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Endothelin-1 regulation of immediate early gene expression in cardiac myocytes: negative feedback regulation of interleukin 6 by Atf3 and Klf2.

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Introduction

Activation of intracellular signalling pathways by endothelin-1.

Cardiac myocytes are terminally-differentiated cells which withdraw from the cell cycle soon after birth. Subsequent growth results from an increase in size of individual myocytes (i.e. hypertrophy) rather than an increase in cell number. Various stimuli promote cardiac myocyte hypertrophy including peptide growth factors which signal through receptor protein tyrosine kinases (e.g. platelet-derived growth factor, PDGF (Clerk *et al.*, 2006)) and Gq protein-coupled receptor (GqPCR) agonists such as ▪-adrenergic agonists or endothelin-1

(ET-1) (Sugden and Clerk, 1998). These promote both morphological changes (increase in cell size and myofibrillar content) and changes in gene expression [e.g. increased expression of immediate early genes (IEGs) including c-*jun* or c-*fos*] associated with the hypertrophic response (Sugden and Clerk, 1998; Clerk *et al.*, 2007a).

In cardiac myocytes, ET-1 acts principally through ET_A receptors. These couple through heterotrimeric Gq proteins leading to activation of phospholipase C$ which promotes hydrolysis of phosphatidylinositol 4,5 *bis*phosphate to produce inositol 1,4,5 *tris*phosphate and diacylglycerol (DAG) (Clerk and Sugden, 1997). This leads to activation of DAG-responsive PKCs and, in cardiac myocytes, ET-1 particularly activates the novel PKC isoforms, nPKC* and nPKC, (Clerk *et al.*, 1994). ET-1 also stimulates small G proteins, with increased GTP-loading of Ras, Rac1 and RhoA (Chiloeches *et al.*, 1999; Clerk *et al.*, 2001; Brown *et al.*, 2006). All these events can be detected within the first 1 min after stimulation. Downstream of these very early signalling elements, mitogen-activated protein kinases (MAPKs) are activated and these include extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs) and p38-MAPKs (Bogoyevitch *et al.*, 1993; Bogoyevitch *et al.*, 1994; Bogoyevitch *et al.*, 1995; Clerk *et al.*, 1998). Activation of all MAPKs is associated with hypertrophic growth of cardiac myocytes, but evidence from transgenic mice particularly implicates ERK1/2 in beneficial (compensated) cardiac hypertrophy (Sugden and Clerk, 1998; Bueno and Molkentin, 2002). Other cells also possess ET_A receptors including fibroblasts and glioma cells. For example, in rat C6 glioblastoma cells as in cardiac myocytes, ET-1 activates phospholipase C$, PKC, and ERK1/2 leading to upregulation of c-*jun* and c-*fos* (Yin *et al.*, 9920; Ambar, Sokolovsky, 1993; Chen, 1993; Leach *et al.*, 1999). In these cells, ET-1 promotes DNA synthesis and cell cycle progression rather than hypertrophy, and ET-1 may promote tumorigenic states (MacCumber *et al.*, 1990;

Shichiri *et al.,* 1991; Clozel and Salloukh, 2005). The acute signalling events triggered by ET-1 presumably lead to the changes in gene/protein expression associated with cardiac myocyte hypertrophy or cell cycle progression in proliferating cells. However, activation of intracellular signalling elements such as PKC and ERK1/2 occurs rapidly and, at least in cardiac myocytes, this activation is transient. Thus, activation of ERK1/2 by ET-1 in cultured cardiac myocytes is maximal at ~5 min, returning almost to basal levels within ~30 min (Clerk *et al.,* 1994; Clerk *et al.,* 1996). How these early transient signals are propagated and lead to the phenotypic responses over many hours or even days is not often considered.

Regulation of gene expression by ET-1.

In any cellular response, activation of pre-existing transcription factors (TFs) downstream of intracellular signalling events leads to the increased expression IEGs. Because IEG expression is driven by pre-existing TFs, there is no requirement for *de novo* protein synthesis and the response is not inhibited by protein synthesis inhibitors such as cycloheximide. Some IEGs encode "structural" proteins such as ion channels or components of the cytoskeleton which directly influence the cellular phenotype. However, many IEGs constitute a "regulatory" component, encoding transcriptional regulators including TFs. At least some of the IEGs encode negative regulators of gene expression which feed back to limit the IEG response, whereas other combinations of TFs regulate downstream, second-phase gene expression. Since second phase genes require synthesis of IEG proteins, this phase of the response is inhibited by cycloheximide. In contrast, preventing the expression of a negative feedback protein by cycloheximide results in enhanced expression of IEG mRNAs. Potentially, second phase TFs regulate expression of third-phase genes further downstream, etc. (see Fig. 1 and (Clerk *et al.,* 2007a)). As the response progresses, the

proportion of "regulatory" to "structural" proteins declines as the new steady-state phenotype develops. Throughout this time, cells remain responsive to the environment although the response is probably modulated by changing expression of cell surface receptors and intracellular signalling components. The cells also influence the environment by producing extracellular matrix components and secreted factors which signal to other cells in the vicinity.

IEGs (e.g. c-*fos*, c-*jun*, *egr1*) are upregulated in cardiac myocyte hypertrophy (Dorn *et al.*, 2003; Sugden and Clerk, 1998), and it is assumed that they regulate expression of hypertrophic genes including classical markers of hypertrophy (e.g. atrial natriuretic factor, ANF). However, our knowledge has previously been limited to a few established IEGs and it is not clear how the expression of these genes/proteins leads hypertrophy. To start to clarify these issues, we have been using microarrays to determine the changes in gene expression induced by ET-1 in primary cultures of neonatal rat ventricular cardiac myocytes (NRVMs) over the first 4 h (as the initial phases of the response develop) and to distinguish IEGs from downstream gene expression (Cullingford *et al.*, 2008b). Our data demonstrate a multiphasic response consistent with the model described above and in Fig. 1. Early IEGs are induced within 30 min, with later IEGs appearing at 1 - 2 h. Surprisingly, the earliest of the second phase genes are detected within 1 h. At these early times, many of the genes are associated with transcriptional regulation or with intercellular or intracellular signalling and are not classically associated with hypertrophy. This indicates that this phase is an intermediate stage in the response. Several families of TFs were subject to regulation by ET-1 within the first hour, and these may be "master-switches" for downstream gene regulation. For example, there are significant changes in expression of many of the Krüppel-like factors (Klfs), suggesting that Klfs play a major role in the response (Cullingford *et al.*, 2008a;

Cullingford *et al.,* 2008b). One example of a potential feedback inhibitor of IEG expression is activating transcription factor 3 (Atf3) which is implicated in downregulation of expression of interleukin 6 (IL6) in the context of endotoxin-induced inflammation (Gilchrist *et al.,* 2006). Here, we present data for the regulation of three IEGs which are responsive to ET-1 in cardiac myocytes: Atf3, Klf2 and IL6.

Materials and methods

Preparation of neonatal cardiac myocytes and adenoviral infection

Myocytes were dissociated from the ventricles of neonatal Sprague-Dawley rat hearts using collagenase and pancreatin as previously described (Iwaki *et al.,* 1990; Bogoyevitch *et al.,* 1995). Non-myocytes were removed by pre-plating onto uncoated tissue culture dishes (30 min, 37/C). The (non-adherent) cardiac myocytes were collected and plated in serum-containing medium at a density of 1.4×10^3 cells/mm^2 on Primaria tissue culture dishes precoated with 1% (w/v) gelatin. After 18 h, serum was withdrawn for 24 h prior to use. Agonists were added directly to the tissue culture medium. For adenoviral infection, cardiac myocytes were serum-starved for 4 h before adding 200 :l of virus stock in PBS to the medium. Cardiac myocytes were incubated for a further 48 h before experimentation.

Semi-quantitative and quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was prepared and semi-quantitative RT-PCR performed as previously described (Clerk *et al.,* 2007b), using 100 pmol forward and reverse primers as follows: Atf3 (accession no. NM_012912): forward 5'- GCTGCCAAGTGTCGAAACAAG -3', reverse

5'-CAGTTTTCCAATGGCTTCAGG-3', 388 bp product; IL6 (accession no. NM_012589) : forward 5'-CCGGAGAGGAGACTTCACAG-3', reverse 5'-GAGCATTGGAAGTTGGGGTA -3', 428 bp product; glyceraldehyde 3' phosphate dehydrogenase (Gapdh): forward 5'-ACCACAGTCCATGCCATCAC-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3', 452 bp product). For Gapdh, samples were subjected to 21 cycles of denaturation (94/C, 30 s), annealing (59/C, 30 s) and extension (72/C, 30 s). For Atf3 and IL6, samples were subjected to 27 cycles of amplification. Bands were detected under UV light and sizes were estimated by comparison to a **N**X174 RF DNA *Hae* III digest DNA ladder (ABgene) or a 100 bp ladder (Invitrogen). Densitometric analysis was performed using Imagemaster 1D Prime, version 3.0 (GE Healthcare).

Quantitative PCR (QPCR) was performed using a 7500 Real-Time PCR System (Applied Biosystems). A master-mix containing (per reaction) 12.5 :l Sybr-Green Jump Start Taq Readymix (Sigma-Aldrich) and 5 :l oligonucleotides (5 pmol each of forward and reverse primers) was aliquoted into Optical 96-well reaction-plates (Applied Biosystems), and the cDNA template added (7.5 :l, 1/15 dilution in Milli-Q filtered water). PCR conditions for all primer pairs were 50/C for 2 min, 95/C for 10 min, followed by 40 cycles of 95/C for 15 s and 59/C for 60 s. Following QPCR, a dissociation curve analysis was performed to check for aberrant amplification products. QPCR analysis of Gapdh was performed in each 96-well plate as an endogenous control and the relative quantitation protocol was used. Primers for QPCR were as follows: Atf3 (accession no. NM_012912): forward 5'-GAGCGAAGACTGGAGCAAAA-3', reverse 5'-AAGGTGCTTGTTCTGGATGG-3', 181 bp product; IL6 (accession no. NM_012589) : forward 5'-CCGGAGAGGAGACTTCACAG -3', reverse 5'-CAGAATTGCCATTGCACAAC-3', 136 bp product; epiregulin (Ereg; accession no.

NM_021689): forward 5'-ACTGCAGATGTGAAGTGGGC-3', reverse 5'-GAGGAAAACGAGAATCACGG-3', 113 bp product); leukaemia inhibitory factor (Lif; accession no. NM_022196): forward 5'-TCAACTGGCTCAACTCAACG-3', reverse 5'-ACCATCCGATACAGCTCGAC -3', 128 bp product); c-Jun (accession no. NM_021835): forward 5'-GATCATCCAGTCCAGCAATG-3', reverse 5'-TATTCTGGCTATGCAGTTCAG -3', 140 bp product).

Western blotting

Cardiac myocyte nuclear extracts were prepared and immunoblotted as described previously (Markou *et al.*, 2008). Blots were probed with primary rabbit polyclonal antibodies specific for Atf3 (Santa Cruz Biotechnology Inc., Cat. no. sc-188; 1/1000 dilution) or Atf2 (Santa Cruz Biotechnology Inc., Cat. no. sc-187; 1/1000 dilution), followed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (DakoCytomation; 1/5000 dilution). Immunoreactive bands were detected by enhanced chemiluminescence (Santa-Cruz Biotechnology Inc.) and the bands analysed by semi-quantitative scanning densitometry (Imagemaster 1D Prime version 3.0).

Chromatin immunoprecipitation (ChIP)

Following stimulation, cardiac myocytes (16×10^6 cells per sample) were fixed in formaldehyde [1 % (v/v) final concentration, 10 min]. The reaction was terminated by addition of glycine (125 mM final concentration, 10 min). Cells were washed with ice-cold PBS, scraped into PBS containing protease and phosphatase inhibitors [0.2 mM leupeptin, 0.01 mM trans-epoxy-succinyl-L-leucylamido-(4-guanidino)-butane (E64), 5 mM dithiothreitol, 0.3 mM phenylmethylsulphonyl fluoride, 0.002 mM microcystin] and collected

by centrifugation (3,000 g, 4/C, 5 min). Cells were lysed in 0.5 ml Buffer A [50 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% (v/v) NP40, 10% (v/v) glycerol] supplemented with protease and phosphatase inhibitors (15 min). Nuclei were pelleted (3,000 g, 4/C, 5 min), resuspended in 200 :l Buffer B [50 mM Tris-HCl pH 8.0, 1% (w/v) SDS, 5 mM EDTA] and the DNA was sheared into 200-800 bp fragments by sonication [5 × 30 sec, 4/C, amplitude 30% (Sonics Vibra-Cell™ sonicator with a 2 mm probe) followed by 2 min recovery in ice-water]. Following centrifugation (4,000 g, 4/C, 5 min), the supernatants were taken. A volume (35 :l) was reserved to assess the input and the remaining sample diluted 1/10 in RIPA buffer [50 mM Tris-HCl pH 8.0, 0.5% (v/v) NP40, 200 mM NaCl, 0.5 mM EDTA]. Samples were precleared with protein A-Sepharose (20 :l of 50% (v/v) suspension in RIPA buffer; 15 min) then incubated without (controls) or with 10 :g Atf3 rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc., Cat. no. sc-188X) with rotation (4/C, overnight). Protein A-Sepharose was added [100 :l; 50% (v/v) suspension in RIPA buffer containing 1 :g/ml sonicated salmon sperm DNA] and samples incubated with rotation (4/C, 2 h). The beads were pelleted by centrifugation (1,000 × g, 4/C, 3 min) and washed (4/C, 3 min, with gentle mixing by rotation) in 1 ml high salt buffer [20 mM Tris-HCl pH 8.0, 0.1% (w/v) SDS, 1% (v/v) NP40, 2 mM EDTA, 500 mM NaCl] followed by 10 mM Tris-HCl pH 8.0 containing 1 mM EDTA. Immune complexes were eluted (10 min, 65/C) in 250 :l elution buffer [10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% (w/v) SDS], samples were centrifuged (200 g, 1 min) and supernatants collected. The elution procedure was repeated and the supernatants pooled. Crosslinks in input and immunoprecipitated samples (500 :l) were reversed by incubation (65/C, overnight) with NaCl (0.2 M final concentration). Samples were incubated (5 min, 4/C) with 500 :l phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) and the phases were separated by centrifugation (15,300 g, 4/C, 10 min). The upper

aqueous phases were retained and DNA precipitated by incubating with 500 :l isopropanol (-80/C, 3 h). Following recovery by centrifugation (15,300 g, 10 min, 4°C), DNA was washed [70% (v/v) ethanol] and resuspended in 25 :l Milli-Q water for subsequent PCR. PCR reactions (2 :l DNA in 20 :l final volume) were performed in 1 × Buffer IV® (AB Gene, 25 :l) containing Taq polymerase (1 U), dATP, dCTP, dGTP and dTTP (0.2 mM each) and 50 pmol primers. Promoter-specific primers for rat IL6 were: forward, 5'-TGCTCAAGTGCTGAGTCACT-3'; reverse 5'-AGACTCATGGGAAAATCCCA-3'). PCR amplification conditions (32 cycles) were: denaturing, 95/C, 45 s; annealing, 52/C, 45 s; extension 72/C, 1 min. The resulting RT-PCR products were analysed by ethidium bromide-agarose gel electrophoresis and the bands captured under UV illumination.

Production and preparation of adenoviruses

Replication-deficient adenoviruses expressing full-length rat Atf3 antisense RNA (AS-Atf3) or shRNA for Klf2 were prepared using the AdEasy™ XL Adenoviral Vector System (Stratagene). For Atf3, the coding sequence was isolated by PCR from rat cDNA using Pfu polymerase and primers designed to the 5' ATG start site and 3' stop codon regions. Primers were designed to include sites for restriction enzymes for insertion into the multiple cloning site of the pShuttle-CMV vector. Control samples were prepared with empty vector. For Klf2 knockdown by siRNA, pShuttle vectors were prepared containing shRNA-generating U6 promoter and annealed oligodeoxynucleotides for Klf2 shRNA. A 19 base sequence for an siRNA-generating construct against Klf2 (5'-AGACCTACACCAAGAGTTC-3') was selected using the siRNA Target Finder and Design tool (www.ambion.com/techlib/misc/siRNA_finder.html), siDESIGN center (http://www.dharmacon.com/designcenter/DesignCenterPage.aspx), and based on Lim *et al.*,

2005. A random control sequence was also used (5'-ATGAACGTGAATTGCTCAA-3'; targeted to luciferase (Dekker *et al.*, 2005)). Complementary oligodeoxynucleotide pairs were synthesized (Integrated DNA Technologies) containing the sense sequence, a loop linker sequence and antisense sequence. Flanking sequences were included to generate BamH1 and HindIII restriction endonuclease overhangs on pairing for cloning into the pShuttle vector with the U6 promoter. Oligodeoxynucleotides (1 nmol each) were annealed in annealing buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$; 20 :l final volume) by heating to 95/C (5 min) then cooling to room temperature over 4 h. Following ligation into pShuttle, sequences and orientation were confirmed. The pShuttle plasmids were linearized and transformed into BJ5183-AD-1 competent cells for homologous recombination between the shuttle vector and pAdEasy-1. Recombinant pAdEasy-1 was produced in bulk in a recombination-deficient bacterial cell line (e.g. XL10-Gold®). Purified recombinant pAdEasy-1 plasmid DNA was linearised and transfected into HEK293 cells to generate recombinant adenoviruses. After 7 - 10 days, cells were collected by centrifugation (600g, 5 min) and washed (10 ml PBS), then resuspended in 2 ml of PBS and subjected to freeze-thaw cycles. Following centrifugation (600 g, 5 min), virus-containing supernatants were collected.

Results and Discussion

Identification of candidate IEG mRNAs likely to be subject to negative feedback regulation by IEG proteins.

We previously identified 45 IEG mRNAs upregulated in cardiac myocytes by ET-1

(100 nM) within 30 min of stimulation (i.e. early IEGs) (Cullingford *et al.*, 2008b). To identify IEG mRNAs which may be under negative feedback control by other IEG proteins, we selected mRNAs which were maximally upregulated at 0.5 - 1 h but whose expression decreased by 2 h (Table 1). Since cycloheximide inhibits the translation of negative feedback IEG proteins, any IEG mRNAs which are subject to negative feedback by these proteins should exhibit enhanced expression in the presence of cycloheximide, particularly as the response decays (i.e. at 2 h). For 22 of the 31 mRNAs selected, the increase in expression induced by ET-1 was further enhanced by cycloheximide (20 μM) by at least 5-fold, with an enhancement of >10-fold for activity regulated cytoskeletal-associated protein (Arc), Atf3, cholesterol 25-hydroxylase (Ch25h), early growth response 2 (Egr2), epiregulin (Ereg), c-Fos, immediate early response 2 (Ier2), IL6, c-Jun, Krüppel-like factor 2 (Klf2) and Lif (Fig. 2). Of these, Atf3 negatively regulates its own expression (Wolfgang *et al.*, 2000) and suppresses expression of IL6 induced in macrophages by lipopolysaccharide (Gilchrist *et al.*, 2006). Klf2 is also implicated in negative regulation of IEG expression (Amit *et al.*, 2007). We therefore focused on the potential role of Atf3 and Klf2 in termination of IEG expression.

Fig. 2

Table 1.

Early IEGs upregulated by ET-1 in cardiac myocytes. Data from Cullingford et al. 2008b were mined to identify early IEGs whose mRNAs were upregulated within 0.5 h of stimulation with ET-1 (100 nM), with maximal increases in expression at 0.5 - 1 h and significant decreases in expression by 2 h. Data were collated to present the fold stimulation (relative to unstimulated controls) for cardiac myocytes exposed to ET-1 for 0.5, 1 or 2 h, or to ET-1 for 1 or 2 h in the presence of 20 μM cycloheximide (CX). The increase in expression of Group A mRNAs by ET-1 at 2 h was enhanced >10-fold by cycloheximide. Group B mRNAs exhibited a lesser

enhancement. Within each group, IEGs are listed alphabetically. Results are means for n = 3 (ET-1 0.5 h; CX+ET-1, 1 or 2 h), n=6 (ET-1 2 h) or n=8 (ET-1, 1 h).

Gene symbol	ET-1 0.5 h	ET-1 1 h	ET-1 2 h	CX+ET-1 1 h	CX+ET-1 2 h
Group A					
Arc	7.4	5.4	1.7	18.8	19.7
Atf3	4.0	10.6	2.8	24.7	36.6
Ch25h	8.5	9.2	1.3	18.6	23.1
Egr2	12.7	8.5	1.4	25.2	42.1
Ereg	2.0	3.1	1.3	5.1	15.4
c-Fos	27.9	9.8	1.3	52.2	67.4
Ier2	5.3	3.4	1.7	23.2	29.4
Il6	4.6	5.6	2.4	22.1	53.4
c-Jun	2.8	2.6	1.2	15.0	20.6
Klf2	3.5	3.2	1.0	8.7	13.3
Lif	3.8	4.9	2.6	12.0	26.2
Group B					
Btg2	4.7	6.1	2.8	13.4	15.7
Cyr61	4.2	5.9	3.7	19.7	15.7
Dusp1	4.0	3.1	2.4	12.0	16.5
Egr1	4.6	4.5	1.0	6.1	7.6
Has2	2.5	7.5	2.4	5.4	9.8
Junb	4.1	1.7	1.9	9.3	8.9
Klf4	2.0	3.6	2.7	5.1	6.4
Klf6	2.4	4.2	2.1	7.5	11.8
Nfil3	2.2	3.4	2.9	5.0	9.4
Nfkbiz	2.3	3.0	2.3	10.7	17.7
Nr4a1	13.6	17.5	3.7	22.4	28.9
Nr4a2	3.7	5.1	1.4	10.4	13.5
Nr4a3	8.4	26.0	13.8	43.3	65.5
Phlda1	2.6	6.2	4.1	5.2	10.9
Plk2	2.4	3.5	1.9	6.3	6.9
Ptgs2	5.1	10.6	3.4	19.3	31.1
Rgs2	2.9	5.1	3.5	4.3	6.1
Slc25a25	3.3	3.4	1.9	7.0	15.7
Tnfaip3	2.5	2.4	1.6	7.1	14.0
Zfp36	5.8	2.8	2.3	14.0	16.8

Regulation of expression of Atf3 and IL6 in cardiac myocytes.

Fig. 3

We validated the microarray data for Atf3 and IL6 using semi-quantitative RT-PCR. ET-

1 (100 nM) promoted a very rapid (within 15 min) and potent increase in Atf3 mRNA expression in cardiac myocytes, with maximal expression at ~0.5 h (Fig. 3A). An increase in protein expression was detected within 1 h of stimulation (data not shown). In a previous microarray study, we also detected simultaneous increases in expression of Atf3 and IL6 in cardiac myocytes by H_2O_2 (as an oxidative stress) (Clerk *et al.*, 2007c). The upregulation of Atf3 in response to 0.2 mM H_2O_2 (Fig. 3B) was delayed relative to that induced by ET-1, with maximal expression of the mRNA at 1 - 2 h and maximal protein expression at ~2 h (data not shown). IL6 mRNA expression was rapidly increased by either ET-1 (Fig. 3C) or H_2O_2 (Fig. 3D) with similar kinetics for upregulation as Atf3 (Fig. 3, A and B). However, downregulation of IL6 mRNA appeared to be more rapid than that of Atf3 mRNA with almost complete attenuation of the response to ET-1 within 1 h.

To determine whether the increase in Atf3 expression induced by ET-1 downregulates the increase in expression of IL6 in cardiac myocytes, cardiac myocytes were infected with adenoviruses encoding full-length antisense RNA to Atf3 to prevent the increase in expression of Atf3 mRNA. With this system, we obtained ~70% inhibition of the increase in Atf3 protein induced by ET-1 (Fig. 4A). The knockdown of Atf3 was specific since there was no effect on expression of the related transcription factor Atf2 (Fig. 4A). Consistent with the proposed role for Atf3 in inhibiting IL6 mRNA expression, knockdown of Atf3 protein by antisense RNA caused superinduction of IL6 mRNA expression by ET-1 (Fig. 4B). We hypothesized that other IEG mRNAs may be subject to regulation by Atf3 in a similar manner as IL6. Of the IEGs we identified (Table 1), Ereg and Lif showed the most similar pattern to IL6 for increased expression by ET-1 and enhancement of expression by cycloheximide (Fig. 2). Interestingly, of the genes tested, Atf3 antisense RNA enhanced the increase in expression of Ereg (Fig. 4C) and Lif (Fig. 4D), but not c-Jun (Fig. 4E), c-Fos or Ier2 (data not shown).

Fig. 4

An Atf3 consensus sequence has been identified in the mouse IL6 promoter (Gilchrist *et al.*, 2006). A similar sequence is present in the rat IL6 promoter (Fig. 5, A and B). To determine whether Atf3 may exert a direct negative regulatory effect on IL6 transcription by binding to this sequence, we used chromatin immunoprecipitation (ChIP) with antibodies to Atf3 to isolate DNA fragments associated with the protein and performed RT-PCR across the putative binding site in the IL6 promoter (Fig. 5B). ET-1 did indeed increase the association of Atf3 with the IL6 promoter at 1 h (Fig. 5C), indicating that Atf3 acts directly on the IL6 promoter to downregulate its mRNA expression.

Fig. 5

Regulation of Klf2 and its potential role in downregulating IL6 expression.

Of the other IEGs in our selected group, Klf2 also negatively regulates IEG mRNA expression (Amit *et al.*, 2007). Like Atf3, expression of Klf2 mRNA is detected very rapidly (within 15 min) with maximal expression at ~30 min and expression levels decline thereafter (Cullingford *et al.*, 2008a). To investigate the role of Klf2 in downstream signalling, cardiac myocytes were infected with adenoviruses encoding shRNA for siRNA knockdown of Klf2 expression prior to stimulation with ET-1. We obtained ~56% inhibition of Klf2 mRNA expression in the absence of any effect on the related protein Klf6 (Fig. 6A). Interestingly, knockdown of Klf2 enhanced the increase in expression of IL6 (Fig. 6B) or Ereg (data not shown) induced by ET-1 in the absence of any effect on Ptgs2 (cyclooxgenase 2). Both IL6 (Fig. 5A) and Ereg promoter regions contain consensus binding sites for Klfs. It remains to be determined whether Klf2 directly binds to the promoters to inhibit expression of IL6 or Ereg, or if the effect may be indirect.

Fig. 6

The role of negative feedback on IL6 expression in modulating cardiac function.

IL6 family cytokines (which includes cardiotrophin-1 and Lif) stimulate gp130 receptors (Müller-Newen, 2003) to activate JAK/STAT signalling and signalling through the ERK5 cascade (Heinrich *et al.*, 2003). Cardiotrophin 1 or Lif act via gp130 receptors to promote a form of cardiac myocyte hypertrophy in which sarcomeres are laid down in series rather than in parallel, resulting in myocyte elongation (Wollert *et al.*, 1996; Nicol *et al.*, 2001). However, serum levels of IL6 are elevated in pathological hypertrophy, and high, sustained expression of IL6 may contribute to the development of heart failure (Mann, 2003). It is therefore possible that part of the response induced by ET-1 may be mediated by IL6. However, limiting its production is probably necessary to prevent the deterioration of cardiac function associated with high level, sustained production of IL6. Thus, negative feedback modulators such as Atf3 and Klf2 are potentially key components of a system designed to facilitate the beneficial aspects of cardiac myocyte hypertrophy. Dysregulation of these systems may contribute to the development of heart failure.

Summary

Overall, our studies are consistent with Atf3 acting directly on the IL6 promoter to inhibit further transcription of IL6 mRNA following stimulation with ET-1, given that Atf3 protein binds directly to the promoter (Fig. 5) and knockdown of the protein enhances IL6 mRNA expression (Fig. 4). Klf2 also appears to operate in a negative feedback system to limit IL6 mRNA expression since its knockdown also enhances IL6 mRNA expression (Fig. 6), although further studies are required to determine whether its effects are directly mediated at the level of the IL6 promoter. Interestingly, Ereg appears to be co-regulated with IL6 with a similar pattern of expression in response to ET-1 and similar enhancement of expression by cycloheximide (Fig. 2), Atf3 knockdown (Fig. 4) or Klf2 knockdown (Fig. 5). However, for both IL6 and Ereg, other

factors may further contribute to the downregulation of mRNA expression following the increase induced by ET-1, possibly by increasing its rate of degradation. It is interesting that ET-1 also increased expression of Zfp36 (Table 1) which is associated with regulation of mRNA stability (Carrick *et al.*, 2004). This and other factors may be required to limit the expression of IL6, Ereg and other selected IEGs responding to stimuli such as ET-1, and ensure that they are expressed only transiently. Understanding how these early changes associated with intercellular or intracellular signalling and gene/protein expression relate to the changing environment of a cell will help us understand how a complex phenotypic response such as cardiac hypertrophy may develop *in vivo* and how such an initially beneficial response may deteriorate into (for the heart) cardiac failure.

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Legends for figures

FIG. 1. Model of regulation of gene expression. Various stimuli regulate the phosphorylation status of pre-existing proteins leading to transcription of IEGs and changes in mRNA stability/translation. IEG proteins may be "structural" (directly influencing cell function) or "regulatory" encoding further transcription/ translation factors. These feed back negatively to terminate IEG expression, and feed forward positively to drive second phase gene expression. Second phase genes encode further "structural" and "regulatory" proteins to modulate third phase genes etc. The cells continually adjust their responsiveness with different cell surface receptors. Cells also modulate their environment through secretion of extracellular matrix components and autocrine paracrine factors. Further stimuli feed into the cell to modulate the functional changes until eventually a new steady state is reached.

FIG 2. Upregulation of immediate early genes in cardiac myocytes by ET-1. Cardiac myocytes were unstimulated or exposed to 100 nM ET-1 for 0.5, 1 or 2 h in the absence (solid circles, solid lines) or presence (open circles, dashed lines) of 20 ：M cycloheximide and RNA expression assessed globally using Affymetrix rat genome 230 2.0 microarrays. The data were mined from Cullingford *et al.*, 2008b to identify IEGs with maximal expression at 0.5 - 1 h, a decline in expression by 2 h and whose expression at 2 h was enhanced >10-fold by cycloheximide. Results are expressed relative to unstimulated controls and are means for n=3 (0.5 h), n=8 (1 h) or n=6 (2 h) independent myocyte preparations.

FIG. 3. Regulation of expression of Atf3 and IL6 mRNA by ET-1 or H_2O_2 in cardiac myocytes. Neonatal cardiac myocytes were exposed to 100 nM ET-1 (A and C) or 0.2 mM H_2O_2 (B and D) for the times indicated and RNA prepared. Expression of Atf3 mRNA (A and B) or IL6 mRNA

C and D) was assessed by semi-quantitative RT-PCR. Representative images from a single experiment are shown for Atf3 or IL6 mRNA in the upper panels with images for the corresponding Gapdh mRNA in the centre panels. Densitometric analysis of the data are presented in the lower panels and are means ± SEM for n=3 (H_2O_2) or n=4 (ET-1) independent myocyte preparations. Control PCR reactions were carried out in the absence of reverse transcriptase (- RT). #$p<0.01$, * $p<0.001$ relative to unstimulated controls, one way ANOVA with Tukey post-hoc test.

FIG. 4. Knockdown of Atf3 expression enhances the increase in expression of IL6, Ereg and Lif (but not c-Jun) by ET-1. Cardiac myocytes were infected with "empty vector" adenoviruses as controls or with adenoviruses encoding full-length antisense RNA to Atf3 (AS-Atf3), then unstimulated or exposed to 10 nM ET-1 for 1 h. A, Cardiac myocyte extracts were immunoblotted with antibodies to Atf3 or Atf2. Representative images are shown in the left panel (Atf3, upper image; Atf2, lower image) with densitometric analysis of Atf3 expression in the right panel. Results are means ± SEM for n=3 independent myocyte preparations. B-D, RNA was prepared and expression of IL6 (B), Ereg (C), Lif (D) or c-Jun (E) mRNAs analysed by QPCR. Results are expressed relative to unstimulated cardiac myocytes infected with empty vector viruses and are means ± SEM for n=3 independent myocyte preparations. * $p<0.001$ relative to ET-1 stimulated, empty vector controls, one way ANOVA with Tukey post-hoc test.

Fig. 5. Atf3 binds directly to the IL6 promoter. A, Schematic diagram of potential transcription factor binding sites in the IL6 promoter. B, Sequence of the rat IL6 promoter. The transcriptional start is indicated in bold italics, the putative Atf/Cre consensus sequence for binding of Atf3 is in bold and outlined and the positions of two potential Sp1/Klf binding sites

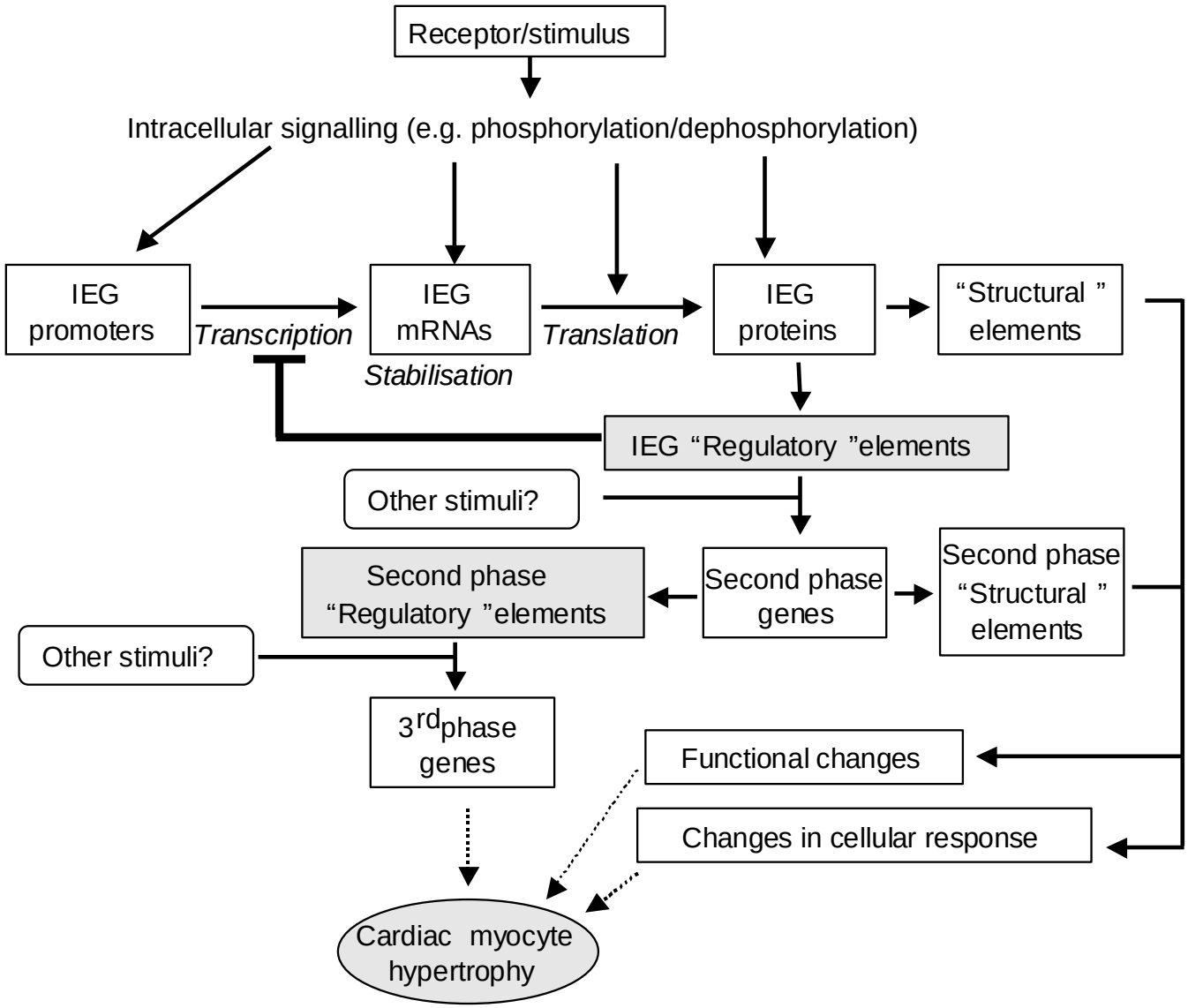
are in italics. Forward and reverse primers used for ChIP analysis are indicated in bold. C, Chromatin immunoprecipitation of Atf3 with PCR amplification of the rat IL6 promoter as indicated in B. Cardiac myocytes were unstimulated or exposed to 100 nM ET-1 for 1 h and Atf3 immunoprecipitated (Atf3 IP). Additional control samples were exposed to ET-1 and processed in the absence of Atf3 immunoprecipitating antibodies (no Atf3 IP). RT-PCR was performed for input DNA and immunoprecipitated DNA. Representative images are shown in the left panel [immunoprecipitated (ChIP) DNA, upper image; input DNA, lower image] with densitometric analysis in the right panel. Results are means ± SEM for n=3 independent preparations of myocytes. * $p<0.001$ relative to unstimulated controls with Atf3 IP or to ET-1 no Atf3 IP, one way ANOVA with Tukey post-hoc test.

FIG. 6. Knockdown of Klf2 expression enhances the increase in expression of IL6 by ET-1. Cardiac myocytes were infected with adenoviruses for siRNA to a random protein (luciferase, Rndm) as controls or with adenoviruses for siRNA to Klf2 (siKlf2), then unstimulated or exposed to 100 nM ET-1 for 1 h. RNA was extracted and mRNA expression of Klf2 (A), Klf6 (B) or IL6 (C) assessed by QPCR. Results are expressed relative to Rndm unstimulated controls and are means ± SEM for n=3 independent myocyte preparations. * $p<0.001$ relative to Rndm + ET-1, one way ANOVA with Tukey post-hoc test.

Key words

Immediate early genes, interleukin 6, activating transcription factor 3, Krüppel like factor 2, epriregulin, endothelin-1, gene expression, siRNA, antisense, microarrays, chromatin immunoprecipitation, transcription factors, cycloheximide, signal propagation, cardiac myocytes.

Clerk et al. Figure 1.



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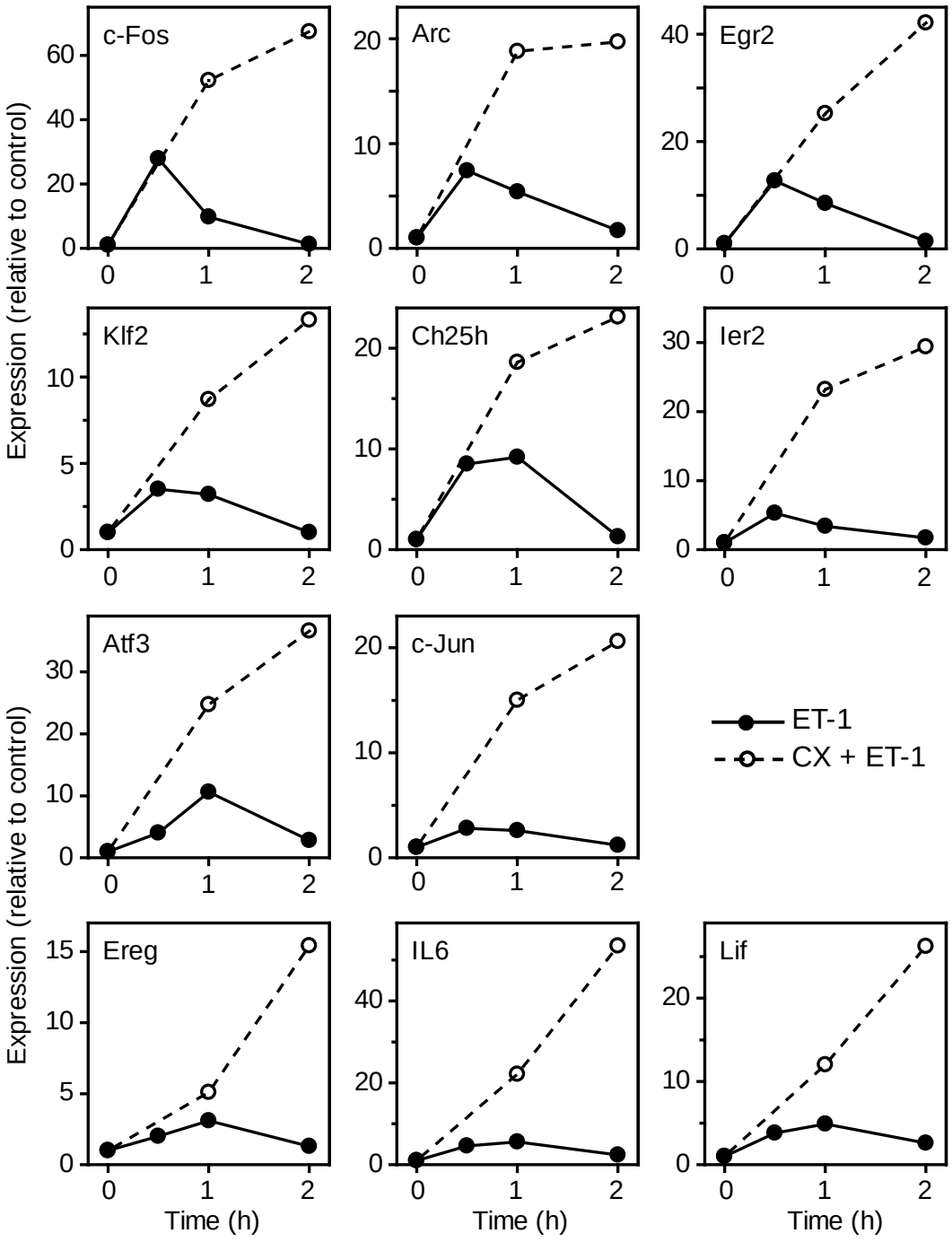

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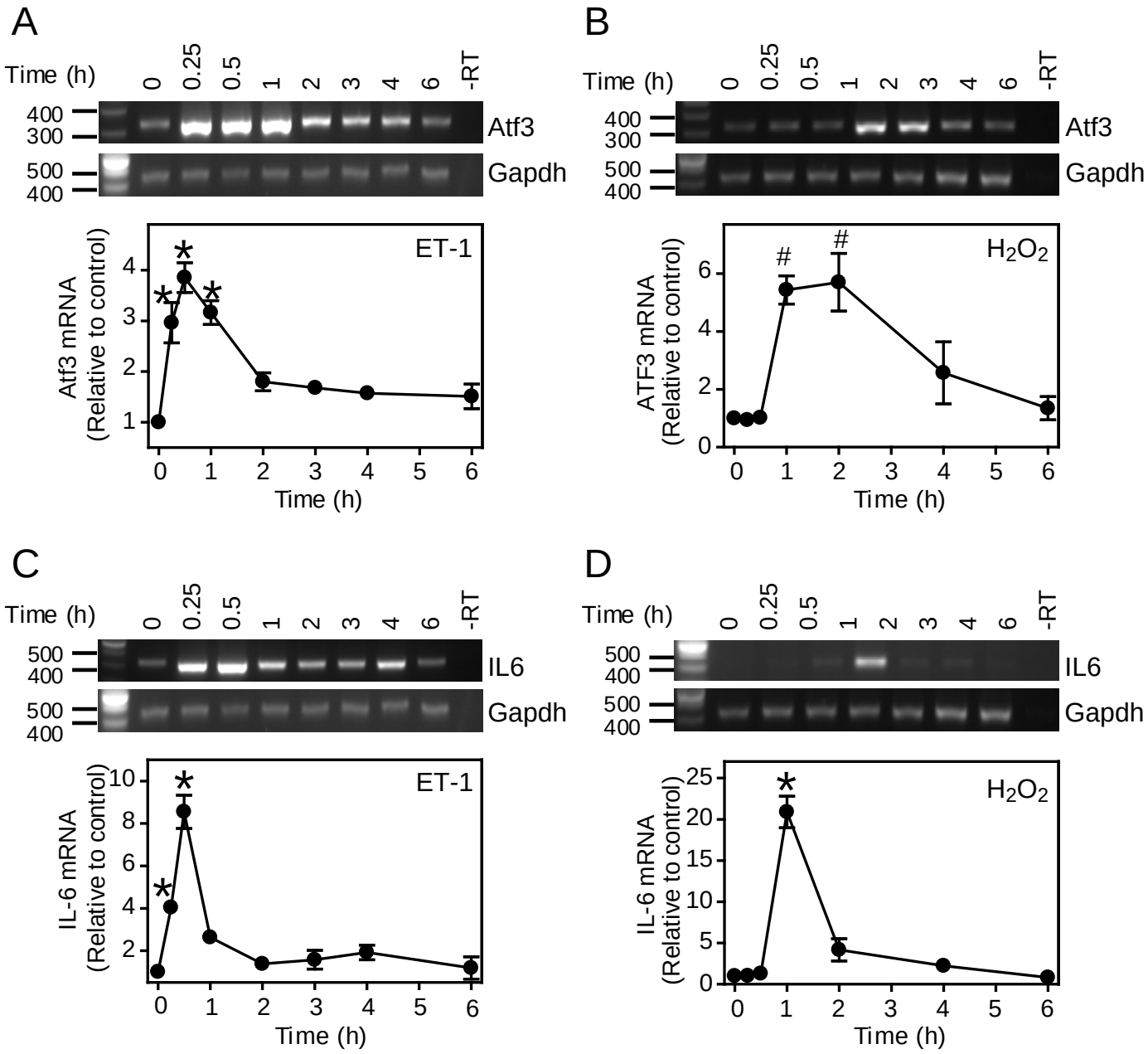

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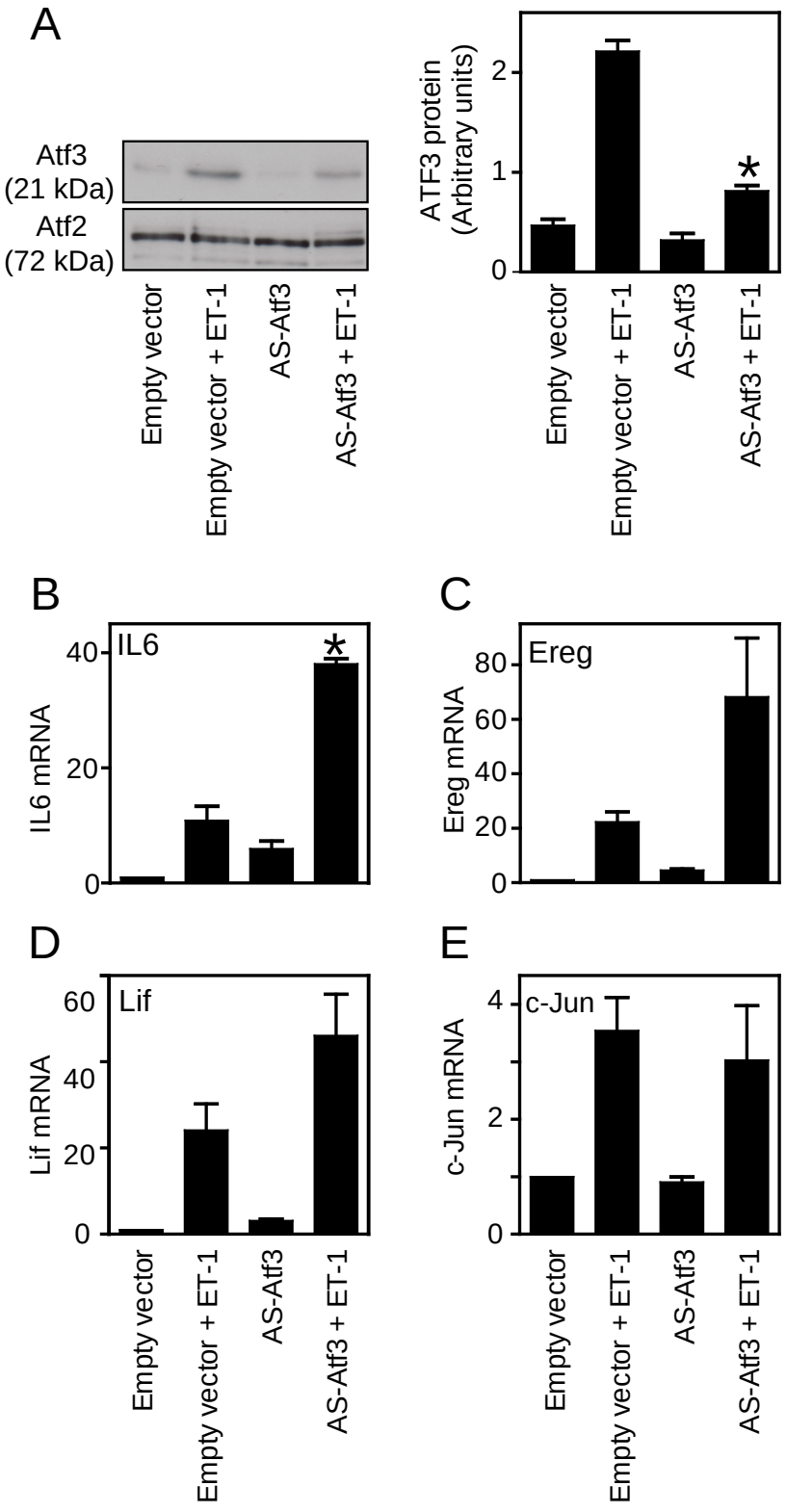

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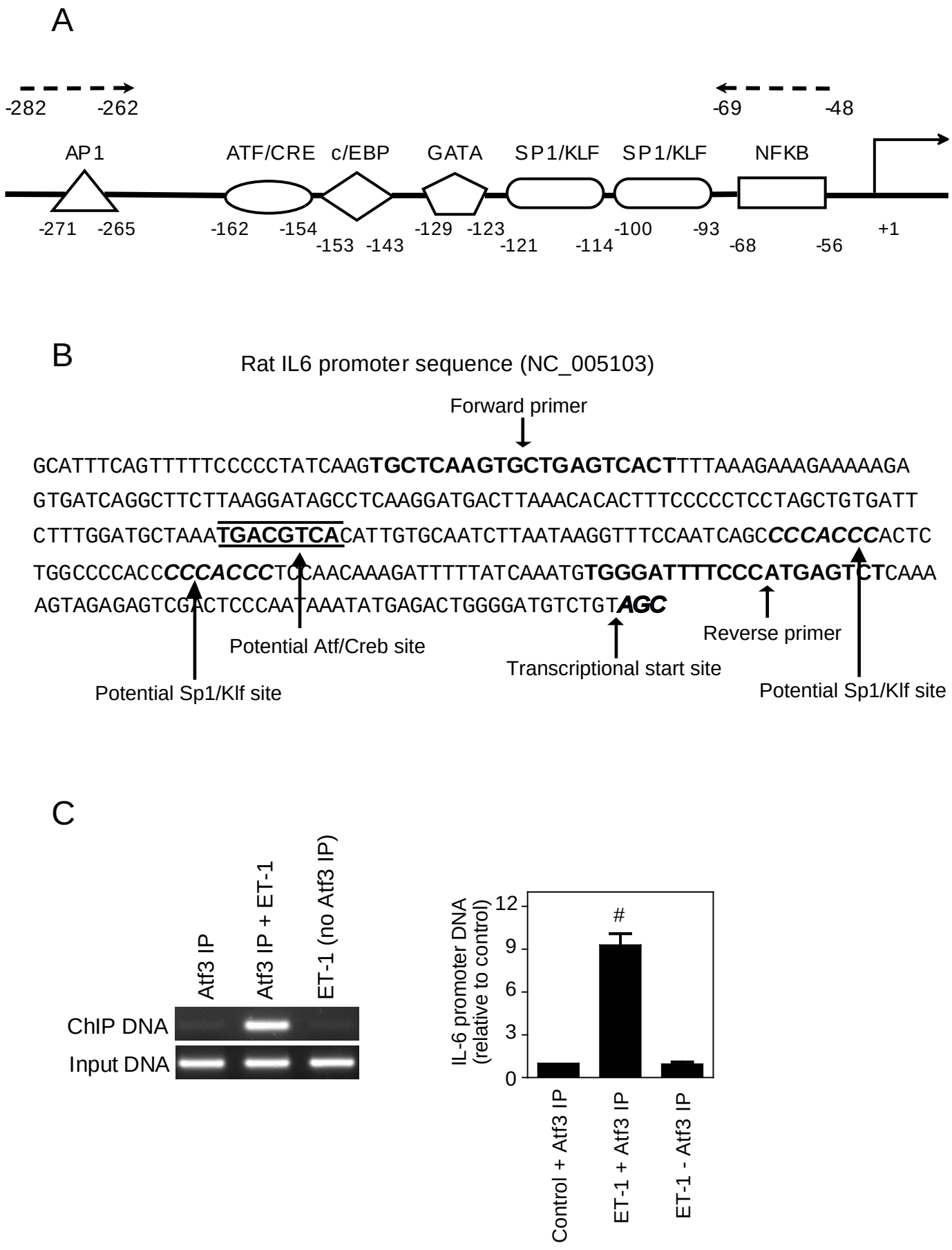

Clerk et al. Figure 6.