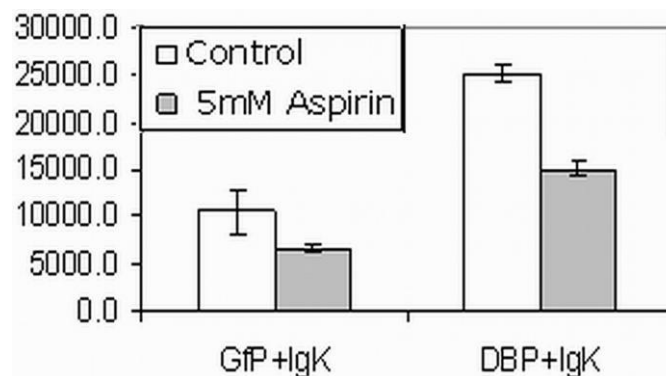


Figure 5c: Effect of aspirin on NF- κ B activation mediated by PYK2 in FDCP-Mix cells. It shows that PYK2 transactivates IgK promoter 1.5 fold compared to GFP vector, while equimolar concentrations of PYK2 (high PYK2) transactivates IgK nearly 2 fold. The PYK2 mediated IgK activation is completely inhibited by 5mM aspirin. The graph shows the mean with standard deviation of a single experiment performed in triplicate.

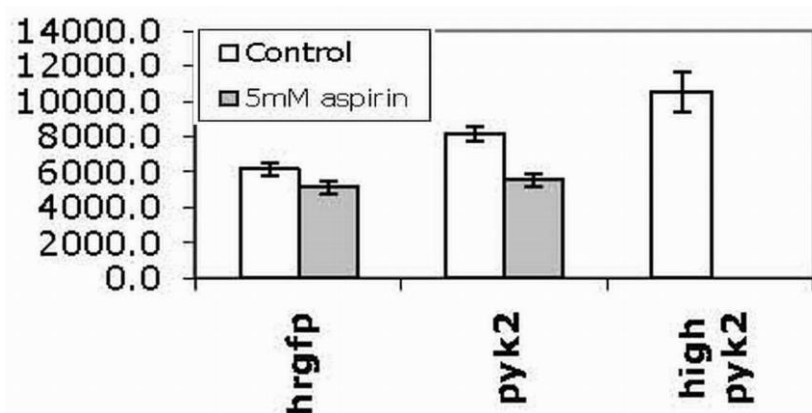


Figure 5d: Effect of SD-SGK1 on IgK promoter activity in 293 cells. It shows that cotransfection of GFP with SD-SGK1 (a constitutive form of SGK1) activates IgK promoter nearly 4 fold, while cotransfection of DBP with SD-SGK1 leads to a 2 fold reduction in the Luc activity compared to the Luc activity seen with GFP and SD-SGK1.

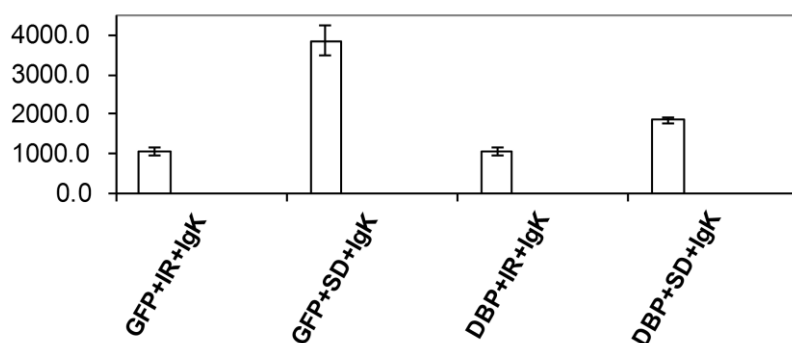


Figure 5e: Effect of SGK1 and SD-SGK1 on IgK promoter in FDCP-Mix cells. It shows that SGK1 activates NF- κ B by 1.5 fold compared to empty IRES2 vector, while SD-SGK1 (a constitutive form of SGK1) inhibits NF- κ B activity by nearly 2 fold. The graph shows the mean +/- SD of a single experiment performed in triplicate.

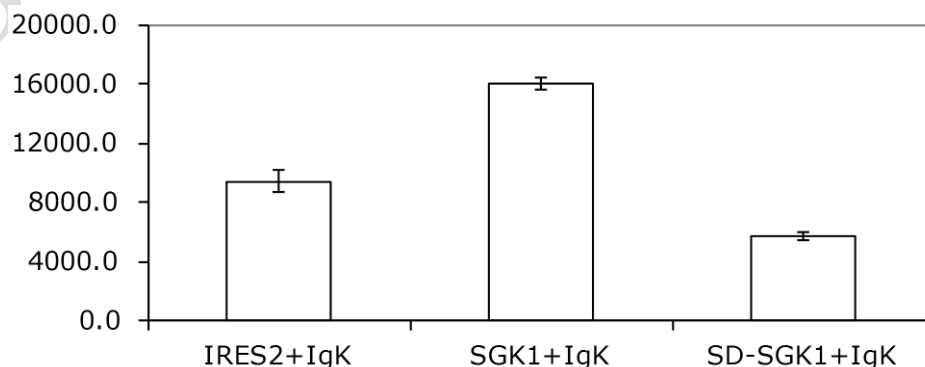


Figure 6a: Effect of DBP and AntiDBP on TRE promoter in FDCP-Mix cells. It shows that DBP transactivates TRE promoter more than 3 fold, while AntiDBP transactivates it more than 2 fold compared to GFP vector.

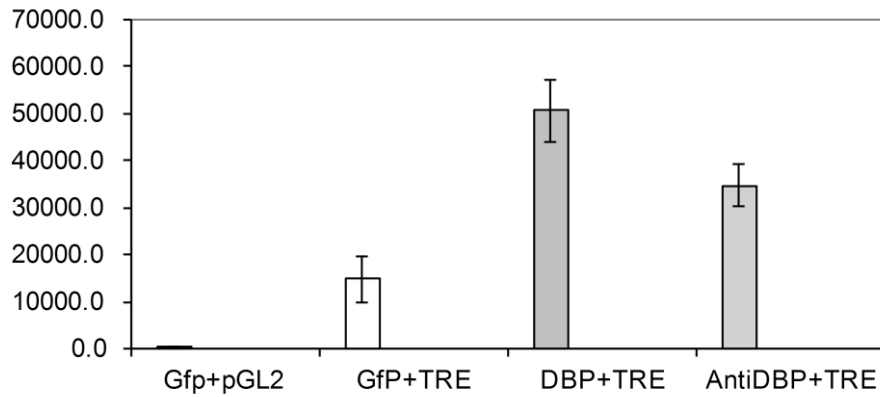


Figure 6b: Effect of PYK2 on TRE promoter in FDCP-Mix cells. It shows that when FDCP-Mix cells are transfected with either normal amounts (10µg) of hrGFP and PYK2 or equimolar concentration (16µg) of PYK2 construct, there is no detectable activation of TRE promoter. The data represent mean +/- SD of a single experiment performed in triplicate.

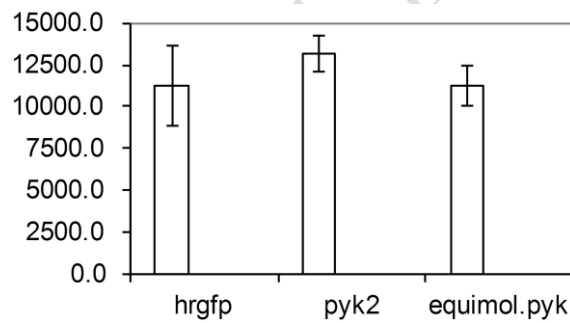


Figure 6c: Effect of SGK1 and SD-SGK1 on TRE promoter in FDCP-Mix cells. Neither transfection of SGK1 nor SD-SGK1 transactivate TRE promoter detectably. The data represent mean +/- SD of a single experiment performed in triplicate.

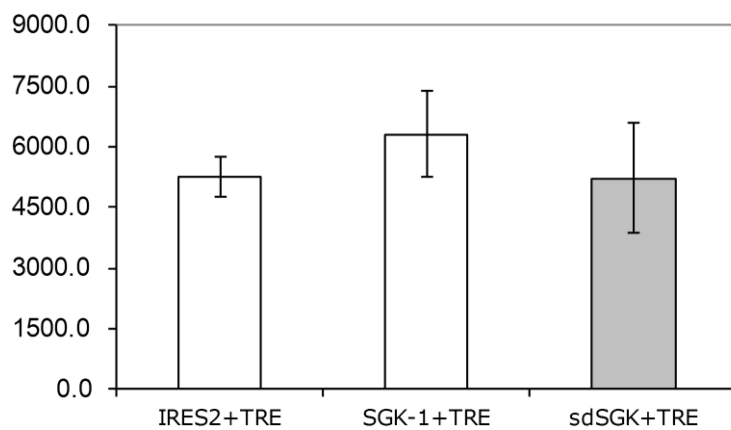


Figure 6d: Effect of AntiPYK2 on TRE promoter in FDCP-Mix cells. It shows that AntiPYK2 expression in FDCP-Mix cells reduces the basal TRE activity 3-4 fold. It also shows that basal TRE activity increases by 2 fold when cells are exposed to 100ng/ml PMA for 6hrs. Expression of AntiPYK2 also reduces the TRE activity mediated by PMA. The data represent mean +/- SD of a single experiment performed in triplicate.

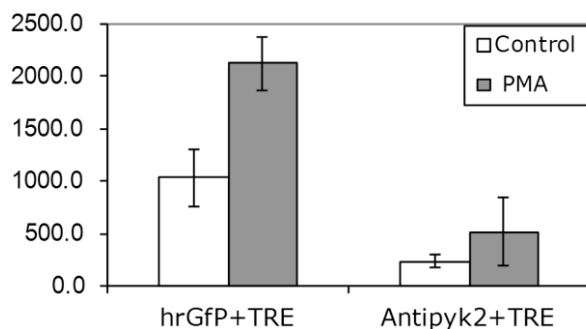


Figure 6e: DBP inhibits TRE activation mediated by PYK2 in 293 cells. It shows that GFP/PYK2 cotransfection leads to a 4-5 fold activation of TRE promoter compared to basal TRE activity (GFP+hr), while cotransfection of DBP with PYK2 reduces this activation to nearly 1.5 fold. The data represent mean +/- SD of a single experiment performed in triplicate.

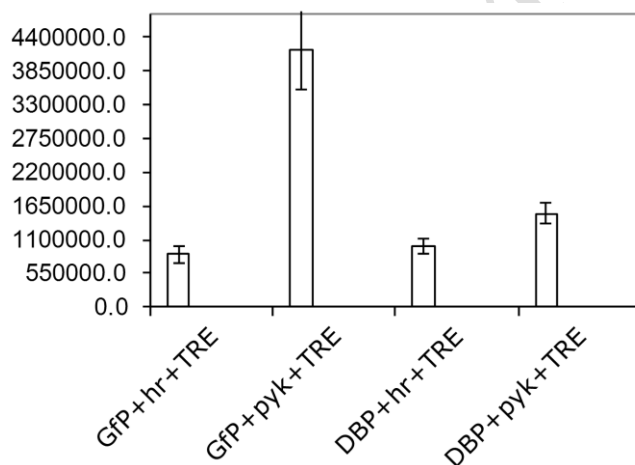


Figure 6f: Effect of cotransfection of DBP and SD-SGK1 on TRE promoter in 293 cells. When GFP is cotransfected with SD-SGK1 (SD), there is a 2 fold activation of TRE promoter compared to the basal TRE activity (GFP+IR+TRE). Cotransfection of DBP with SD-SGK1 does not inhibit the 2 fold TRE activation mediated by SD-SGK1. The data represent mean +/- SD of a single experiment performed in triplicate.

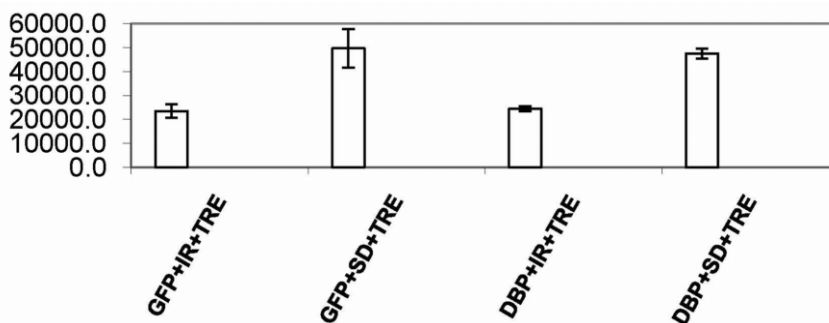


Figure 6g: DBP inhibits TRE activation mediated by PMA in 293 cells. It shows that PMA activates TRE more than 7 fold when GFP transfected cells are treated with 100ng/ml of PMA for 15hrs (30hrs post transfection) compared to the basal TRE activity. When DBP transfected cells are treated with PMA, there is 5 fold reduction in TRE activity. However, DBP alone inhibits the basal TRE activity 3 fold compared to the basal TRE activity seen with GFP.

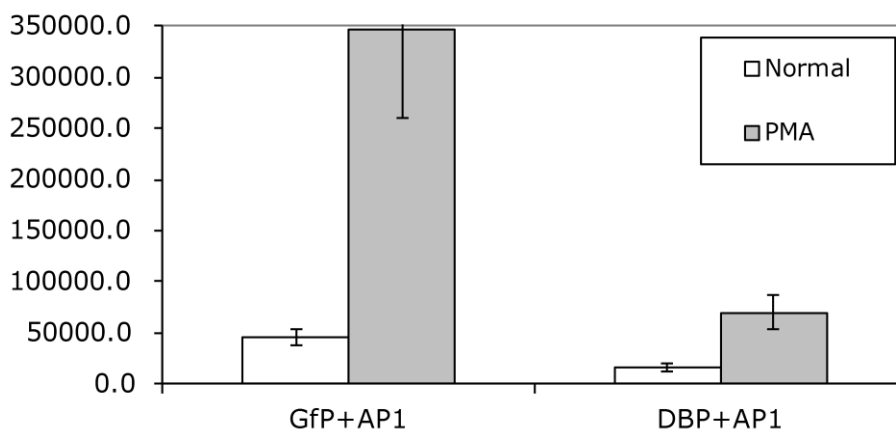


Figure 7a: Cotransfection of DBP with PYK2 activates HIF-1 under normoxia. Cotransfection of DBP with PYK2 activates HIF-1 reporter (EP) nearly 4 fold compared to the basal HIF-1 activity seen with the control GFP/hrGFP cotransfection. However, the cotransfection of GFP/PYK2 inhibits basal HIF-1 activity more than 3 fold. The data represent mean +/- SD of a single experiment performed in triplicate.

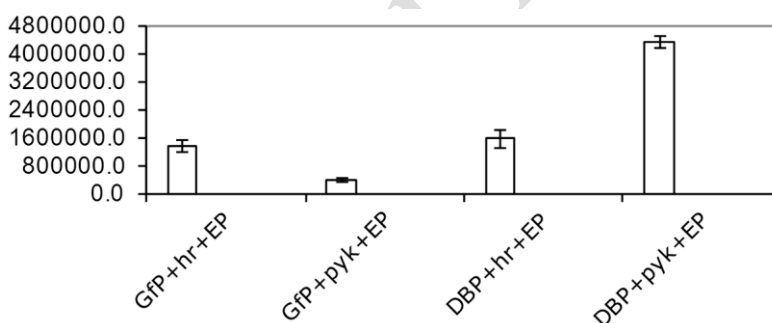


Figure 7b: Cotransfection of DBP with DYRK3 activates HIF-1 under normoxia in 293 cells. Cotransfection of DBP with DYRK3 activates HIF-1 reporter (EP) nearly 6 fold compared to the basal HIF-1 activity seen with GFP/hrGFP transfection. The cotransfections of GFP/DYRK3 and DBP/hrGFP activate HIF-1 by 1.5 and 2 fold respectively compared to the basal HIF-1 activity. The data represent mean +/- SD of a single experiment performed in triplicate.

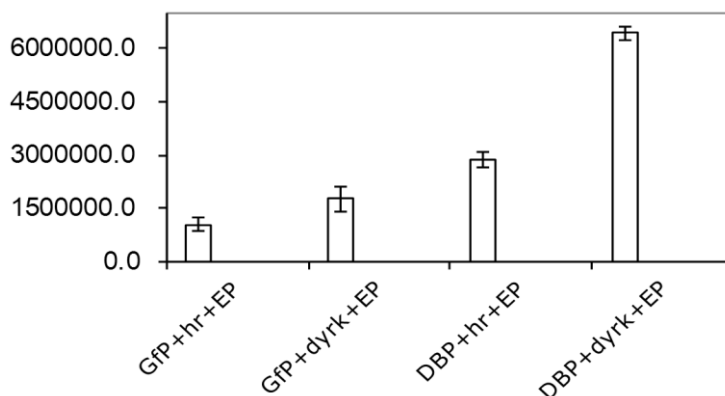


Figure 7c: Effect of PMA on HIF-1 activity mediated by DBP in 293 cells. The HIF-1 activity mediated by DBP/hrGFP is not affected by 75ng/ml of PMA when treated for 7hrs. The graph shows the mean +/- SE of a single experiment performed in triplicate.

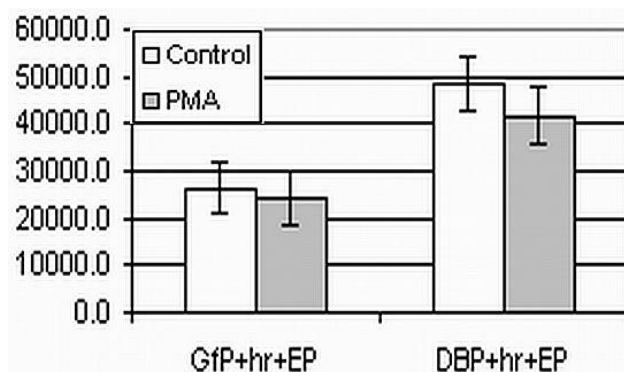


Figure 7d: Effect of PMA on HIF-1 activity mediated by DBP in 293 cells. When DBP/PYK2 cotransfected cells are exposed to PMA (75ng/ml) for 7hrs, the HIF-1 activity is reduced to near basal level of activity seen with GFP/hrGFP cells with or without PMA treatment. However, the inhibitory effect of PMA is much less pronounced in DBP/DYRK3 transfected cells. The graph shows mean +/- SD of a single experiment performed in triplicate.

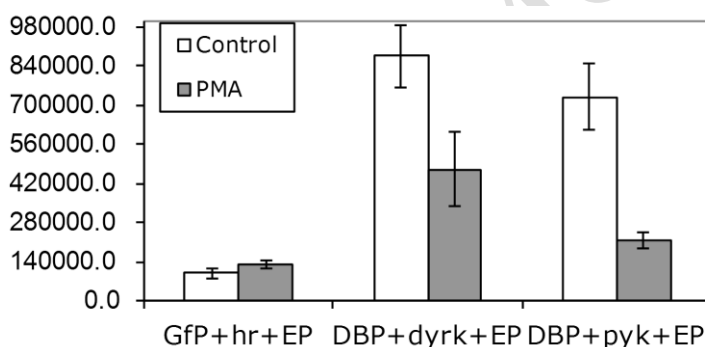


Figure 7e: Effect of DBP on HIF-1 activity under hypoxia in 293 cells. It shows that there is a dip in the HIF-1 activity mediated by DBP/ DYRK3 combination when 293 cells are exposed to hypoxia at 27hrs post-transfection for 9hrs (Hypo 36hrs) while exposing the similar DBP/DYRK3 transfected cells to hypoxia at 36hrs post-transfection for 9hrs (Hypo 45hrs) does not cause any reduction in HIF-1 activity. However, exposing DBP/PYK2 transfected cells to hypoxia at an earlier or later time point has no effect on HIF-1 activity.

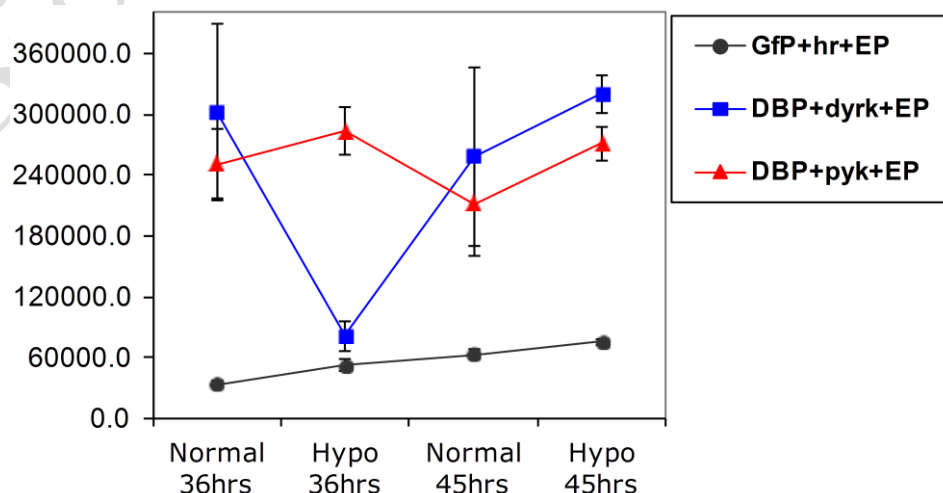


Figure 7f: Effect of DBP on HIF-1 activity under high cell density in 293 cells under normoxia. It shows that DBP inhibits HIF-1 activity nearly 6 fold compared to basal HIF-1 activity seen with GFP/hrGFP transfection. However, DBP/PYK2 and DBP/DYRK3 transfections do not activate HIF-1 under the same conditions.

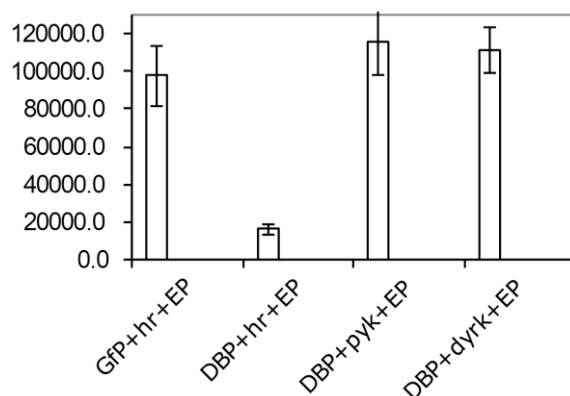


Figure 7g: Effect of media change on HIF-1 activity mediated by DBP. It shows that when confluent 293 cells are diluted by 2.5 fold with fresh DMEM media with 10% FCS, there is an activation of HIF-1 by DBP to nearly 3 fold compared to the basal HIF-1 activity seen with GFP/hrGFP, while DBP/PYK2 transfected cells inhibit HIF-1 by 5 fold. However, addition of fresh media has no effect on DBP/DYRK3 transfected cells.

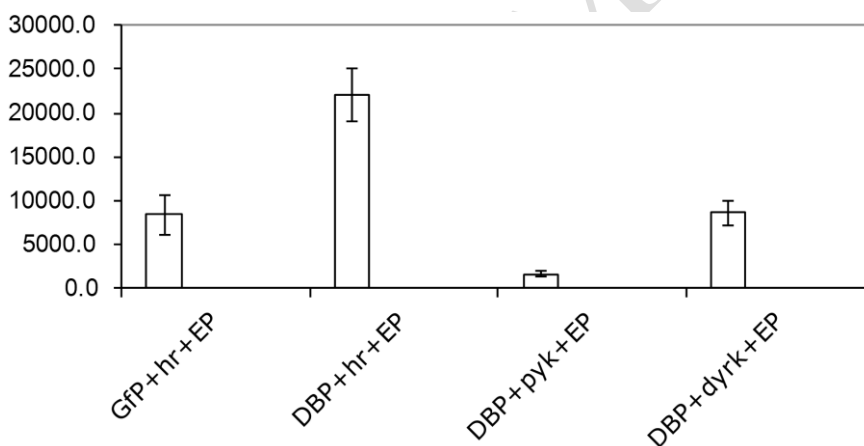


Figure 7h: Effect of LiCl on HIF-1 activity mediated by DBP in 293 cells under hypoxia. When DBP/DYRK3 cotransfected cells are exposed to 10mM LiCl, there is a 2 fold reduction in HIF-1 activity. However, LiCl and NaCl have no effect on basal HIF-1 activity seen with GFP/hrGFP transfected cells. The data represent mean +/- SD of a single experiment performed in triplicate.

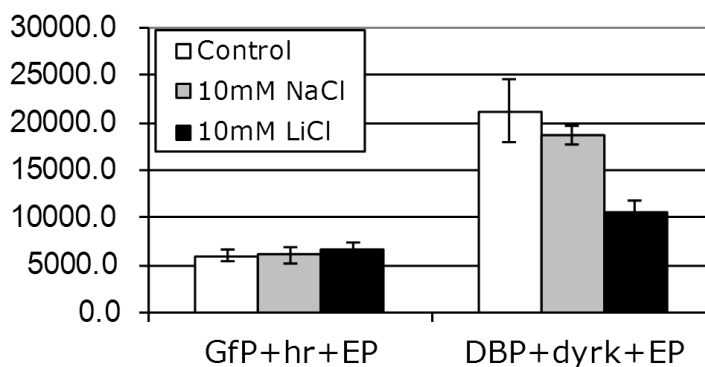


Figure 7i: Effect of elastase inhibitor on HIF-1 activity mediated by DBP in 293 cells under normoxia. Elastase inhibitor (N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone) at the concentration of 0.02mM reduces the HIF-1 activity mediated by DBP nearly 2 fold compared to the HIF-1 activity mediated by DBP in the absence of inhibitor. The results show mean +/- SD of a single experiment performed in triplicate.

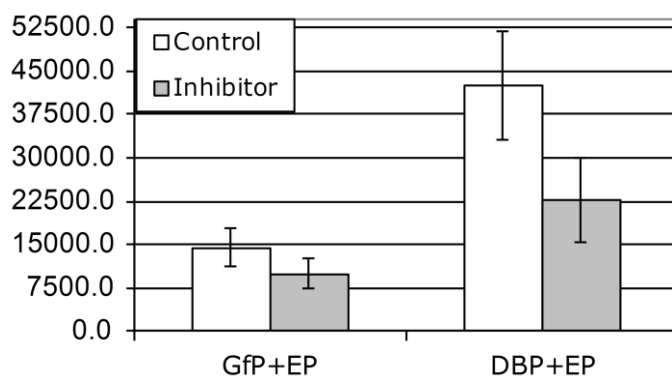


Figure 7j: Effect of PYK2 on HIF-1 activity in FDCP-Mix cells. FDCP-Mix cells transfected with PYK2 results in a 1.5 fold activation of HIF-1 reporter under normoxia with no further increase following exposure to hypoxia for 6hrs. When control hrGFP transfected cells are exposed to 500µM ATP for 6hrs, there is a 1.5 fold potentiation of HIF-1 activity under normoxia. The data represent mean +/- SD of a single experiment performed in triplicate.

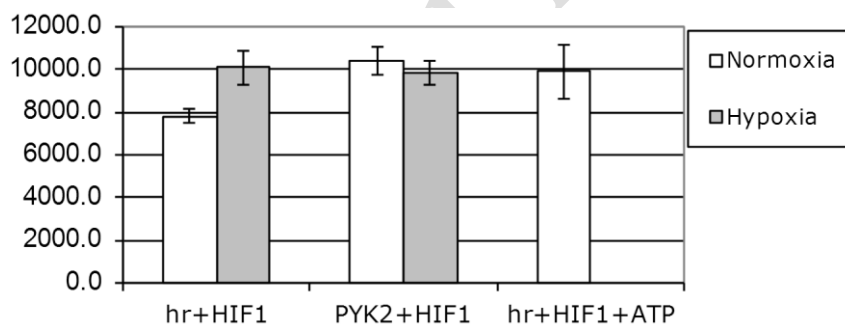


Figure 8a: Coactivation of c-fms promoter by PU.1 with PYK2. When FDCP-Mix cells are cotransfected with PU.1 alongwith PYK2, there is more than 1.5 fold activation of c-fms promoter compared to the c-fms promoter activity seen with PU.1/hrGFP transfected cells. The results represent mean +/- SD of a single experiment performed in triplicate.

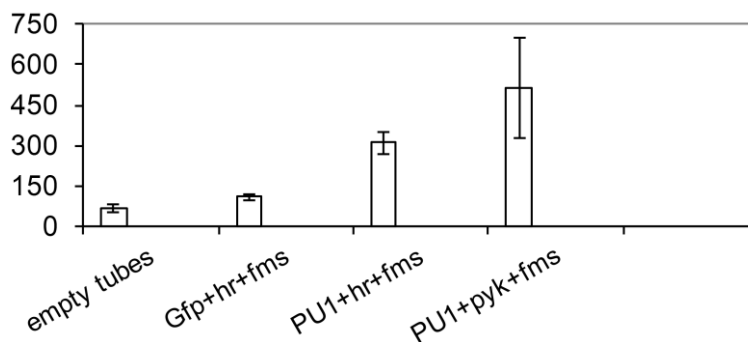


Figure 8b: Coactivation of c-fms promoter by PU.1 with SGK1 or SD-SGK1. When FDCP-Mix cells are cotransfected with PU.1 alongwith SGK1 or SD-SGK1, there is a 1.5 fold activation of c-fms promoter compared to the c-fms promoter activity seen with PU.1/ires2egfp transfected cells. The results represent mean +/- SD of a single experiment performed in triplicate.

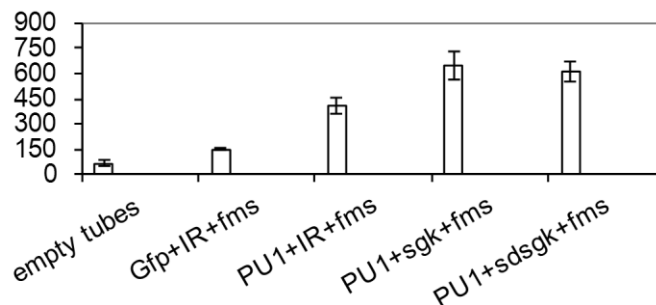


Table 1: Showing different types of biological rhythms and their characteristic period with an example.

Name of the rhythm	Period length	Example	References
Circadian	24 hr rhythm	Secretion of melatonin	Pevet et al., 2002
Ultradian	50-120 min or 20 hrs (less than 24hrs)	Secretion of insulin	Simon and Brandenberger, 2002
Infradian	3 days or 7 days, 21 days (longer than 24hrs)	Release of leuteinizing hormone	Lewy et al., 1999
Circalunar	28 days	cycles of salivary testosterone	Celec et al., 2002
Circannual/seasonal rhythms	1 yr or 12 yrs	Seasonal occurrence of cardiac arrest	Herlitz et al., 2002

Table 2: Showing the metabolic and nonmetabolic genes regulated by DBP.

Target Gene	Function	References
Factor IX	Blood clotting	Begbie et al., 1999
Factor VIII	Blood clotting	McGlynn et al., 1996
Phosphoenol-pyruvate carboxykinase (PEPCK)	Gluconeogenesis	Roesler et al., 1992
Aldolase B isoenzyme	Glycolysis	Gregori et al., 1993 Vallet et al., 1995
Alcohol dehydrogenase (class I)	Detoxication	van Ooij et al., 1992
Alpha (2,6) Sialyltransferase	Sialylation	Svensson et al., 1990; 1992
Cytochrome P450 steroid 15alpha-hydroxylase (Cyp2a4 and Cyp2a5)	Hydroxylation reactions (in the metabolic pathways of testosterone and estradiol)	Lavery et al., 1999
Cholesterol 7 alpha hydroxylase (C7alpha H)	Cholesterol metabolism	Lee et al., 1994
CYP2C6 (P450 enzyme)	Detoxication	Yano et al., 1992
CYP2E1 ((P450 enzyme)	Detoxication	Liu and Gonzalez, 1995
Serine protease inhibitor (SPI 2.1, 2.3)	Inhibition of serine proteases	Rossi et al., 1992
Angiotensinogen	Precursor of angiotensin II	Narayanan et al., 1998
Insulin-like growth factor binding protein-1 (IGFBP-1)	Regulation of Insulin-like Growth Factor (IGFs)	Babajko and Groyer, 1993
Albumin	Calcium chelation and osmotic control	Mueller et al., 1990

Table 3: It Shows the prominent metabolic features of HSCs.

Increase in the self-renewing potential of HSCs in response to hypoxia (Ivanovic et al., 2002).	High Proliferative Potential (HPP) with low proliferative activity (quiescence) is seen with low DNA content observed by using Hoechst dyes (Reddy et al., 1997)
Loss of glucose transporters is an early event of erythroid differentiation (Mathew et al., 1994; it may suggest a differential glucose metabolism in HSCs when viewed with the hypoxic effect)	Low mitochondrial activity observed with Rhodamine dye known as Rho low cells (Reddy et al., 1997)

Table 4: It shows some of the known cyclic hematopoietic processes and diseases.

Cyclic phenomena	Notes
Cyclic neutropenia (CN) Cyclic thrombocytopenia (CT) Polycythemia vera (PV) Chronic granulomatous disease (CGD)	Cyclic hematopoietic disorders known to harbour 21days cycle in humans and it is probably altered in these diseases (Haurie et al., 1998)
Circadian expression of human erythropoietin (Wide et al., 1989) and human granulocyte colony stimulating factor (hG-CSF, Jilma et al., 1999)	This may suggest that survival and proliferation of HSCs may be affected by the circulating concentrations of hematopoietic growth factors in the blood.
DNA synthesis in bone marrow is circadian stage dependent (Smaaland et al., 1991)	

Table 5: It shows the parental vectors/materials given by the researchers.

Name	Materials
Prof. Ueli Schibler, Geneva	pET-mDBP, Anti-DBP antibody
Dr. A. Israel, Paris	3X IgK cona luc (IgK)
Prof. Peter Herrlich, Jena	5X TRE TATA luc (TRE)
Prof. Ivan Dikic, Frankfurt	pRK5 hPYK2
Prof. Walter Becker, Aachen	pGEX-hDYRK3
Prof. Friedemann Horn, Leipzig	293 HEK cells and PMA
Prof. Ronald Wenger, Zurich	pGLHIF1.3 EPOwt (EP) and pGL3Tf 3xHBS (EP)
Prof. Florian Lang, Tuebingen	pSGK1-IRES2eGFP and pSD-SGK1-IRES2eGFP

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