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Review

Regulation of the human thioredoxin gene promoter and its key substrates: A study of functional and putative regulatory elements

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ABSTRACT

Background: The thioredoxin system maintains redox balance through the action of thioredoxin and thioredoxin reductase. Thioredoxin regulates the activity of various substrates, including those that function to counteract cellular oxidative stress. These include the peroxiredoxins, methionine sulfoxide reductase A and specific transcription factors. Of particular relevance is Redox Factor-1, which in turn activates other redox-regulated transcription factors.

Scope of review: Experimentally defined transcription factor binding sites in the human thioredoxin and thioredoxin reductase gene promoters together with promoters of the major thioredoxin system substrates involved in regulating cellular redox status are discussed. An *in silico* approach was used to identify potential putative binding sites for these transcription factors in all of these promoters.

Major conclusions: Our analysis reveals that many redox gene promoters contain the same transcription factor binding sites. Several of these transcription factors are in turn redox regulated. The ARE is present in several of these promoters and is bound by Nrf2 during various oxidative stress stimuli to upregulate gene expression. Other transcription factors also bind to these promoters during the same oxidative stress stimuli, with this redundancy supporting the importance of the antioxidant response. Putative transcription factor sites were identified *in silico*, which in combination with specific regulatory knowledge for that gene promoter may inform future experiments.

General significance: Redox proteins are involved in many cellular signalling pathways and aberrant expression can lead to disease or other pathological conditions. Therefore understanding how their expression is regulated is relevant for developing therapeutic agents that target these pathways.

1. Introduction

The thioredoxin (Trx) system is one of the most important antioxidant systems in the cytoplasm of a cell, with a corresponding system in the mitochondria. The cytoplasmic system consists of Trx, which is a 12 kDa redox active protein, and thioredoxin reductase (TrxR), a selenoprotein [1]. The active site of Trx contains two active cysteine residues that undergo reversible oxidation to form a disulfide bond, during the process of transferring reducing equivalents to a disulfide substrate. TrxR then recycles the oxidised Trx protein to a reduced state using NADPH as the electron donor [2]. To date TrxR is the only known enzyme capable of reducing Trx and thus it is an essential component of the Trx system. There are Trx systems in both the cytoplasm and mitochondria. The cytoplasmic system contains Trx-1 and TrxR1, while the corresponding Trx system in the mitochondria is comprised of Trx-2 and TrxR2. For the purposes of this review the terms Trx and TrxR will refer to Trx-1 and TrxR1. Through a reversible redox reaction involving TrxR, Trx can regulate the activity of specific protein substrates in many pathways, including transcription factors [3], apoptotic signalling [4], ribonucleotide reductase [5] and members of the peroxiredoxin family, which degrade hydrogen peroxide [6]. Misregulation of Trx and its substrates can lead to many pathological conditions, including cancer [7,8], and thus their potential transcriptional coregulation is of great interest and relevance to the design of therapeutic agents.

Key transcriptional regulators of the human Trx gene promoter have been identified [9–13]. However it is not clear if the Trx gene is

Abbreviations: AP-1, Activator protein-1; APE, Apurinic/apyrimidinic endonuclease; ARE, Antioxidant response element; ChIP, Chromatin immunoprecipitation; CRE, cAMP response element; CREB, CRE binding protein; Egr-1, Early growth response factor-1; EMSA, Electrophoretic mobility shift assay; Ets, E26 transformation specific; FOXO, Forkhead O; GSH, Glutathione; HIF, Hypoxia inducible factor; Keap1, Kelch-like ECH-associated protein 1; NF-κB, Nuclear Factor-κB; Nrf2, Nuclear factor-erythroid 2 p45-related factor 2; MiTF, Microphthalmia associated transcription factor; MRE, Metal response element; MSR, Methionine sulfoxide reductase; NADPH, Nicotinamide adenine dinucleotide phosphate; NGF, Nerve growth factor; Oct-1, Octamer binding protein; p53, Tumour protein 53; PRDX, Peroxiredoxin; PPAR, Peroxisome proliferator-activated receptor; PPRE, PPAR response element; RA, Retinoic acid; RAR, Retinoic acid receptor; RARE, RA response element; RXR, Retinoid X receptor; Ref-1, Redox Factor-1; ROS, Reactive oxygen species; Sp1, Specificity protein 1; tBHQ, *tert*-Butylhydroquinone; Trx, Thioredoxin-1; TrxR, Thioredoxin reductase 1; TSS, Transcription start site

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coregulated with genes that encode the key substrates for Trx needed for an effective cellular response against oxidative stress. Therefore we selected the human cytoplasmic Trx system genes (Trx and TrxR) and a number of key substrates including all of the peroxiredoxins, methionine sulfoxide reductase A (MSRA), and Redox Factor-1 (Ref-1) to review current knowledge regarding which transcription factor binding sites have been experimentally validated to regulate these gene promoters. We then searched the promoter sequences for other putative transcription factor binding sites.

Peroxiredoxins (PRDXs) are 20–30 kDa thiol-specific antioxidant proteins that scavenge reactive oxygen species (ROS) and form part of the cellular response to oxidative stress [6]. Currently six human PRDXs (PRDX1–6) have been described that degrade H_2O_2 and other peroxides using the thiol groups of their cysteines as the catalytic site. PRDX1–4 contain two conserved cysteines in their active site and utilise Trx as their reductant [6]. PRDX5 is also a substrate for Trx [14] but is classified as an atypical 2-cys PRDX that prefers to reduce alkyl hydroperoxides and peroxynitrite [15]. PRDX6 contains a single conserved cysteine and unlike the other PRDXs utilises GSH but not Trx to catalyse the reduction of H_2O_2 [16,17]. It is included as part of this study to compare its promoter structure to those PRDXs that are regulated by Trx. PRDX1, PRDX2 and PRDX6 are located in the cytoplasm [6], PRDX3 contains a mitochondrial targeting sequence [18] and PRDX4 is secreted outside the cell [19]. PRDX5 is found in the cytoplasm, mitochondria and peroxisomes [20,21].

Another substrate for Trx is MSRA, which reduces oxidised methionine residues [22]. When methionine is oxidised, it can form two different stereoisomers; the Met-S-SO form, which is reduced by MSRA and the Met-R-SO form, which is reduced by MSRB [23]. This oxidation forms an internal disulfide bond in the MSRA protein that is subsequently reduced by the Trx system [24]. The gene for MSRA contains two transcription start sites (TSSs) located 40 kb apart. Therefore two distinct promoters regulate the expression of MSRA: the 'upstream' promoter regulates MSRA1 transcription and the 'downstream' promoter regulates MSRA2 and MSRA3 expression. Products encoded from MSRA1 are located in the mitochondria and those from MSRA2 and MSRA3 are localised in the cytoplasm and nucleus, respectively [25,26]. MSR activity appears to decrease during ageing [27,28], but as yet the regulatory mechanisms are undefined. In addition, a reduced level of MSRA is associated with various neurodegenerative disorders including Alzheimer's disease [29] and Parkinson's disease [30]. Since MSRA is a Trx substrate and is associated with these oxidative stress linked pathological conditions MSRA was selected for inclusion in this current review.

Redox Factor 1 (Ref-1) is a 36.5 kDa protein also known as apurinic/apyrimidinic endonuclease (APE1) and was first described as an important enzyme in the base excision repair (BER) pathway. This pathway is activated to repair apurinic/apyrimidinic (AP) sites, which often occur when ROS causes DNA damage [31]. APE1 catalyses the second step of the pathway, which is to cleave the phosphodiester backbone after the removal of damaged bases by glycosidases in step 1. The cleavage occurs immediately 5′ to an AP site and creates an abasic 5′-deoxyribose phosphate and a 3′ hydroxyl, which is utilised by DNA polymerase β and DNA ligase to continue the repair [31]. Ref-1 also has a redox activity that resides in a separate N-terminal domain of the protein [32]. Ref-1 activity has been shown to enhance the DNA binding ability of transcription factors such as nuclear factor-kappa B NF-κB [33,34], tumour protein 53 (p53) [35,36], hypoxia inducible factor-1α (HIF-1α) [37,38] and activator protein-1 (AP-1) [33,39]. Trx has been shown to bind to Ref-1 *in vitro* and through mammalian two–hybrid assays in Cos-7 cells [40]. Other studies have further implicated Trx as cooperating with Ref-1 within the nucleus of mammalian cells to enhance AP-1 activity [3,41], although there have been surprisingly few studies reported that focus on the physiological relevance of the Trx–Ref-1 interaction. More recently, Ref-1 was shown to have a redox chaperone activity, independent of its own redox activity, whereby it facilitates the reduction of transcription factors by other reducing agents, including Trx [33].

This report focuses on Trx, TrxR, and their major substrates, the PRDX family, MSRA and Ref-1, as they are significant contributors to the antioxidant response and there are potential redundancies or cross regulation between several of these proteins. Since Trx and many of these substrates are upregulated in tumours, or involved in other pathologies, it is possible that a coordinated approach to target their gene expression regulation may present a more effective therapeutic intervention. However we first need to know which transcription factors are involved in antioxidant promoter regulation in response to a specific stimulus. We will discuss the experimental evidence that supports the binding of specific transcription factors to specific sites in the gene promoters and then identify potential binding sites using *in silico* searches. While not all of the putative binding sites will be functional, the potential sites found in these searches may guide the design of future specific functional studies in combination with current knowledge regarding regulation of these gene promoters.

2. Methodology

Promoter regions of eleven human genes that express redox control proteins were selected for this study. They include Trx, TrxR, MSRA1, MSRA2, PRDX1, PRDX2, PRDX3, PRDX4, PRDX5, PRDX6, and Ref-1. All nucleotide sequences were obtained from the NCBI and ENSEMBL databases (Table 1) and cover from −1100 to +200 bp relative to the main TSS. Experimentally mapped TSSs reported in the literature were used where available, otherwise TSSs were assigned using the NCBI and ENSEMBL databases to compare sequenced transcripts to genomic sequence. Consensus transcription factor binding sites in their promoter regions were identified utilising the MatInspector programme [42] of the Genomatix web site (www.genomatix.de), with the Ci-value set to 80%. In particular we focussed on the following transcription factor binding sites, listing first the most studied sites in these gene promoters with respect to oxidative stress: antioxidant response element (ARE), Ets binding site, forkhead element, AP-1, E-box, retinoic acid receptor element (RARE), PPAR response element (PPRE), cAMP response element (CRE), TATA box, specificity protein 1 (Sp1), octamer binding protein (Oct-1) NF-κB, binding site, early growth response factor-1 (Egr-1) sites and the metal response element (MRE). These putative transcription factor-binding sites were cross-referenced with the experimentally validated sites described in the literature.

3. The antioxidant response element (ARE)

Oxidative stress can result from many stimuli or cellular pathologies leading to the activation of different signalling pathways. Consequently, different transcription factors are activated, which bind to quite specific DNA binding elements in gene promoters. The most common DNA element associated with cellular oxidative stress is the antioxidant response element (ARE). This element is found in many phase II detoxifying gene promoters and gene expression is regulated by the binding of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) [43,44]. In unstimulated cells an inhibitor called Kelch-like ECH-associated protein 1 (Keap1) binds to Nrf2 and targets it for degradation *via* the ubiquitination pathway [45]. During oxidative stress Keap1 is modified such that it no longer interacts with Nrf2, allowing Nrf2 to move into the nucleus [46]. Nrf2 has been shown to bind to an ARE in four promoters out of the eleven, Trx, TrxR, PRDX1 and PRDX6. The reported sites are listed in Tables 2a and 2b and depicted in Fig. 1.

3.1. The Trx gene promoter

Trx, as expected, is induced in response to various oxidative stress stimuli and a potential ARE was first described in the Trx gene promoter in 2001 [10]. Hemin, which is an oxidised form of heme, was utilised as

Table 1
Overview of the redox genes used for this study. Sequences were obtained utilising the NCBI search engine, http://www.ncbi.nlm.nih.gov/ and ENSEMBL search engine, http://www.ensembl.org/.

	Official symbol	GenBank accession numbers		Gene ID	Chr.	Map position	ENSEMBL code
		(mRNA)	(Gene cds)				
Thioredoxin	TXN	BC003377	AF548001	7295	9	9q31.3	ENSG00000136810
Thioredoxin reductase	TXNRD1	NM_003330	AF247671[a]	7296	12	12q23-q24.1	ENSG00000198431
MSRA1	MSRA	BC054033	AY958432	4482	8	8p23.1	ENSG00000175806
MSRA2	MSRA	AAY17430	AY958432	4482	8	8p23.1	ENSG00000175806
Peroxiredoxin 1	PRDX1	BC021683	DQ297142	5052	1	1p34.1	ENSG00000117450
Peroxiredoxin 2	PRDX2	BC039428	DQ231563	7001	19	19p.13.2	ENSG00000167815
Peroxiredoxin 3	PRDX3	BC022373	DQ298752	10935	10	10q25-q26	ENSG00000165672
Peroxiredoxin 4	PRDX4	BC016770	NG_012563	10549	X	Xp22.11	ENSG00000123131
Peroxiredoxin 5	PRDX5	BC110983	DQ247769	25824	11	11q13	ENSG00000126432
Peroxiredoxin 6	PRDX6	BC035857	DQ230990	9588	1	1q25.1	ENSG00000117592
Ref-1	APEX1	S43127	AF488551	328	14	14q11.2-q12	ENSG00000100823

[a] Core promoter sequence.

the inducer as it causes free radical formation in the cell, resulting in oxidative stress [47]. The hemin responsive region of the Trx promoter was located through luciferase reporter assays and the sequence defined as similar to the ARE and AP-1 consensus binding sites [10]. Mutations were incorporated into the potential ARE sequence to define residues important for transcription factor binding. Over-expression of Nrf2 protein increased promoter activity in response to hemin, whereas expression of the dominant negative mutant of Nrf2 suppressed promoter activity [10]. EMSAs using nuclear extracts of hemin induced cells showed that Nrf2/small Maf proteins were present and could bind to this predicted ARE sequence. In contrast, under unstimulated conditions Nrf2 was not detected in nuclear extracts tested in EMSAs, whereas NF-E2p45/small Maf proteins were present and could bind to this ARE *in vitro* [10].

Two other compounds, *tert*-butylhydroquinone (tBHQ), and sulforaphane (SF) were also shown to induce Trx expression through this same ARE in gene reporter assays [11,12]. tBHQ is a major metabolite of butylated hydroxyanisole (a phenolic antioxidant) and is itself oxidised to electrophilic compounds [48]. SF is a naturally occurring isothiocyanate found in certain plants, such as broccoli. Electrophiles and isothiocyanates can activate phase II detoxifying enzymes by targeting specific cysteine residues of Keap1 that inhibit the Keap1 mediated ubiquitination of Nrf2, thereby allowing Nrf2 to bind to AREs [49,50]. For both compounds EMSAs were used to show that Nrf2 and small Maf proteins were induced, which could bind to the ARE in the Trx promoter *in vitro* [11,12]. A recent chromatin immunoprecipitation (ChIP)-sequencing (ChIP-Seq) study confirmed that Nrf2 binds to the Trx promoter in response to SF in human lymphoblastoid cells [51]. This *in vivo* binding corresponded to the region of the Trx promoter previously characterised to contain the ARE using gene reporter assays and EMSAs. Since ChIP is regarded as a more robust tool for defining transcription factor binding *in vivo*, this recent study [51] in combination with previous studies [11,12] provides strong evidence to support that the Nrf2 transcription factor binds to the ARE in the Trx promoter in response to oxidative stress stimuli, leading to an induction of Trx expression.

3.2. The core TrxR gene promoter

TrxR is essential for Trx system functionality and it is critical for a cell to upregulate TrxR in situations that also require a rapid increase in Trx expression, such as during oxidative stress. Therefore it is not surprising that TrxR promoter activity is also upregulated through an ARE. Two adjacent putative AREs close to the TSS were examined for their potential to be functional [52]. Both ARE sites appear to be involved in TrxR promoter induction by SF since mutation of either ARE reduced promoter induction [52]. When cadmium was used as an inducing agent only the distal ARE was required for TrxR promoter activity, since mutating the proximal ARE did not reduce promoter induction [53]. ChIP assays confirmed that Nrf2 bound to the core TrxR promoter *in vivo* upon

Table 2a
Characterised transcription factor binding sites in human Trx and TrxR gene promoters.

Gene	TF that binds	DNA site	Position	Method	Stressing agent	Cell line and origin	Ref
Trx	Nrf2/small maf	ARE	−446 to −436	EMSA, PRA, DM, ChIP	hemin tBHQ sulforaphane	K562 (erythroleukaemia) K-1034 (retinal pigment epithelial), lymphoblastoid	[10] [11] [12] [51]
Trx	Fos/Jun	AP-1	−446 to −440	EMSA	PMA	K562 (erythroleukaemia)	[10]
Trx	CREB	CRE	−257 to −233	DM, PRA, EMSA	Nerve Growth Factor	PC12 (rat pheochromocytoma)	[110]
Trx	TBP	TATA	−30 to −23	DM, PRA		MDA-MB-231	[120]
Trx	Sp1	Sp1 (GC box)	−171 to −158 −148 to −138 −118 to −104	DM, PRA, EMSA		MDA-MB-231	[120,122]
Trx	RAR/RXR	RARE	−506 to −501 −454 to −446 −421 to −415	DM, PRA, EMSA, FP	Vitamin A	Bronchial epithelial cells	[9]
Trx	PPARa/RXRa	PPRE	−2116 to −2108	DM, PRA, EMSA	PPARa	Macrophages	[103]
TrxR	Nrf2	2 x ARE (distal and proximal)	−59 to −49 −47 to −37	DM, PRA, EMSA, ChIP	Sulforaphane	HepG2 (hepatoma) lymphoblastoid	[52] [51]
TrxR	Nrf2	ARE (distal)	−59 to −49	DM, PRA, EMSA, ChIP	Cadmium TPA	BAEC (arterial endothelial cells) HepG2 (hepatoma)	[53] [52]
TrxR	Oct-1	POU domain	−98 to −91	DM, PRA, EMSA		A549 and HeLa	[123]
TrxR	Sp1, Sp3	Sp1 (GC box)	−19 to −12 +65–+105	DM, PRA, EMSA		A549 and HeLa	[123]

EMSA (electrophoretic mobility shift assay); DM (deletion or mutation); PRA (promoter reporter assays); FP (footprinting); ChIP (chromatin immunoprecipitation).

Table 2b
Characterised transcription factor binding sites in human redox gene promoters.

Gene	TF that binds	DNA site	Position	Method	Stressing Agent	Cell line and origin	Ref.
MSRA2	RARA	RARE	−690 to −683 −684 to −675 −655 to −645	DM, RPA	Retinoic acid	D407 (retinal pigment epithelium)	[105]
PRDX1	Nrf2	2 x ARE (proximal and distal) ARE	−537 to −527 −1430 to −1420 −89 to −79	DM, PRA, EMSA, ChIP ChIP	Reoxygenation after hypoxia; sulforaphane	A549 (lung cancer) lymphoblastoid	[55] [51]
PRDX1	Ets1 & Ets2	Ets binding site	−247 to −237	DM, PRA, EMSA, ChIP	30 min $H_2O_2$2/12 h recovery; 4 h hypoxia/12 h Reoxy	PC3 (prostate cancer) KB (epidermoid cancer)	[67]
PRDX2	FOXO3A	FOXO	−15 to −8	PRA	Nipradilol	NTM5 and GTM3 cell line	[73]
PRDX3	FOXO3A FOXO3A	FOXO FOXO	−224 to −217 −201 to −194	DM, PRA, EMSA, ChIP	Serum withdrawal	Primary human cardiac fibroblast HEK293 (Embryonic kidney)	[72]
PRDX3	c-Myc	E-box	−135 to −128	PRA, ChIP	Arsenic trioxide	U-937 (leukaemic monocyte lymphoma) NB4 (acute promyelocytic)	[86]
PRDX5	Ets1 & Ets2	Ets binding site	−39 to −30	DM, PRA, EMSA, ChIP	30 min H_2O_2/12 h recovery; 4 h hypoxia/12 h Reoxy	PC3 (prostate cancer) KB (epidermoid cancer)	[67]
PRDX6	Nrf2	ARE	−307 to −297	DM, PRA, ChIP	H_2O_2; sulforaphane	A549 (lung cancer) lymphoblastoid	[54] [51]
Ref-1	MiTF	E-box	−147 to −138 (most) −85 to −79 −65 to −56	DM, RPA, ChIP	H_2O_2	MNT1 (melanoma); SK-Mel-28	[92]
Ref-1	Jun/ATF2	CRE	−691 to −671	DM, RPA, EMSA	H_2O_2	CHO-9	[111]
Ref-1	Egr-1	Egr-1	−63 to −54	RPA, EMSA, ChIP	PMA, H_2O_2	HOBIT (osteoblastic)	[138]
Ref-1	Sp1	Sp1	−173 to −157	DM, RPA, ChIP	Camptothecin	HCT116 (human colon carcinoma)	[128]

EMSA (electrophoretic mobility shift assay); DM (deletion or mutation); PRA (promoter reporter assays); ChIP (chromatin immunoprecipitation).

cadmium stimulation, but no Nrf2 was bound in untreated cells [53]. The recent ChIP-Seq study confirmed that Nrf2 binds to this region of the TrxR promoter in response to SF in human lymphoblastoid cells [51]. Therefore the evidence strongly supports the hypothesis that one mechanism for how Trx and TrxR expression can be coordinately upregulated during oxidative stress is through Nrf2 binding to an ARE sequence in their promoters.

3.3. The PRDX gene promoters

The PRDX family degrades excess H_2O_2 in a cell and therefore it is to be expected that the PRDX genes are also upregulated by oxidative stress. To date AREs have been characterised in the PRDX1 and PRDX6 gene promoters [54,55]. In fact the PRDX1 gene promoter contains multiple potential AREs [55].

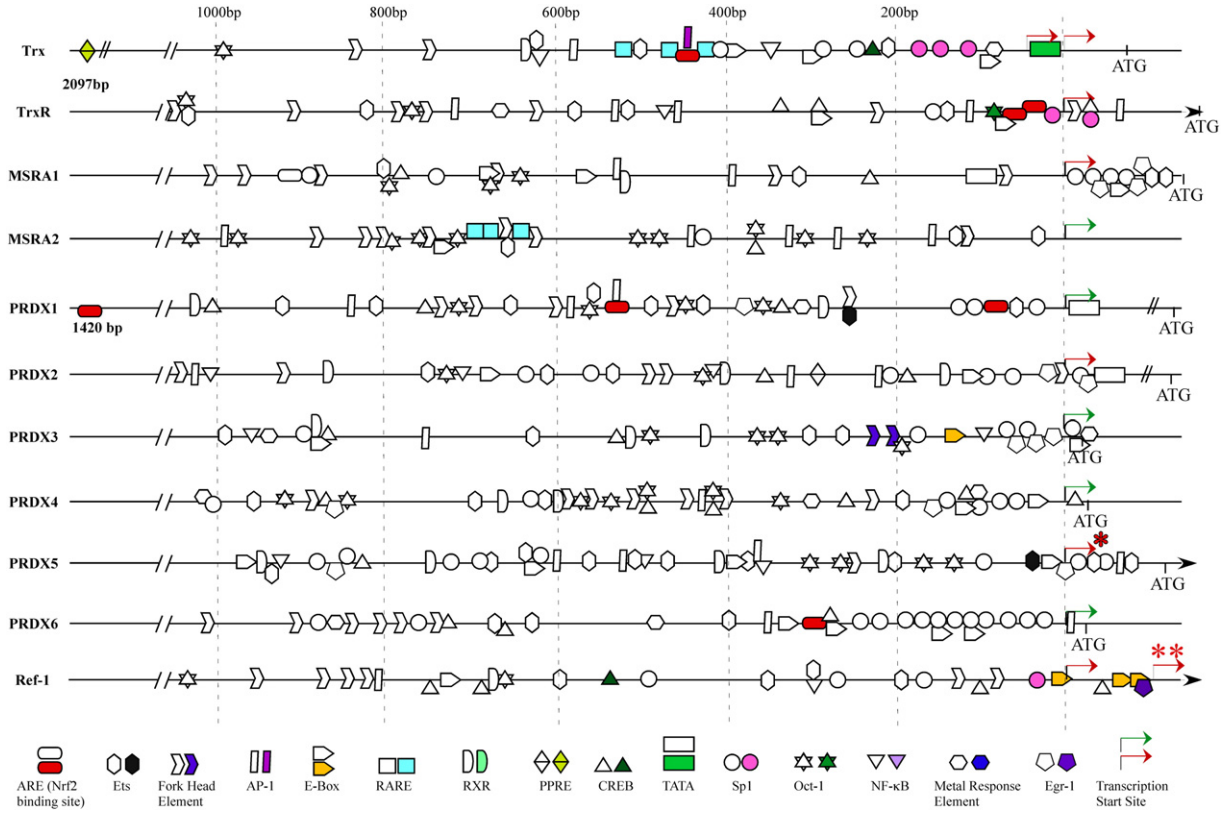

Fig. 1. Transcription factor binding sites in the promoter regions of redox-related gene targets. Diagrammatic representation of transcription factor binding motifs (characterised and putative) of the 5′ region of redox related genes. The sequences shown span approximately −1100 bp to +200 bp. Transcription start sites (TSSs) are denoted by arrows: the reported TSS is in red and predicted TSS is in green. The characterised transcription factor binding sites are shown in solid colour, while the putative sites found in the Genomatix search are colourless. *6 TSSs are located within −130 bp upstream of the ATG start codon. **This TSS for Ref-1 is selected as a reference for the position of transcription factor binding sites as listed in Table 2b.

PRDX1 is upregulated in many cancers [56,57] and these increased levels have a functional role since knockdown of PRDX1 expression results in significant growth reduction and reduced metastases of lung cancer cells [58]. These high PRDX levels were postulated to arise due to the unstable oxygenation conditions present in tumours [55]. Many tumours grow in hypoxic conditions, with oxygen availability below critical levels [59]. However there is a sporadic and variable oxygen supply throughout these tumours, caused by abnormalities in the tumour vasculature [60]. This results in transitory oxidative stress, as the cells are subjected to periods of reoxygenation following hypoxic growth [61]. Therefore Kim and co-workers [55] grew A549 lung cancer cells under hypoxic conditions for 4 h followed by incubation in normal atmospheric oxygen levels (often referred to as normoxia) to provide a reoxygenation stimulus. Hypoxia did not stimulate the PRDX1 promoter but 6 h of subsequent growth under normoxia gave the highest increase in promoter activity.

PRDX1 promoter deletion mutants and luciferase assays were used to map two regions of the promoter as important for induction, with both regions containing elements that are similar to consensus AREs [55]. ChIP assays confirmed that Nrf2 bound to both elements during the reoxygenation stimuli. The proximal ARE was also required for basal level expression, but mutating the distal ARE had no effect on basal level expression [55]. The PRDX1 promoter was also responsive to tBHQ, as seen for the Trx gene promoter. However this induction was not mapped to a specific ARE [55]. As a complication to identifying which ARE is bound by Nrf2 during oxidative stress, the recent ChIP-Seq study detected Nrf2 bound to the PRDX1 promoter in response to SF in lymphoblastoid cells, but this binding did not correspond to either of the ARE previously shown by ChIP to bind Nrf2 [51]. Nrf2 binding was located to a region about 80 bp upstream from the TSS and focused on a sequence detected in our Genomatix *in silico* search as a consensus ARE. It is possible that this putative ARE binds Nrf2 in response to different stimuli or in different cells. Further experimentation will be required to understand the contribution of these different potential ARE sites, to the different stimuli.

A functional ARE was also defined in the PRDX6 gene promoter by incubating A549 cells with 400 μM H_2O_2 for 8 h [54]. Deletion of the ARE abolished induction by H_2O_2 while PRDX6 promoter activity was upregulated by cotransfection with Nrf2. ChIP assays confirmed Nrf2 binding to the ARE and that this binding was increased with H_2O_2 treatment. As with the PRDX1 promoter, the PRDX6 promoter was also upregulated when cells were stimulated with tBHQ [54]. Together these results support the possibility that both PRDX1 and PRDX6 gene expression may be co-regulated through the Nrf2 pathway in response to oxidative stress.

3.4. Coordinated regulation

Overall, four of the redox gene promoters (Trx, TrxR, PRDX1, and PRDX6) contain AREs that have been shown by ChIP to be bound by Nrf2 during similar oxidative stress stimuli [51,53–55]. This includes the classical inducers of the Nrf2 pathway, such as tBHQ and SF, and also during reoxygenation after hypoxia. In addition the PRDX3 and PRDX5 gene promoters are also activated by the Nrf2 pathway in response to quercetin, a flavonoid, although specific AREs have not yet been characterised [62]. Thus Nrf2, potentially through an ARE, may induce four of the six PRDX gene promoters, which together with the Nrf2 regulation of Trx and TrxR, supports the speculation that there is co-regulation of the antioxidant response.

The ARE is clearly an important element for the response to oxidative stress stimuli. However formulating a consensus sequence that represents all functional AREs has been difficult. The extended ARE consensus sequence published by Wasserman and Fahl in 1997 [63] was based on the core TGACnnnGC sequence originally defined in 1991 [64]. Table 4 shows the sequences of the binding sites identified by either ChIP study or *in silico* searching. The Genomatix *in silico* search found potential AREs in 5 of the redox gene promoters (Trx, TrxR, PRDX1, PRDX6 and MSRA1) of which ChIP has verified Nrf2 binding in all except MSAR1. The potential ARE in the MSRA1 gene promoter has yet to be assessed using different oxidative stress stimuli. Interestingly for PRDX1 the two distal AREs were originally shown by ChIP to bind Nrf2 in response to reoxygenation [55] but the recent ChIP-Seq study [51] identified Nrf2 bound to another site in response to SF. The site bound by Nrf2 in response to SF is the only putative ARE identified in the Genomatix *in silico* search of the PRDX1 promoter (Table 4).

4. E26 transformation specific family (Ets)

Hypoxia inducible factor (HIF)-1α is the most studied transcription factor that regulates gene expression during hypoxia. It, along with HIF-2α, is stabilised by hypoxia and rapidly degraded when oxygen is present (recently reviewed in [65]). However another class of transcription factors, the E26 transformation specific (Ets) family, also activates many target genes required in hypoxia, including angiogenesis and metastasis genes [66]. Ets-1 is upregulated by HIF-1α and it also cooperates with HIF-1α and -2α to upregulate certain genes during hypoxia. However Ets-1 can also function independent of HIF-1α and upregulates some genes during hypoxia, even in the absence of HIF factors [66].

Both the PRDX1 and PRDX5 gene promoters were significantly induced in human PC3 prostate cancer cells by either H_2O_2 or reoxygenation after growth in hypoxia [67]. In contrast only minimal induction of PRDX2 and PRDX3 was observed after recovery from either hypoxia or H_2O_2. No induction was observed for PRDX4 under either stimulus or after recovery. Mapping and mutational studies identified consensus Ets-binding sites (Fig. 1, Tables 2a, 2b). Co-transfection of constructs over-expressing either Ets-1 or Ets-2 with the PRDX1 and PRDX5 promoter constructs resulted in increased promoter activity. ChIP experiments confirmed that both Ets-1 and Ets-2 bind to the PRDX1 and PRDX5 gene promoters in PC3 cells [67].

The results of these studies together with those previously described in Section 3.3 by Kim and co-workers [55,67] show that reoxygenation upregulates the PRDX1 gene promoter through two pathways, Nrf2 binding to the ARE and Ets binding to the Ets site. Together with the likely involvement of the ARE in Trx and TrxR upregulation during reoxygenation [68], this supports the importance of the antioxidant response since two different transcription factors can mediate the same stimulus. Given the role of Ets during hypoxia [66], it can be speculated that the Ets pathway may play a role in regulating redox control during hypoxia and reoxygenation, to complement the Nrf2 pathway of activation.

Although only the PRDX1 and PRDX5 gene promoters have functionally characterised Ets sites, a number of putative Ets binding sites were obtained during the Genomatix search (Fig. 1). In fact, every redox gene promoter contains at least three putative Ets sites (Table 3), which suggests there is a possibility that Ets may also regulate other redox gene promoters. While Ets did not bind to the PRDX2, 3 and 4 promoters during reoxygenation [67], an involvement during a different stimulus or in a different cell line cannot be ruled out.

5. Forkhead and serum deprivation

Forkhead proteins are transcription factors of the winged helix domain and are characterised by a conserved DNA binding domain [69]. There are 19 subgroups and the Forkhead O (FOXO) is one of the largest subgroups consisting of four families (FOXO1, FOXO3, FOXO4, FOXO6). FOXO3a overexpression inhibits tumour growth *in vitro* and tumour size *in vivo* and the FOXO family is regarded as tumour suppressor genes [69,70]. However FOXO3a also coordinates resistance to oxidative stress during serum withdrawal by activating transcription of the manganese superoxide dismutase gene [71] and recently it has been linked to the upregulation of PRDX2 and PRDX3 [72,73] (Fig. 1, Tables 2a, 2b).

Table 3
Transcription factor binding sites in human redox gene promoters. A summary of reported transcription factor binding sites from the literature and putative sites utilising the Genomatix search engine. The number of gene promoters in which a binding site is found is shown in the bottom row.

		ARE	Ets	Forkhead	AP-1	E-box	RARE/RXR/PPRE	CRE	TATA	Sp1	Octamer	NF-κB	MRE	Egr-1	Total
Trx	Studied	1	0	0	1	0	4	1	1	3	0	0	0	0	11
	Putative	0	3	2	1	3	1	0	0	3	1	2	1	0	17
TrxR	Studied	2	0	0	0	0	0	0	0	2	1	0	0	0	5
	Putative	0	5	7	5	2	0	3	0	1	2	1	1	0	27
MSRA1	Studied	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Putative	1	4	6	2	3	1	2	1	6	3	0	0	3	32
MSRA2	Studied	0	0	0	0	0	3	0	0	0	0	0	0	0	3
	Putative	0	3	7	5	1	0	1	0	1	10	0	0	0	28
PRDX1	Studied	3	1	0	0	0	0	0	0	0	0	0	0	0	4
	Putative	0	7	5	3	0	2	3	1	3	4	0	1	1	30
PRDX2	Studied	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Putative	0	3	5	3	2	4	2	1	6	2	3	0	2	33
PRDX3	Studied	0	0	2	0	1	0	0	0	0	0	0	0	0	3
	Putative	0	4	0	1	2	3	2	0	5	4	2	2	3	28
PRDX4	Studied	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Putative	0	4	7	1	2	2	6	0	6	7	0	3	2	40
PRDX5	Studied	0	1	0	0	0	0	0	0	0	0	0	0	0	1
	Putative	0	10	1	4	4	4	1	0	8	4	3	0	2	41
PRDX6	Studied	1	0	0	0	0	0	0	0	0	0	0	0	0	1
	Putative	0	3	6	2	4	0	3	0	13	0	0	2	0	33
Ref-1	Studied	0	0	0	0	3	0	1	0	1	0	0	0	1	6
	Putative	0	4	6	1	1	1	4	0	3	2	1	0	0	23
Found in "n" genes		5	11	11	11	10	8	11	4	11	10	6	6	7	

The PRDX2 gene promoter and FOXO3a are both induced by nipradilol, a compound used for treating glaucoma [73]. Oxidative stress has a role in glaucoma pathogenesis and thus upregulating PRDX proteins may be of therapeutic benefit. When FOXO3a was downregulated by siRNA the promoter activity of PRDX2 was significantly reduced, while cell sensitivity to H_2O_2 increased. This suggests that nipradilol upregulates PRDX2 expression through FOXO3a binding to an element in the promoter. A putative forkhead response element is located near the TSS and is conserved in the mouse gene promoter [73], but experimental evidence is required to confirm if this element is functional.

The PRDX3 gene promoter contains two FOXO binding sites situated close together (Fig. 1, Tables 2a, 2b). EMSAs demonstrated that FOXO3a has optimal binding when both sites are present and that this binding was enhanced when cells were serum deprived [72]. ChIP assays confirmed that FOXO3a binds to the PRDX3 promoter *in vivo* [72], but this protocol cannot determine if both sites are bound.

FOXO3a also binds to other gene promoters in which two binding sites are present. For example there are two FOXO3a binding sites in the oestrogen receptor α gene promoter and both must be present for upregulation to occur [74]. ChIP confirmed that FOXO3a binds to this region of the promoter. The *bim* gene promoter also contains two putative FOXO3a binding sites, as shown using *in vitro* EMSAs, but in this case either site alone is sufficient for FOXO3a to upregulate gene promoter activity, as assessed in reporter assays [75].

It is therefore noteworthy that the Genomatix search revealed that all of the gene promoters, except PRDX5, contain closely positioned forkhead consensus sequences (Fig. 1, Table 3), while PRDX5 contains one putative forkhead element (Fig. 1, Table 3). It is possible that forkhead may be another pathway that functions to provide upregulation of antioxidants in response to certain stress stimuli, but more experiments are required to determine the extent to which this pathway regulates the redox genes *in vivo*.

6. AP-1

Trx also regulates the activity of the activator protein-1 (AP-1) transcription factor, through Ref-1 [33,40]. The well-studied subunits of AP-1 are encoded by c-*fos* and c-*jun* and dimerise through a leucine zipper [76]. AP-1 affects gene expression in response to growth factors, cytokines, tumour promoters, carcinogens and increased expression of various oncogenes [77].

Kim and colleagues [10] mapped an AP-1 site in the Trx gene promoter, which overlaps with the ARE (Fig. 1, Tables 2a, 2b). When cells were stimulated with PMA, Jun and Fos were present in nuclear extracts that were shown to bind to the AP-1 site using EMSAs. Under basal level conditions there was no Fos and Jun binding [10]. As yet there has been no ChIP performed to evaluate if this represents *in vivo* binding. However it is intriguing to speculate that the antioxidant response for some stimuli could be enhanced through Trx gene promoter activity being upregulated by a transcription factor (AP-1), which itself is redox regulated [33,39,41]. Further *in vivo* experiments are required to test this hypothesis.

There are putative AP-1 sites present in all redox promoters used in this study (Fig. 1, Table 3). The AP-1 site in the Trx promoter, overlaps with the ARE previously described in Section 3.1 [10,51]. Hayes and colleagues have described four distinct classes of AREs with different consensus sequences, with two classes including an embedded AP-1 sequence (as in the case of Trx) [78]. In the Genomatix search one of the ARE sites in the PRDX1 promoter was depicted as an AP-1 site (Table 4) and whether both AP-1 and Nrf2 can bind in physiological conditions is yet to be determined.

The expression of another redox enzyme, sulfiredoxin, is also potentially regulated by both AP-1 and Nrf2 [79]. Sulfiredoxin reactivates

Table 4
Putative antioxidant response element (ARE) sequences in redox gene promoters. The designation of the site found in the Genomatix search is shown. References are provided for studied sites. The three core TGA bases are underlined as a ce position.

Gene	Strand	Position from TSS	Genomatix designation	Ref	ARE sequence
Trx	–	−436	ARE/AP-1	[10,51]	CAAAGTGCTGAGTAACGGTGACC
TrxR	–	−49	ARE	[51–53]	AGTCAGAATGACAAAGCAGAAAT
TrxR	+	−37	Not found	[51–53]	TGTCATTCTGACTCTGGCAGTTA
PRDX1	+	−1420	Not found	[55]	GATTCCCCTGCCTCAGCCTCCCA
PRDX1	+	−527	AP-1	[55]	CGTGTAACTGAATCAGCCTCCCA
PRDX1	–	−79	ARE	[51]	CGCCGGAATGACTCGGCGCTTTC
PRDX6	+	−297	ARE	[54]	GGGCAACGTGACCGAGCCCCGCA
MSRA1	+	−901	ARE		CTTAATAGTGACCAGGCCTTCGC
Wasserman and Fahl consensus [63]					TMAnnRTGAYnnnGCRwwww
Core consensus [64]					TGACnnnGC
ARE sequence from Nrf2 ChIP-Seq study [51]					TGASTCAGC

peroxiredoxins when their active site cysteine has been hyperoxidated to sulfinic acid [79]. Nrf2 binding to the sulfiredoxin gene promoter was confirmed recently by ChIP [51], while two highly conserved AP-1 sites were shown to be required for TPA induced activation in luciferase reporter assays [80]. Therefore further investigation to determine if any of the putative AP-1 sites is functional in the other redox gene promoters may give a better understanding of AP-1's role in regulating the expression of redox control genes and how AP-1 cooperates with other transcription factors, such as Nrf2, to upregulate redox gene expression.

7. E-box

The E-box is primarily bound by basic helix–loop–helix (bHLH) proteins, including myc. The Myc family of transcription factors regulates many genes involved in cell proliferation, cell cycle progression and metabolism. The Myc family is often over-expressed in cancers [81,82], leading to uncontrolled growth and repression of tumour suppressor genes [83]. As such it has been put forward as a possible target for cancer treatments, although how to target c-Myc in cancer cells without having side effects on healthy cells is still an issue (for reviews see [84] and [85]).

The PRDX3 gene promoter contains a consensus E-box situated just upstream of the TSS [86], which is present in a region previously shown by ChIP analysis to bind c-Myc [87]. The levels of c-Myc binding to the PRDX3 promoter were decreased in acute promyelocytic leukaemia (APL) cells upon treatment with the anti-cancer drug, arsenic trioxide (ATO) [86]. ATO can induce apoptosis at certain doses and it has been proposed that this occurs through the production of ROS, particularly H_2O_2 [88], with the significance being that PRDX3 has been shown to scavenge H_2O_2 [6]. It was reported that ATO treatment resulted in a reduction of cellular c-Myc protein levels, which correlated with the decreased binding of c-Myc to the PRDX3 gene promoter, as assessed by ChIP and with a reduction in PRDX3 gene promoter activity, as measured by gene reporter assays [86]. Since PRDX3 is usually expressed at high levels in cancers, including leukaemia cells [89], the authors therefore suggested that one mechanism by which ATO induces apoptosis in leukaemia cells is through downregulating PRDX3 expression, thereby leading to H_2O_2 accumulation [86]. In a separate study ATO was shown to directly inhibit TrxR, which subsequently led to Trx oxidation [90]. The potential downstream consequences are that the targets of Trx, including PRDX3, would be left in an inactive state. Therefore it could be speculated that ATO may affect PRDX3 in two ways, by decreasing PRDX3 expression through c-Myc downregulation and by decreasing PRDX3 activity through inhibition of TrxR. Nonetheless, given the effective anti-cancer properties of ATO and the numerous targets of the Trx system, there are likely to be several antioxidant proteins affected in addition to PRDX3.

In the mouse, the Prdx5 gene promoter contains four E-boxes, each of which ChIP demonstrated c-Myc binding [91]. Interestingly the binding of c-Myc to one of those boxes is dependent on the presence of Prdx1, and this represents a mechanism by which one Prdx family member can regulate another in a c-Myc dependent manner. The Genomatix search found a number of E-boxes in the human PRDX5 gene promoter (Fig. 1), but as yet none have been reported to bind c-Myc or any other specific transcription factors.

The Ref-1 gene promoter contains three E-boxes situated in close proximity to its multiple TSSs. The melanocyte specific bHLH transcription factor microphthalmia associated transcription factor (MiTF) was shown using ChIP to bind to this region of the promoter in melanoma cells [92]. Luciferase reporter assays showed that the first E-box is critical for MiTF regulation, the second has a minor role and the third is less important. The first box is just 5′ of the major TSS while the other two sites are within the 5′UTR [92]. The MiTF transcription factor also binds to and enhances expression from the HIF-1α promoter in melanoma cells [93]. Thus MiTF can upregulate the expression of both Ref-1 and a potential *in vivo* Ref-1 substrate, HIF-1α [38], leading to the speculation that this may be another example of a coregulated antioxidant response to specific stimuli.

Utilising the Genomatix search, putative E-boxes were found in all of the gene promoters examined in this study, except PRDX1 (Fig. 1, Table 3). As yet none of these E-boxes have been reported to bind c-Myc or any other bHLH transcription factors.

8. Retinoic acid (RARE, PPRE)

Retinoic acid (RA) is the active derivative of retinol and is involved in embryonic development, vision, cell differentiation, organogenesis and immune regulation [94,95]. RA also induces apoptosis, *via* activation of the mitochondrial death pathway [96]. It also generates ROS and modulates the expression of antioxidant enzymes in rat sertoli cells and human retinal pigment epithelial cells [97,98]. In this respect it is interesting that RA increases Nrf2 nuclear accumulation as well as Nrf2 occupancy at AREs, leading to an overall increase in ARE reporter activity [99]. However while RA can upregulate antioxidant gene expression through the Nrf2 pathway, it can also function *via* the retinoic acid response element (RARE).

RA does not directly regulate transcriptional activities of target genes. Instead RA binds to a retinoic acid receptor (RAR) and retinoid X receptor (RXR) heterodimer [100,101]. RARs have three subtypes designated as RARα, RARβ, and RARγ and RXRs also have three subtypes known as RXRα, RXRβ, and RXRγ. The heterodimer then binds to the RARE and activates transcription of target genes [100,101]. RARE/RXR/PPRE sites have been mapped and characterised in the Trx and MSRA2 promoter regions (Fig. 1, Tables 2a, 2b).

8.1. The Trx gene promoter

Four consensus RAREs [9] are present in the Trx gene promoter [9]. However, site-directed mutagenesis revealed that only three of the RAREs are potentially functional [9] (Fig. 1). Mutation of the two proximal RAREs resulted in decreased basal activity of the Trx promoter in reporter assays and decreased activation by RA. Mutation of the third RARE (the most distal shown in Fig. 1) had less effect on gene expression and mutation of the fourth element (not shown in Fig. 1) had no effect. Foot printing activity assays confirmed that proteins bind to the two proximal sites in both RA treated and untreated cells, while the third RARE site is protected only when RA is added. The fourth potential site didn't show any protective foot printing activity [9]. Co-transfection of the Trx promoter with constructs expressing RARα resulted in a 9-fold increase in expression.

Interestingly an AP-1/ARE site is present at −446, in between the two proximal RARE sites. This may explain why the proximal RARE sites contribute to Trx promoter activity in the absence of RA, and why footprinting assays detect proteins bound to this region [9]. The ARE is bound by the NF-E1p45/small Maf complex during basal conditions while specific stimuli induce the binding of either Nrf2 or AP-1 [10]. Since RA increases nuclear accumulation of Nrf2 the co-localisation of the RAREs with the ARE may have functional significance that will be important to test in future experiments.

The peroxisome proliferator-activated receptor (PPAR) is another receptor that binds to the RXR to activate promoter activity. The PPAR has 3 different isoforms, α, γ, and β/δ that play a role in lipid and glucose metabolism, with effects on inflammation and cardiovascular health (reviewed in [102]). To regulate transcription of genes, PPARs bind to RXR to form a heterodimer, which then binds to the PPAR response element (PPRE) in the promoter of target genes [102].

A specific PPAR isoform, PPARα, forms a heterodimer with RXRα and increases Trx promoter activity by binding to a consensus PPRE located between −2185 and −2198 of the promoter [103]. Mutation of this element prevented the promoter from being activated by GW647, an agonist selective for PPARα. EMSAs confirmed that the PPARα/

RXRα heterodimer could bind to this element, but there has been no *in vivo* binding studies performed.

8.2. The MSRA gene promoters

The MSRA gene has two promoters: promoter 1 is the upstream promoter that regulates the MSRA1 transcript [26,104]; promoter 2 is the downstream promoter that regulates MSRA 2/3 transcripts [25,26]. Both MSRA promoters are regulated by RA [105]. Using luciferase reporter assays, a 65 bp region between −693 and −628 (from TSS) in D407 (RPE origin) cells was responsible for MSRA promoter 2 activity. Mutation analysis revealed three consensus RARE sequences within this region that are required for promoter activity, but as yet no element has been experimentally validated to bind a transcription factor. Intriguingly the potential is for three closely positioned sequences to respond to RA, similar to that found for the Trx promoter (Fig. 1).

Transient transfection of RA with MSRA promoter 1 and promoter 2 constructs resulted in an increase in promoter activity [105]. Overexpression of RARα, the most abundant RAR expressed in these cells, gave an increase in activity of both promoters when RA was added, although promoter 2 was more responsive to RA than promoter 1. Co-transfection with siRNA targeting RARα decreased the response of both promoters to RA. These findings suggest that the MSRA gene promoters are regulated by retinoic acid and that promoter 2, with a defined RARE, responds more vigorously to RA [105]. However it has not yet been established if any transcription factors are bound to these promoters *in vivo*, during stimulation.

8.3. Ref-1

Ref-1 is required for RARα to bind to RAREs [106] and since RA regulates several redox gene promoters, this is yet another example whereby one redox protein influences the expression of other redox gene promoters. EMSAs showed that RARs failed to bind to RAREs when Ref-1 function was inhibited in a dose dependent manner through the addition of E3330, a small molecule inhibitor of Ref-1 redox function [106,107]. However in the presence of reduced Ref-1, RARα binding to RARE is increased [106]. To date, no RAR/RXR binding sites have been mapped in the Ref-1 gene promoter; however there is one putative binding site detected in the Genomatix search (Fig. 1) that has not been validated.

The PRDX1–5 gene promoters also contain putative RXR binding sites, as obtained in the Genomatix search (Fig. 1, Table 3). A noteworthy aspect is that multiple putative elements are co-located in close proximity in the PRDX3 and PRDX4 gene promoters. As described above, RAREs are typically located close to each other. Therefore, further investigation may provide a better understanding if this type of arrangement influences the various RAR/RXR heterodimers to bind more effectively to induce transcriptional activity.

9. cAMP response element binding protein (CREB)

cAMP response element binding protein (CREB) is a cellular transcription factor that binds to the cAMP response element (CRE) [108]. As a second messenger, cAMP exists ubiquitously in the human body affecting cell metabolism, apoptosis, and cell proliferation and is also involved in responses to oxidative stress [109]. Potential CREB sites have been studied in the Trx [110] and Ref-1 [111] promoters (Fig. 1, Tables 2a, 2b).

9.1. The Trx gene promoter

Trx expression is induced by nerve growth factor (NGF) in PC12 cells [110]. Luciferase reporter assays identified a region of the Trx promoter, which contains a sequence similar to the CRE consensus [112]. When this region was mutated there was no NGF responsiveness. EMSAs confirmed that proteins bound to this sequence upon stimulation with NGF and an anti-CREB antibody abolished this binding [110]. However there have been no recent experiments reported to confirm whether this CREB binding has any physiological relevance or if CREB can bind to the Trx gene promoter under other stimuli.

9.2. The Ref-1 gene promoter

The Ref-1 gene promoter contains a CRE site that was shown using EMSAs to be bound by a complex consisting of c-Jun and activation transcription factor-2 (ATF-2) and this binding was enhanced when cells were treated with H_2O_2 [111]. An increase in Jun expression preceded that of Ref-1 mRNA induction, whereas ATF-2 expression was only slightly enhanced, suggesting that Jun may be the key component needed for Ref-1 induction by H_2O_2 through the CRE [111]. Ref-1 expression may also be regulated by CREB. Analysis of the data provided by a ChIP on chip analysis [113] revealed a strong signal, with a confidence level showing a p value < 0.001, for CREB binding to the Ref-1 promoter. This proposed binding has not been mapped to a precise site in the Ref-1 promoter.

Although only two of the redox gene promoters have been studied with respect to CREB sites [110,114], search results utilising the Genomatix programme revealed that putative CREB sites are present in all of the gene promoters analysed in this current study (Fig. 1, Table 3). In the ChIP on chip study it was reported that those promoters with predicted CRE sequences (from *in silico* analysis) were statistically over-represented for CREB binding [113]. Our analysis of their deposited data revealed that PRDX3, PRDX5, MSRA, TrxR and Ref-1 showed strong indications for CREB binding (p values less than 0.001) [113]. Whether CREB binding to any of these promoters is important to upregulate gene expression in response to specific stimuli is yet to be established.

10. Core promoter elements

Genes that lack a TATA box in their promoter region contain multiple elements involved in generating transcription activity. Those include GC rich regions and specific sites for binding of transcription factors such as Sp1, Oct-1 and AP-1. Sp1 is a member of the Sp-family of transcription factors [115] and TATA-less promoters often depend on Sp1 binding for transcription initiation [116–118]. Although TATA boxes are important to initiate transcription activity, Sp1 boxes are more abundantly present in gene promoters than are TATA boxes [119].

10.1. The Trx gene promoter

The Trx gene promoter has an unusual arrangement of transcription start sites and contains a TATA box that is functional, but not essential for transcription [120]. The first Trx TSS1 is located at −110 bp from the start of the Trx coding region and before the TATA box [13]. The second TSS2 is located at −75 bp from a consensus TATA box [121] and it is a more traditional transcription site since transcription normally commences downstream of a TATA box. However, both TSSs are utilised in a cell [120]. Luciferase reporter assays showed that transcription initiates even if the TATA element is mutated, suggesting that other transcription factors such as Sp1 are involved for basal level and oxidative stress induced expression.

The Trx gene promoter is extremely GC rich and contains three consensus Sp1 sites to which Sp1 can bind [122]. Co-transfection of a reporter plasmid containing the core Trx promoter (including these three Sp1 sites) with a construct over-expressing Sp1 increased Trx promoter activity more than 2-fold. In addition Trx alone or with the Trx system (comprising Trx, TrxR and NADPH) increases Sp1 DNA binding activity *in vitro* [122] suggesting the possibility that expression of the Trx gene may be regulated by the binding of Sp1, which itself may be regulated by Trx. Further *in vivo* experiments are required to verify this speculation.

10.2. The TrxR core gene promoter

The TrxR core gene promoter does not contain a TATA box or CAAT element, but it has other characterised transcription factor binding sites [123,124]. The two characterised ARE sites are also located within this core promoter [52,53]. There are also consensus Sp1 sites in the core promoter and EMSAs identified two sites where both Sp1 and Sp3 could bind and EMSAs also showed that Oct-1 can bind to sequences located near one of these sites [123]. An interesting aspect is that Sp1 and Oct-1 interact with each other to regulate gene expression [125] and both potentially bind to the core promoter of the TrxR gene [123].

10.3. The Ref-1 gene promoter

The Ref-1 gene promoter is regulated in a complex manner since it has bidirectional functionality with another gene. The gene encoding the O-sialoglycoprotein endopeptidase gene (OSGEP) commences transcription approximately 350 bp upstream from the Ref-1 main TSS, but in the opposite direction [126]. A similar arrangement is also present in the mouse genome [127]. Both promoters lack a TATA box, but contain CpG islands and both genes exhibit similar expression patterns. Luciferase reporter assays demonstrated that the same CCAAT box is required for both Ref-1 and OSGEP basal level expression [126].

Sp1 also regulates expression from the Ref-1 gene promoter. Zaky and colleagues showed that Ref-1 expression is down-regulated by p53 interfering with Sp1 binding to its site [128]. p53 is a tumour suppressor involved in DNA damage control, hypoxia and other tumour related processes [129,130] and is reported to be redox regulated, potentially through the Trx system and Ref-1 [35,36]. The influence of p53 on the Ref-1 gene promoter was assessed in human colon carcinoma cells. When the cells were treated with camptothecin, a genotoxic stress causing agent, the activated p53 downregulated Ref-1 by significantly reducing mRNA and protein levels [128]. ChIP assays showed that Sp1 recruits p53 to the Ref-1 promoter, which interferes with Sp1 binding to its *cis* element, thus decreasing Ref-1 expression [128].

10.4. Other redox gene promoters

Putative TATA boxes were only found in the MSRA1, PRDX1 and PRDX2 gene promoters (in addition to Trx). In contrast there are up to 13 Sp1 sites in each of the 11 gene promoters, although only three genes in this study (Trx, TrxR, and Ref-1) have characterised Sp1 sites to date (Fig. 1, Table 3). Since Sp1 plays an important role in transcription activation when the promoter lacks a TATA box, these Sp1 sites may play a vital role in regulating gene expression under both basal and oxidative stress stimulated conditions. While no Sp1 site has yet been characterised in the human PRDX gene promoters, curcumin induced the binding of Sp1 to a site in the mouse Prdx6 gene promoter, and this binding was verified by ChIP. There is an Sp1 site at the equivalent position in the human gene [131].

According to the Genomatix search Oct-1 potential consensus sites were found in several gene promoters (TrxR, MSRA2, PRDX3-5 and Ref-1) that do not contain a putative TATA box (Fig. 1, Table 3). Although it requires further examination, Oct-1 may also play a role in activating transcription of these other redox genes under either oxidative stress or basal conditions.

11. Other transcription factor binding sites relevant to redox control

11.1. NF-κB

One of the most noteworthy transcription factors is NF-κB, which plays an important role in inflammation and immune response in the cell, as well as regulation of cell growth, differentiation, development and apoptosis [132]. In addition NF-κB is heavily involved in maintaining cellular oxidative stress balance and its DNA binding ability is enhanced by Trx *in vitro* [3,133]. Six gene promoters out of the eleven analysed in this study contain up to three putative NF-κB binding sites in their promoter regions (Table 3). However, no NF-κB site has been functionally characterised in any of these promoters. This does not mean that NF-κB is excluded from functioning as a redox-regulator in these promoters. In fact, two NF-κB-binding elements in the mouse Prdx6 promoter were shown to bind NF-κB during oxidative stress conditions [134] and the mouse Prdx2 promoter is upregulated during muscle differentiation through a NF-κB binding site [135]. Therefore, it is possible that some of these putative NF-κB binding sites in the human redox gene promoters may also function to regulate cellular redox status against a specific oxidative stress stimulus, but these are yet to be defined.

11.2. Early growth response factor-1 (Egr-1)

Egr-1 is a zinc finger transcription factor that controls cell proliferation and apoptosis and is activated when cells are exposed to oxidative stress [136]. The DNA-binding activity of Egr-1 is reported to be redox-regulated. EMSAs, Ref-1 over-expression and co-immunoprecipitation experiments from an osteoblast cell line suggest that Ref-1 may be the reducing factor involved [137]. It is therefore interesting that ChIP experiments showed that Egr-1 binds to a region of the Ref-1 promoter that contains a consensus Egr-1 site situated approximately 80 bp downstream from the major TSS of the Ref-1 gene promoter, and overlapping with the third E-box [137] (Fig. 1, Tables 2a, 2b). Thus the authors proposed an as yet untested model with Ref-1 in an autoregulatory loop, whereby Ref-1 potentially activates Egr-1, which in turn regulates Ref-1 gene expression. In our study seven gene promoters (MSRA1, PRDX1, PRDX2, PRDX3, PRDX4, PRDX5 and Ref-1) contained putative Egr-1 sites, but except for Ref-1 none have been examined for any possible functional significance.

11.3. Metal response element (MRE)

MREs regulate the expression of heavy metal-responsive genes [138]. Recently Stoytcheva and Berry analysed the 25 human selenoprotein encoding genes and determined that 18 of the 25 gene promoters contain a putative MRE, including TrxR [124]. In our study six genes – Trx, TrxR, PRDX1, PRDX3, PRDX4 and PRDX6 – contain one putative MRE site each according to the Genomatix search (Table 3). To date none of these sites have been characterised and while TrxR expression is upregulated by cadmium, the effect was shown to be mediated by Nrf2 binding to an ARE site in the promoter region [53].

12. Conclusion

A rapid response to oxidative stress is required by the cell to prevent damage. The expression of Trx, TrxR and their major substrates is upregulated by many different oxidative stress causing stimuli. We have reviewed the experimentally defined binding sites for transcription factors in gene promoters that regulate expression of a subset of redox control proteins regulated by the Trx system. The ARE is present in several of these gene promoters and is bound by Nrf2 in response to similar oxidative stress stimuli, leading to the speculation that a coordinated response of the redox genes may occur. Other transcription factors also bind to these promoters under similar stress conditions, such as Forkhead and Ets, with the redundancy supporting the importance of the antioxidant response. Some transcription factors are in turn redox regulated, leading to the potential for autoregulatory loops, although as yet physiological evidence is lacking. Many putative transcription factor binding sites are yet to be investigated and where other information suggests a particular transcription factor may be a relevant regulator, the putative sites found in the *in silico* searches may be used to guide additional experiments. Since many of these redox proteins are upregulated during pathological conditions a better

understanding of the transcription factors that bind to their gene promoters may reveal co-regulatory mechanisms that can be targeted as therapeutic strategies. The transfer of the information gained from *in vitro* and cell culture studies to physiological relevance will be challenging, but is necessary to understand the full complexity of the antioxidant response.

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