The tert-butylhydroquinone-mediated activation of the human thioredoxin gene reveals a novel promoter structure

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Thioredoxin is a redox-active protein that plays multiple roles in regulating cell growth, cell signalling and apoptosis. Here, we have demonstrated that a complex mechanism involving multiple regulatory elements is involved in the tBHQ [tert-butylhydroquinone or 2,5-di-(t-butyl)-1,4-hydroquinone]-mediated activation of the thioredoxin gene. Luciferase assays, utilizing various wild-type and mutated thioredoxin promoter fragments, revealed roles for the ORE (oxidative stress responsive element), ARE (antioxidant responsive element), three Sp1 (specificity protein 1)-binding sites and the TATA box in the activation of the thioredoxin gene by tBHQ. The ORE required the presence of the ARE to elicit its response, whereas the independent removal of three Sp1-binding sites and the TATA box also decreased activation of the thioredoxin gene, with mutation of the TATA box having the greatest effect. Real-time RT (reverse transcriptase)–PCR analysis also revealed varying roles for two TSSs (transcription start sites) in the activation of the thioredoxin gene by tBHQ. Transcription was initiated from both TSSs; however, different response rates and fold inductions were observed. Together, these results suggest that the thioredoxin gene is controlled by a novel arrangement of two overlapping core promoter regions, one containing a TATA box and the other TATA-less. Altering the intracellular levels of thioredoxin in a breast cancer cell line also influenced the induction of thioredoxin transcription in response to tBHQ. Stable transfections with a redox-inactive thioredoxin mutant produced 3.6 times higher induction levels of thioredoxin transcription compared with control cells, indicating an intrinsic form of control of promoter activity by the thioredoxin system itself.

Key words: antioxidant responsive element, oxidative stress, specificity protein 1 (Sp1), tert-butylhydroquinone, thioredoxin, transcriptional activation.

INTRODUCTION

ROS (reactive oxygen species) are produced naturally by all aerobic organisms and in response to various stimuli including UV irradiation, X-rays and chemical carcinogens and cause both beneficial and detrimental responses within the cell. Beneficially, ROS are involved in normal cellular processes including intracellular signal transduction [1] and redox regulation of transcription factors [2]; however, they can also cause damage to biomolecules such as proteins [3], lipids [4] and DNA [5]. Additionally, excessive levels of ROS within the cell characterize the state of oxidative stress.

Oxidative stress disrupts the balance of the redox status in the cell and this can cause various cellular dysfunctions and cell death. One of the key systems involved in the maintenance of the intracellular redox state is the thioredoxin system [6]. The thioredoxin system comprises thioredoxin and thioredoxin reductase and plays an important role in many cellular functions including the synthesis of deoxyribonucleotides [7], redox control of transcription factors [8], cell growth and cancer [9] and protection against oxidative stress. The thioredoxin system protects the cell against oxidative stress by scavenging ROS through a variety of mechanisms. Directly, thioredoxin can quench singlet oxygen and scavenges hydroxyl radicals [10], whereas indirectly thioredoxin reduces thioredoxin peroxidase [11], an enzyme that scavenges hydrogen peroxide. Thioredoxin reductase is also capable of directly acting upon and reducing hydrogen peroxide [12]. When cells are subjected to oxidative stress, thioredoxin expression is elevated [13] and this up-regulation has resulted in a significant amount of research conducted to study the mechanisms by which the thioredoxin promoter is activated. Most of this research has focused on an ARE (antioxidant responsive element) [14] and an ORE (oxidative stress responsive element) [13] present within the promoter region of the thioredoxin 1 gene.

Activation of the thioredoxin gene through the ARE and the ORE has been investigated in response to many oxidative agents. The ARE has been found to respond to hemin [14], sulforaphane [15] and electrophiles, including tBHQ [tert-butylhydroquinone or 2,5-di-(t-butyl)-1,4-hydroquinone] [16], while the ORE was initially identified as the element responsible for activation of the thioredoxin gene in response to hydrogen peroxide, diamide and menadione [13]. The possibility of combined inductive action by the ARE and ORE has not been previously investigated, nor has the potential of these oxidative elements to function with other regulatory elements present within the thioredoxin promoter region been previously investigated. Characterization of the thioredoxin core promoter region has revealed many elements including three Sp1 (specificity protein 1)-binding sites [17], a TATA box [18] and two TSSs (transcription start sites) [17,18]. Since one of these TSSs is upstream from the traditional TATA box, it is possible that two different regulatory processes result in transcription of the thioredoxin gene in response to different stimuli or cellular development events. If so, this represents an unusual promoter arrangement.

We have investigated the role of the Sp1 and TATA elements, together with the ARE and ORE, in response to tBHQ. We report

Abbreviations used: ARE, antioxidant responsive element; ORE, oxidative stress responsive element; ROS, reactive oxygen species; RT, reverse transcriptase; Sp1, specificity protein 1; tBHQ, tert-butylhydroquinone or 2,5-di-(t-butyl)-1,4-hydroquinone; TFIID, transcription factor IID; TSS, transcription start site.

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here that there are complex interactions between these regulatory elements in the activation of the thioredoxin gene and propose a novel mechanism of gene regulation involving two overlapping core promoter regions. The relationship between oxidative stress, thioredoxin and the thioredoxin promoter was further investigated by demonstrating that altering thioredoxin intracellular levels affects induction of the thioredoxin promoter in response to oxidative stress-causing reagents.

### EXPERIMENTAL

#### Materials, cell lines and cell culture

tBHQ was purchased from Sigma (St. Louis, MO, U.S.A.). MDA-MB-231 (breast cancer cell line) cells were cultured in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin in 5% CO₂ at 37°C. Penicillin and streptomycin were not included in the media used in the transfections, real-time RT (reverse transcriptase)–PCR analysis and for growth of stable thioredoxin-transfected MDA-MB-231 cells.

#### Plasmids

The vectors in the transient and stable transfections were constructed as described below. Note all vectors containing thioredoxin promoter fragments are mapped with respect to the ATG translation start codon.

**pGL3-trxAREUp**

Oligonucleotides AREUp and T7 contained a KpnI site inserted 20 bp upstream of the ARE and amplified — 547 to — 46 bp of thioredoxin promoter region. PCR products were cloned into pGemT-Easy and excised using KpnI/BamHI. Excised fragments were cloned into the pGL3 basic vector using KpnI/BglIII.

**pGL3-trxAREDown**

XhoI was used to excise the — 2400 to — 427 bp fragment from the — 2400 to — 46 bp thioredoxin promoter region cloned into pBSKS. The remaining — 427 to — 46 bp region and pBSKS were re-ligated. The 381 bp thioredoxin promoter fragment was excised using KpnI/BamHI and cloned into pGL3 basic vector using KpnI/BglIII.

**pGL3-trxOREUp, delARE and pGL3-trxOREDown, delARE**

Oligonucleotides delARE and BasLucForward (where Luc is luciferase) were used to insert an XhoI site 15 bp upstream of the ARE and amplify — 1068 to — 524 bp and — 975 to — 524 bp in the thioredoxin promoter region. PCR products were cloned into pGemT-Easy, excised and used to replace the — 1068 to — 427 bp region in pGL3-trxOREUp and the — 975 to — 427 bp region in pGL3-trxOREDown using KpnI/XhoI. This cloning strategy ultimately removed the ARE from pGL3-trxOREUp and pGL3-trxOREDown.

**pGL3-trxAREUp**

Deletion of Sp1 and mutated TATA box constructs

Removal of the Sp1-binding sites and mutation of the TATA box in each of the thioredoxin reporter constructs involved the replacement of the — 376 to — 46 bp region with mutant fragments using SmaI/HindIII. The — 376 to — 46 bp region was cloned into pGL3 basic vector using SmaI and BamHI/BglIII; this construct was further altered to remove three Sp1-binding sites and to mutate the TATA box. Removal of the Sp1-binding sites was described previously [19]. Oligonucleotides mutTATA and BasLucForward mutated the TATA box, introduced a BamHI site 10 bp downstream of the TATA box and amplified the — 376 to — 86 bp fragment. The PCR products were cloned into pGemT-Easy, excised using SmaI/BamHI and cloned into the pGL3 basic vector using SmaI/BglIII.

**pTrx-wt and pTrx-1SS**

The full-length thioredoxin coding sequences encoding either wild-type protein or a mutant protein with both active site cysteine residues substituted with serine residues have been described previously [20]. These sequences were excised and subcloned into pcDNA3 (Invitrogen) under control of the CMV (cytomegalovirus) promoter to generate the plasmids pTrx-wt and pTrx-1SS respectively.

**Thio5′ construct**

A 330 bp SmaI/BamHI fragment containing the minimal thioredoxin promoter was ligated with a 275 bp BamHI/SphI fragment of the human thioredoxin cDNA clone into the pGEM3Z (Promega) vector.

### Transient transfections and luciferase assays

All transient transfections were carried out using Lipofectamine™ 2000 (Invitrogen, Melbourne, Australia) according to the manufacturer’s instructions. After a 6 h incubation at 37°C, 100 μM tBHQ was applied to all MDA-MB-231 cells. Cells were lysed...
RESULTS

Interaction between the ORE and the ARE

To investigate potential interactions between the ARE and the ORE, various lengths of the human thioredoxin promoter region (Figure 1) were cloned into luciferase vectors (Figure 2) and transiently transfected into the MDA-MB-231 breast cancer cell line. The reporter constructs investigated represented all possible combinations of the ORE and the ARE within the thioredoxin promoter region: pGL3-trxOREUp contained 1000 bp of the thioredoxin promoter region with both the ORE and the ARE present; pGL3-trxOREDown contained 950 bp of thioredoxin promoter region with only the ARE present; pGL3-trxAREUp contained 450 bp of thioredoxin promoter region with only the ARE present; pGL3-trxAREDown contained 450 bp of thioredoxin promoter region without either the ORE or the ARE; pGL3-trxOREUp,delARE contained 1000 bp of thioredoxin promoter region with both the ORE and the ARE present; pGL3-trxOREDown,delARE contained 950 bp of thioredoxin promoter region with the ORE present and the ARE deleted; pGL3-trxOREDown,delARE contained 950 bp of the thioredoxin promoter region without either the ORE or the ARE present. All of the constructs were transiently transfected into the MDA-MB-231 breast cancer cells with pGL3-Basic as the control. The luciferase activity of each reporter construct in response to tBHQ was measured and normalized against the untreated samples. Results are expressed as means ± S.D. and P values are indicated in the Figure legends.

The various lengths of the thioredoxin promoter fragments used in the luciferase (Luc) reporter constructs are shown and are mapped with respect to the ATG start codon. The presence or absence of the ORE and the ARE is indicated. Also shown are the three Sp1 sites and the TATA box, in front of the luciferase reporter gene. All of these constructs contained the Sp1 sites and TATA box. MDA-MB-231 breast cancer cells were transfected with the luciferase reporter constructs depicted, and treated with 150 µM tBHQ for 5, 12 and 24 h and samples were collected for mRNA extraction and cDNA synthesis. This dosing experiment was performed in triplicate.

Each of the reporter constructs was transiently transfected into the MDA-MB-231 breast cancer cell line. The reporter constructs investigated represented all possible combinations of the ORE and the ARE within the thioredoxin promoter region: pGL3-trxOREUp contained 1000 bp of the thioredoxin promoter region with both the ORE and the ARE present; pGL3-trxOREDown contained 950 bp of thioredoxin promoter region with only the ARE present; pGL3-trxAREUp contained 450 bp of thioredoxin promoter region with only the ARE present; pGL3-trxAREDown contained 450 bp of thioredoxin promoter region without either the ORE or the ARE; pGL3-trxOREUp,delARE contained 1000 bp of thioredoxin promoter region with both the ORE and the ARE present; pGL3-trxOREDown,delARE contained 950 bp of thioredoxin promoter region with the ORE present and the ARE deleted; pGL3-trxOREDown,delARE contained 950 bp of the thioredoxin promoter region without either the ORE or the ARE present. All of the constructs were transiently transfected into the MDA-MB-231 breast cancer cells with pGL3-Basic as the control. The luciferase activity of each reporter construct in response to tBHQ was measured and normalized against the untreated samples. Results are expressed as means ± S.D. and P values are indicated in the Figure legends.

Interaction between the ORE and the ARE

To investigate potential interactions between the ARE and the ORE, various lengths of the human thioredoxin promoter region (Figure 1) were cloned into luciferase vectors (Figure 2) and transiently transfected into the MDA-MB-231 breast cancer cell line. The reporter constructs investigated represented all possible combinations of the ORE and the ARE within the thioredoxin promoter region: pGL3-trxOREUp contained 1000 bp of the thioredoxin promoter region with both the ORE and the ARE present; pGL3-trxOREDown contained 950 bp of thioredoxin promoter region with only the ARE present; pGL3-trxAREUp contained 450 bp of thioredoxin promoter region with only the ARE present; pGL3-trxAREDown contained 450 bp of thioredoxin promoter region without either the ORE or the ARE; pGL3-trxOREUp,delARE contained 1000 bp of thioredoxin promoter region with both the ORE and the ARE present; pGL3-trxOREDown,delARE contained 950 bp of thioredoxin promoter region with the ORE present and the ARE deleted; pGL3-trxOREDown,delARE contained 950 bp of the thioredoxin promoter region without either the ORE or the ARE present. All of the constructs were transiently transfected into the MDA-MB-231 breast cancer cells with pGL3-Basic as the control. The luciferase activity of each reporter construct in response to tBHQ was measured and normalized against the untreated samples. Results are expressed as means ± S.D. and P values are indicated in the Figure legends.
these constructs contained the Sp1 sites and TATA box. Induction of luciferase expression from these reporter constructs in response to tBHQ was measured (Figure 2). Induction was measured 18 h after stimulation with tBHQ to allow our results to be compared with those published previously for the thioredoxin promoter by other workers [16].

A significant increase in gene expression in response to tBHQ was produced by the reporter constructs pGL3-trxOREUp, pGL3-trxOREDown and pGL3-trxAREUp, with a 16-, 10- and 12-fold induction respectively in gene expression. These findings showed that the presence of both the ORE and the ARE in pGL3-trxOREUp resulted in the highest fold induction of gene expression in response to tBHQ (16-fold), and that the removal of the ORE in pGL3-trxOREDown lowered this induction to 10-fold. Luciferase activity from the ARE-containing pGL3-trxAREUp resulted in a 12-fold induction of gene expression, contrasting with the removal of the ARE in pGL3-trxAREDown, which produced a substantially lower 2-fold induction. This significant decrease in induced gene expression clearly demonstrates the importance of the ARE to oxidative stress-mediated thioredoxin activation; however, the ORE also plays some role in the response to tBHQ.

The reporter constructs pGL3-OREUp.delARE and pGL3-OREDown.delARE were used to determine if any interactions were occurring between the ORE and the ARE, or if their responses to tBHQ were independent of each other. Removal of the ARE from both reporter constructs saw a significant decrease in gene expression induction, compared with the results achieved from the reporter construct without either the ARE or the ORE, pGL3-trxAREDown. This suggested that without the ARE in the thioredoxin promoter region, the ORE was unable to induce gene expression above basal levels, indicating that the ORE either did not have a role in oxidative stress-induced gene expression or that the ORE requires the ARE for its regulation. To provide additional evidence for an interaction between the ORE and the ARE, further analyses of the fold inductions produced by pGL3-OREUp and pGL3-trxOREDown in response to tBHQ revealed a consistently higher ratio of increased induction in the presence of the ORE. Five independent experiments using transfections performed in triplicate consistently produced a higher tBHQ fold induction in gene expression from pGL3-trxOREUp compared with pGL3-trxOREDown, with the presence of the ORE increasing fold induction by an average 1.6 times.

Based on the above results, it appears that the ARE can function independently of the ORE; however, the ORE can enhance the effectiveness of the ARE in the activation of the thioredoxin gene in response to oxidative stress caused by tBHQ.

Figure 3  Analysis of the ORE, Sp1-binding sites and TATA box in tBHQ activation of the thioredoxin gene

Maps of the thioredoxin promoter fragments used in the luciferase (Luc) reporter constructs are shown depicting the absence or presence of the ORE, ARE, TATA box or Sp1-binding sites. MDA-MB-231 breast cancer cells were transfected with the luciferase constructs as illustrated, and treated with 100 µM tBHQ. Values shown are the average fold inductions of luciferase activity from each reporter construct in response to tBHQ, calculated from luciferase expression in untreated cells. Two independent experiments were each conducted in triplicate. Results shown are expressed as the means ± S.D. ANOVA statistical analyses were used, followed by post hoc comparisons using Tukey’s procedure. The symbol ‘*’ indicates statistically significant difference compared with pGL3-trxOREUp (P < 0.03) and ‘**’ indicates P < 0.08. The symbol ‘#’ indicates statistically significant difference compared with pGL3-trxOREDown (P < 0.12), while ‘##’ represents statistically significant difference compared with pGL3-trxOREDown (P < 0.02).

The reporter constructs utilized in these experiments represented various combinations of the ORE, ARE, Sp1-binding sites and the TATA box (Figures 3 and 4). Expression from these constructs in MDA-MB-231 cells revealed a role for the Sp1-binding sites, and to a greater extent, the TATA box, in the oxidative stress activation of the thioredoxin gene. Removal of the Sp1-binding sites lowered the combined inductive action of the ORE and the ARE from 16-fold (pGL3-trxOREUp) to 9-fold (pGL3-trxOREUp.delSp1); mutation of the TATA box, however, diminished induction significantly (pGL3-trxOREUp.mutTATA) (Figure 3). The OREDown construct, which does not contain the ORE element, was also further analysed by either removing the Sp1 sites or mutating the TATA box. Induction with tBHQ was decreased from 10-fold (pGL3-trxOREDown) to 5-fold when the Sp1 sites were removed (pGL3-trxOREDown.delSp1), to 2-fold when the TATA box was mutated (pGL3-trxOREDown.mutTATA) (Figure 3).

These findings suggest a role for both the Sp1-binding sites and the TATA box in the oxidative stress activation of the thioredoxin gene. Action of the ORE and the ARE with Sp1 and the TATA box revealed a dependence of these responsive elements on the traditional regulatory elements. Activity from the ORE was also investigated independently of the ARE, with removal of the Sp1-binding sites and mutation of the TATA box in pGL3-OREUp.delARE and pGL3-OREDown.delARE (results not shown). No significant induction was achieved from any of these constructs, further emphasizing the dependence of the ORE on the ARE for its oxidative stress response.

It is also important to note that all of the constructs utilized in the experiments described above still retained some promoter activity, as shown in Figure 4. The mutation of the TATA box reduced expression levels in uninduced cells, but was still clearly
functioning as a promoter. As reported previously [14], deletion of the ARE also reduced basal expression levels, as did deletion of the Sp1 sites [19] but in all cases the promoter was still functional and yielded luciferase values of statistical significance above that of the pGL3 basic vector. In addition, the highest fold inductions were observed from constructs that had the highest basal level of expression, thus emphasizing how powerful the induction of the thioredoxin promoter is in response to stimulation with tBHQ.

Transcription initiation from TSS1 (the first TSS) and TSS2 in response to tBHQ

Two TSSs have been identified in the thioredoxin promoter region. The TSS1 is located at or around −110 bp [17], while TSS2 (the second TSS) is located downstream at −74 bp [18]. While alternative TSSs are not uncommon, particularly in TATA-less promoters, in the thioredoxin promoter one site is upstream from a TATA box while the other site occurs 28 bp downstream from the TATA box in the traditional position. Other researchers have reported the existence of other transcripts originating from the TSS1 region [22] and as shown above the TATA box clearly has a role in regulating transcription, providing further support that TSS2 (originally mapped by primer extension [18]) is also a valid transcription initiation site. To investigate whether transcription is induced from one or both TSS regions in response to oxidative stress, we performed real-time RT–PCR on extracts from MDA-MB-231 breast cancer cells treated with 150 µM tBHQ for various lengths of time.

Two different forward primers were used to amplify the transcripts generated from both TSSs. As shown in Figure 1, the forward primer Trx.TSS1 amplified transcripts beginning only at TSS1; the forward primer Trx.TSS2, however, amplified transcripts beginning at both TSS1 and TSS2. Hence, we were unable to direct the amplification of transcripts beginning only at TSS2, since the TSS2 sequence is also located in the transcript generated from TSS1. Therefore the number of transcripts originating from the downstream start site was determined by subtracting the number of longer transcripts (calculated from results obtained with the forward primer Trx.TSS1) from the numbers obtained with the forward primer Trx.TSS2. The amplification efficiency of the two primer pairs was measured to be statistically similar for these subtraction analyses to be statistically valid.

The results (Figure 5) indicate a maximum 3.4-fold induction of transcription from the upstream transcription initiation region TSS1 in response to tBHQ at 12 h post-oxidative stress. Induction from the downstream transcription initiation region occurred at a
significantly higher level with approximately a maximum 14-fold induction level. These results indicate a role for both TSSs in the induction of thioredoxin transcription in response to oxidative stress, although the downstream transcription initiation site is utilized in most of the induced transcripts.

While precise comparisons between real-time RT–PCR data of endogenous mRNA expression and data obtained with reporter activity assays are difficult to make due to time-lags experienced after construct transfection [23], the same trends would be expected. Thus the tBHQ induction experiments were repeated for the most highly induced reporter constructs but using a much shorter time frame of 6 h. Fold induction values for the constructs pGL3-trxOREUp, pGL3-trxOREDown and pGL3-trxAREUp were statistically significantly lower at 5 h than at 18 h (Figure 6). This correlates with the real-time RT–PCR data, which shows that the maximum level of endogenous mRNA induction occurs at 18 h and that at 6 h a maximum rate has not yet occurred.

While none of the constructs used for the reporter assays can solely direct transcription initiated from TSS2, mutation of the TATA box should have no or little effect on TSS1 transcripts (as they initiate upstream of the TATA box). Therefore the induction of two constructs that have a mutated TATA box (pGL3-trxOREUp.mutTATA and pGL3-trxOREDown.mutTATA) was also tested. Fold induction for these constructs was found to have no statistically significant difference between 6 and 18 h (Figure 6), which again correlates with the real-time RT–PCR data where the long transcript (TSS1) expression levels were increased at 6 h and then did not increase much above those levels, whereas the shorter transcripts (TSS2) continued to increase to the highest induced levels at 18 h (Figure 5).

### Thioredoxin promoter activity in response to altered thioredoxin functionality

To investigate how altered levels of intracellular thioredoxin affect the responsiveness of the thioredoxin gene promoter to oxidative stress, the MDA-MB-231 breast cancer cell line was stably transfected with a control vector or constructs expressing either a wild-type human thioredoxin (pTrx-wt) or a redox-inactive form of human thioredoxin (pTrx-1SS). These stably transfected cell lines were then transiently transfected with a luciferase reporter construct containing the full thioredoxin promoter (pGL3-trxOREUp) and luciferase assays were used to monitor the activity of the promoter in these transfected cells when subjected to tBHQ-induced oxidative stress.

Three clones of each stably transfected cell line were subjected to numerous luciferase assays using the pGL3-trxOREUp construct. Activation of the thioredoxin gene promoter occurred in all cell lines in response to tBHQ and the data are presented in Figure 7 as a ratio of the average induction in the stably transfected pTrx-wt or pTrx-1SS-transfected clones as compared with the control (pcDNAIII) transfected clones (defined as 1). Results were obtained from four independent transient transfections utilizing three clones from each stable transfection. ANOVA statistical analysis was used, followed by post hoc comparisons using Tukey’s procedure. Results shown are expressed as the means ± S.D. and the symbol *** indicates significant difference between the Trx.1SS construct-transfected clones and the control plasmid transfected clones with $P < 0.007$.
were observed from pGL3-trxOREUp in both the control and ptrx-wt cell lines; however, the ptrx-1SS cell lines produced an average 3.6 times higher fold induction in response to tBHQ than the control or pTrx-wt-transfected cell lines. These findings suggest that cells with impaired thioredoxin functionality display greater sensitivity to tBHQ induction of thioredoxin promoter activity than do cells without such impairment.

**DISCUSSION**

In the present study, we have shown that the ARE and the ORE in the thioredoxin promoter region interact, not only with each other but also with other regulatory promoter elements, to activate thioredoxin expression in response to tBHQ-induced oxidative stress. Previous studies demonstrated the activation of thioredoxin through the ARE in response to tBHQ [16] and through the ORE [13] in response to hydrogen peroxide. Here, we report that the tBHQ-induced activation of the thioredoxin gene can also involve the ORE. Our luciferase assays revealed that the ARE can function independently of the upstream ORE; however, the ORE required the presence of ARE within the thioredoxin promoter region to induce luciferase gene expression. This action by the ORE requires further investigation to determine if the ORE functions to enhance binding of factors at the ARE, or if the ORE is part of a more complex regulation of the thioredoxin gene in response to tBHQ-induced oxidative stress. While the factors binding to the ARE element in response to tBHQ activation have been identified to be Nrf-2 (NF-E2 related factor 2)/small mal heterodimers [14], the protein factors that bind to the ORE [13] have not yet been identified.

Activation of the thioredoxin gene in response to oxidative stress was also found to rely on the TATA box, and to a lesser extent, on three Sp1-binding sites located in the thioredoxin promoter region. It appears as though the transcription factors that activate gene expression from the ORE and the ARE largely require the TATA box to initiate this transcription. In TATA-less promoters, Sp1 generally assumes the role of transcription initiation [24] and previous investigations using luciferase assays showed that the removal of the three Sp1-binding sites [19] from a thioredoxin promoter fragment (~376 to ~46 bp) significantly lowers basal transcription levels. Although the present study showed that the Sp1-binding sites were not as important to the oxidative stress induction as the TATA box, they do play some role in the induction. Also, it has been found that oxidative stress activates Sp1 by enhancing its acetylation [25], further supporting a role for the Sp1-binding sites in the oxidative stress-induced activation of the thioredoxin gene promoter.

Further investigations using real-time RT–PCR methods to examine the induction of thioredoxin transcription in response to oxidative stress revealed roles for two TSS regions located at −110 bp [17] and −74 bp [18] in the thioredoxin promoter region. The TSS1 is located at −110 bp and is 72 bp downstream of three Sp1-binding sites, but upstream from the TATA box. The TSS2 is located at −74 bp, which is 28 bp downstream of a TATA box. Our results showed that transcription from TSS1 is induced in response to tBHQ by a maximum 3.4-fold induction at 12 h post-oxidative stress, which is similar to the induction levels observed at 5 h. Transcription from TSS2 was found to exhibit a maximum 14-fold induction of transcription in response to tBHQ at 12 h post-oxidative stress. Since TSS2 lies downstream from the TATA box, it would be consistent if most of the transcription in response to oxidative stress is induced from TSS2, as the TATA box was shown to be crucial to tBHQ-mediated thioredoxin transcription. The regulatory elements upstream of TSS1 are three Sp1-binding sites and while removal of these Sp1-binding sites lowers the transcriptional response, mutation of the TATA box has a far greater effect on decreasing transcription induction. This was also apparent when constructs that lacked the TATA box were assessed for luciferase activity at 6 h after tBHQ induction. These constructs displayed the same low fold induction at 6 h as that observed at 18 h, thus correlating with the real-time RT–PCR data obtained for the long (TSS1-initiated) transcripts. In contrast, the highly inducible constructs, which can direct both long and short (TSS2-initiated) transcripts, had not yet reached maximal induction levels at the 6 h time measurement.

The real-time RT–PCR data indicate that while both TSSs are involved in the tBHQ activation of the thioredoxin gene, most of the thioredoxin transcription in response to tBHQ arises from TSS2 that is potentially under the control of both the upstream TATA box and Sp1-binding sites. Transcription initiation from TSS1, although apparently lower than that from TSS2, possibly occurs from a TATA-less promoter region that is controlled by Sp1-binding sites. Thus these findings suggest that the thioredoxin promoter region may comprise two overlapping promoter regions, one containing a TATA box and one TATA-less.

The structure of core promoter regions has often been overlooked when gene regulation mechanisms are studied for inducible genes. The core promoter is often assumed to be of little importance in dictating diverse gene expression patterns due to a belief that the core promoter has a relatively fixed structure. In addition, a common misconception still prevalent today is that most of the core promoters contain a TATA box, whereas it is more likely to occur in less than 40% of human genes [26, 27]. However, overall gene regulation mechanisms are now believed to be far more complex with the upstream factors proposed to interact differently with the core promoter region depending on its structure [28, 29], which may include a TATA box, TATA-less structures, CpG islands (dispersed regions of DNA with a high frequency of the CpG dinucleotide), INR (Initiator) and/or DPE (downstream core promoter element) elements, without any one element being universally present. One suggested explanation is that the conformation of the TFIIID (transcription factor IID) complex is altered depending on which core promoter elements are present and this in turns alters the activation potential of the gene by upstream enhancer elements [30]. The proteins that bind to these upstream elements regulate many genes including the possibility of regulating more than one adjacent gene. By interacting differently with the various core promoter elements, some differential gene regulation can be achieved. The thioredoxin gene thus exhibits an unusual arrangement in having two different forms of core promoter structure overlapping with each other. If there is a change in conformation of the TFIIID transcription complex binding to different elements within a core promoter, it may enable the thioredoxin promoter to be efficiently responsive to multiple transcription factors capable of binding to upstream promoter elements when stimulated by cellular events. Depending on their preference for particular conformations, these upstream binding factors could act via one or both of the core transcription complexes binding to the thioredoxin promoter, thereby allowing the thioredoxin gene a greater opportunity to be strongly activated in preference to other gene promoters containing a less preferred core promoter structure.

Other genes also exhibit differential promoter usage, but in most cases the two promoters are separated by some distance, and are not completely overlapping as in the case of the thioredoxin promoter. One other example, however, the MHC class I gene promoter [31], also exhibits an unusual promoter arrangement with two TSS regions situated closely together. In this promoter, basal level expression is initiated predominantly through the upstream
sites, whereas activated transcription is initiated from the down-stream sites. In addition, different promoter elements were found to have different roles in basal versus activated transcription and it was further suggested that the composition of basal and activated transcriptional complexes may be distinct. For this gene promoter, Sp1 was only required for basal level expression and not for activated transcription, whereas for the thioredoxin gene promoter, the Sp1 sites appear to play some role in both basal and oxidative stress-induced transcription.

The promoter region of the mouse eIF-1A [eukaryotic (translation) initiation factor 1A] gene [32] also contains two closely situated TSSs that are regulated differently. However, these sites are separated by 628 bp, representing distal and proximal promoter regions, whereas TSS1 and TSS2 in the thioredoxin promoter region are only separated by 36 bp and thus represent overlapping promoter regions. Studies on the promoter of the mouse eIF-1A gene [32] reveal a switch in promoter utilization during pre-implantation development with the TATA-less promoter becoming more efficiently utilized relative to the TATA-containing promoter. The switch in promoter usage is postulated to be a result of DNA methylation, producing transcriptionally inactive chromatin, increased levels of Sp1 during pre-implantation development and/or the increased expression of a co-activator protein that bridges Sp1 with the TATA-binding protein during transcription initiation. Alternatively, it could be due to a difference in conformation of the transcription complexes bound to the two promoter regions, as proposed above.

The observed use of the upstream transcription initiation region TSS1 in the thioredoxin promoter region in response to tBHQ possibly correlates with an increase in Sp1 activation that accompanies oxidative stress [25]. Further studies are required to investigate the use of the two TSSs in the thioredoxin promoter region so as to confirm the alternate use of the TATA-containing and TATA-less promoters, not only in response to oxidative stress, but during development and cancer progression. In addition, by acting through alternative core promoter elements, this differential gene regulation occurs without any change to the coding potential of the thioredoxin gene and without expending any extra energy in alternative splicing or alternate promoter usage that involves transcription of longer 5'-untranslated regions. It is possible that the thioredoxin promoter has evolved to contain two different strategies for initiating thioredoxin expression, perhaps in response to different stimuli or to provide redundant mechanisms of gene activation. Studies on the thioredoxin promoter are therefore not solely of interest to researchers studying this antioxidant system, but also to researchers studying the mechanisms by which a core promoter is activated by multiple stimuli.

With an increased understanding of how oxidative stress initiates expression of the thioredoxin gene, our investigation progressed to study how altering the functionality of the thioredoxin system itself affected the ability of the cell to regulate thioredoxin promoter activity. MDA-MB-231 breast cancer cells were stably transfected with constructs expressing wild-type thioredoxin, and a redox-inactive thioredoxin mutant. The responsiveness of these cells to tBHQ was subsequently tested by transiently transfecting the stable clones with a thioredoxin reporter construct containing both the ARE and the ORE.

Our findings indicated that the clones expressing the redox-inactive thioredoxin mutant had the more responsive thioredoxin promoter, as the highest magnitude of expression induction was observed in these cells. Previous studies [33] show that this particular redox-inactive thioredoxin mutant competes with wild-type thioredoxin for thioredoxin reductase and consequently reduces the functional capacity of the thioredoxin system in the cell. While all clones exhibited tBHQ-mediated induction, there was no difference in the level of the response to tBHQ between the control cells or the cells expressing wild-type thioredoxin. This could be explained by the fact that breast cancer cell lines including MDA-MB-231 cells are known to express high levels of thioredoxin [34] and so the control cells may have more than adequate levels of thioredoxin system functionality and therefore incorporating the wild-type thioredoxin construct (pTrx-wt) makes no difference to tBHQ stimulation of thioredoxin promoter activity. Thus the responsiveness of the thioredoxin gene promoter to tBHQ-induced oxidative stress may be modulated by the prevailing functionality of the thioredoxin system itself within the cell.

In summary, the results obtained from this investigation present evidence for a complex mechanism of thioredoxin gene regulation in response to tBHQ that involves two overlapping promoter regions that incorporate two TSSs, three Sp1-binding sites and a TATA box, in conjunction with an upstream ARE and ORE. We observed significant fold induction of transcription through the ARE and the ORE in response to tBHQ, suggesting an involvement of the ORE in the tBHQ-mediated activation of the thioredoxin gene. Furthermore, the crucial role of the TATA box in facilitating induction of thioredoxin transcription in response to tBHQ and to a lesser extent the importance of three Sp1-binding sites was evident. Additionally, two TSSs are interwoven into this mechanism of gene regulation, inducing transcription of thioredoxin at different rates and to different degrees of magnitude, suggesting the presence of two promoter regions. These regions appear to overlap and represent a TATA-containing promoter region and an upstream TATA-less promoter region that may be alternatively utilized in the response to oxidative stress. Further studies are required to confirm the function of these two overlapping promoter regions in the control of the thioredoxin gene.

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REFERENCES
