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Occupancy and synergistic activation of the *FMR1* promoter by Nrf-1 and Sp1 *in vivo*

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Fragile X syndrome is due to mutation of the *FMR1* gene. The most common mutation is an expansion of a CGG repeat in the 5′ UTR that triggers dense DNA methylation and formation of a heterochromatin-like structure which lead to transcriptional silencing. *In vitro* experiments have identified several transcription factors, including Sp1, Nrf-1 and USF1/2, as potential regulators of normal *FMR1* promoter activity. Using CpG methylation-deficient *Drosophila* cells, we demonstrate *in vivo* that Nrf-1 and Sp1 are strong, synergistic activators of an unmethylated human *FMR1*-driven reporter, while USF1/2 and Max repress this activation. In addition, analyses of transcription factor activity upon DNA methylation of the reporter show that Sp1 activity was largely intact when the promoter was densely methylated, but Nrf-1 transactivation was very sensitive to dense methylation. Notably, Nrf-1 transactivation was relatively insensitive to methylation of cytosines only at its binding site. *FMR1* reporter activity is also reduced in HeLa cells after expression of a short interfering RNA directed against endogenous Nrf-1. Using chromatin immunoprecipitation, we demonstrate directly that Sp1 and Nrf-1 occupy the human *FMR1* promoter *in vivo* and these interactions are disrupted in fragile X patient cells. In addition, we discover that Max resides at the *FMR1* promoter and show that USF1/2 but not c-Myc are present at endogenous *FMR1*. These findings provide the first direct *in vivo* evidence identifying the specific transcription factors that regulate *FMR1*.

INTRODUCTION

Fragile X syndrome is the most common form of inherited mental retardation (reviewed in 1). The disease is caused by the lack of functional FMRP, an RNA binding protein. The vast majority of fragile X cases are due to transcriptional silencing of *FMR1*. This occurs through expansion of a CGG repeat in the 5′ UTR of *FMR1* and dense methylation of the CpG island promoter (2–6). There are four *in vivo* footprints in cells expressing *FMR1* (7,8). The proteins that occupy these footprints are unknown but the DNA includes two GC boxes (Sp1 and Sp1-like sites), a palindromic sequence that binds Nrf-1 (also known as α-Pal) *in vitro* and an E-box, CACGTG, which binds USF1 and USF2 *in vitro* (9). A promoter fragment containing 272 base pairs upstream of the transcription start site and 193 bases downstream of the start site that includes these four footprints is sufficient for high level expression in reporter assays in HeLa cells (10). Associated with the expansion of repeats and methylation of the *FMR1* promoter is the condensation of the chromatin and absence of *in vivo* footprints in the promoter demonstrating the loss of DNA binding by transcription factors (8,7,11,12). Mutation of these sites in reporter plasmids results in a loss of promoter activity (9,10,13).

Although they have been implicated by the presence of their respective binding sites, the exact involvement of these transcription factors and the mechanism by which they fail to bind to their sites in the *FMR1* promoter in fragile X cells, is not known (7,8). While the activity of some transcription factors, such as Sp1, have been shown to be methylation-insensitive (14), transcriptional silencing may occur through direct inhibition of activator binding by methylation, as has been shown for the cAMP-responsive element binding protein (CREB) (15). Indeed, it has been reported that methylation of cytosines in the Nrf-1 recognition site reduces its *in vitro* binding to the *FMR1* promoter with a commensurate reduction in transcriptional activation (9).

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An indirect mechanism for transcriptional silencing of genes may result through the binding of methyl-CpG binding proteins (MBDs) which in turn, recruit repressive complexes including histone deacetylases (HDACs) and histone methyltransferases and form chromatin structures that render promoter DNA inaccessible to transcriptional activators (16–19). Methylation of the *FMR1* promoter following CGG expansion renders *FMR1* DNA in fragile X cells poorly accessible to restriction endonuclease digestion (12). In addition, histones H3 and H4 are hypoacetylated in fragile X cells, implicating HDACs and their partners, MBDs, in *FMR1* silencing (11). *FMR1* transcription can be reactivated in fragile X cell lines with 5-azadC which causes passive demethylation of the DNA and with a combination of 5-azadC and trichostatin A, which inhibits HDACs, though reactivation is not to normal levels (11,20–23). In patient cells, *FMR1*-associated histones are hypermethylated at lysine 9 of histone H3, a feature of heterochromatin (20). The relative contribution of direct inhibition of transcription factor binding by DNA methylation of *FMR1* and the indirect establishment of repressive chromatin through methyl cytosine binding proteins is unclear.

Drosophila Schneider 2 (SL2) cells have been used to study several human promoters (24–29). They are advantageous for this purpose because they lack homologs of several human transcription factors, yet can support transcription driven by ectopically expressed human versions of these proteins (24–29). *Drosophila* naturally lacks the Sp family of proteins, which are implicated in *FMR1* transcription (25,30). In addition, the known *Drosophila* homolog of Nrf-1, *ewg* is not expressed in SL2 cells (31,32). These cells have also been used to study upstream stimulatory factors (USF) proteins (29). Thus, SL2 cells provide a system in which to systematically test the functions of transcription factors at the human *FMR1* promoter (24–32).

Kudo (25) has successfully used cultured *Drosophila* SL2 cells to study DNA methylation-mediated, silencing of transcription. These cells are advantageous for this purpose because they are deficient in significant CpG methylation, DNA methyltransferases and methyl cytosine binding proteins, yet they possess chromatin remodeling and histone modifying enzymes (33–35). This enables the separation of the direct effects of DNA methylation from the effects of methyl cytosine binding proteins on transcription factor activity *in vivo* (25).

Here we show that Nrf-1 and Sp1 are potent and synergistic activators of transcription from an *FMR1*-driven reporter in *Drosophila* SL2 cells. We find that synergy is sensitive to selective methylation of Nrf-1's binding site but Nrf-1 still activates transcription efficiently from the methylated site. Dense methylation of the transfected DNA, as in fragile X syndrome, represses Nrf-1-dependent activation but Sp1 still activates transcription. We targeted endogenous Nrf-1 using RNA interference in HeLa cells, showing it is important for at least 50% of *FMR1* promoter activity. We also find that Max and USF1/2 repress Nrf-1 and Sp1 activation of the *FMR1* promoter in Schneider 2 cells. Finally, we directly show that the transcription factors Nrf-1, Sp1 and E-box binding proteins USF1/2 and Max bind the endogenous *FMR1* promoter in normal cells but not fragile X cells.

RESULTS

Sp1 and Nrf-1 work synergistically to activate *FMR1*

In vivo footprinting suggests that at least four DNA-binding proteins associate with the wild-type *FMR1* promoter (Fig. 1) (7,8). The conserved sequence of these sites and *in vitro* DNA binding assays suggest that the operative transcription factors could include Sp1/Sp3, c-Myc, Max, USF1/2, Nrf-1, CREB and AP2 (7–9,13,36). We tested the activity of five such candidate transcription factors, Sp1, USF1 and 2, Max and Nrf-1, by co-transfecting expression vectors encoding the human versions of these proteins along with an *FMR1*–firefly luciferase reporter plasmid into *Drosophila* SL2 cells. To control for transfection efficiency, a plasmid containing the *Renilla* luciferase gene driven by the *Drosophila* actin promoter (pRL-dA5C) was included in all transfections and firefly luciferase activity was normalized to *Renilla* luciferase.

In the absence of added transcription factor expression vectors, *FMR1* promoter activity in SL2 cells was no more active than a vector lacking a promoter (data not shown). Introducing an α–Pal/Nrf-1 expression vector activated the *FMR1* promoter 18-fold (Fig. 2, bar 2 versus bar 1) but did not activate the promoterless vector (data not shown). Transfection of an Sp1 expression vector had a much larger effect and increased transcription of the co-transfected *FMR1* promoter >100-fold (Fig. 2, bar 3 versus bar 1), but did not increase luciferase activity from the empty vector (data not shown). Co-expressing Nrf-1 and Sp1 synergistically activated the *FMR1* promoter >250-fold (Fig. 2, bar 4 versus bars 2 and 3), showing that Nrf-1 and Sp1 can act together to stimulate *FMR1* transcription.

Sp1 and Nrf-1 transactivation are relatively resistant to methylation of the Nrf-1 recognition site

Next, we tested the effect of methylating the transfected reporter plasmid upon the activity of the transcription factors. Previous work has shown that extensive methylation of *FMR1* reporter constructs, represses promoter activity in HeLa and COS-1 cells (10,37). More limited methylation of the Nrf-1 site alone represses transcription by half in mammalian cells (9). Since SL2 cells lack functional MBDs, we could dissect repression due to MBDs from direct interference of transcription factor function by DNA methylation (33).

The *FMR1* reporter plasmid was methylated with *Bss*HII methylase. The only target sites on the plasmid for this methylase (recognition site 5′-GCGCGC-3′) were three cytosines (on each strand) in the Nrf-1 recognition motif (Fig. 1). Their resistance to *Bss*HII endonuclease digestion confirmed the complete methylation of these sites (data not shown). Surprisingly, the *Bss*HII methylated reporter was reproducibly 3-fold more active in SL2 cells than the mock methylated plasmid (Fig. 2, bar 1 versus bar 5). Though the basis for this effect is unclear, it is a feature of *Drosophila* SL2 cells, since the *Bss*HII methylated pFMR1-luc construct was repressed to ~50% the activity of a mock methylated reporter plasmid in mammalian PC12 (9) and COS-7 cells (data not shown). When a Nrf-1 expression plasmid was co-transfected, a comparable level of reporter activity was observed for methylated and unmethylated *FMR1* promoter DNA (Fig. 2,

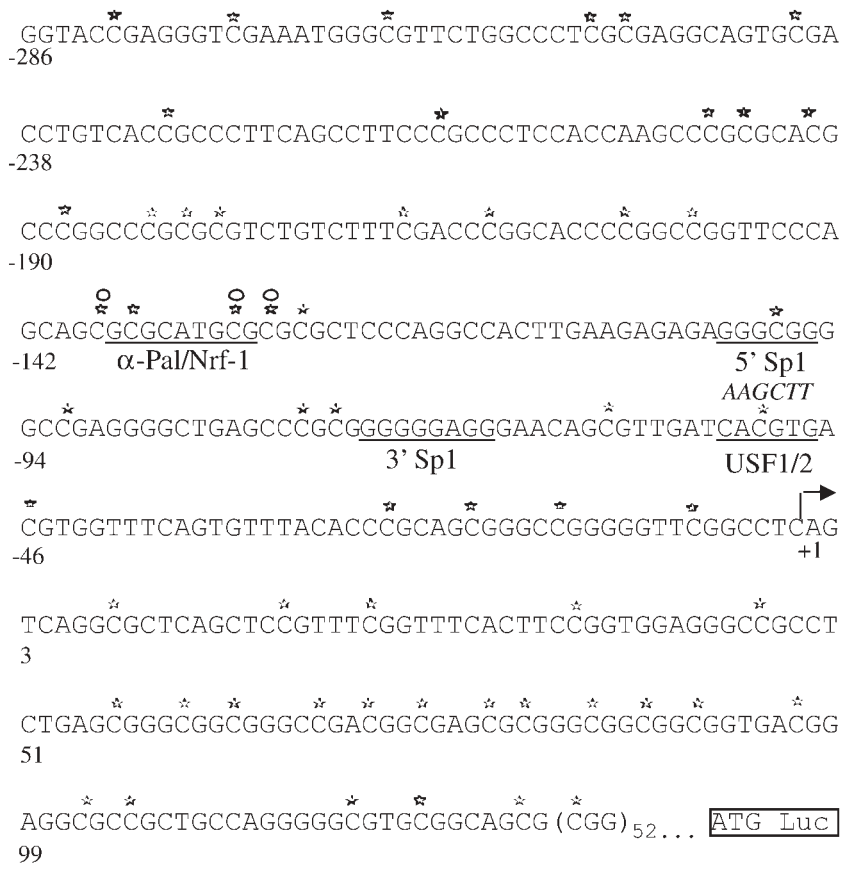


Figure 1. Promoter sequence of human *FMR1*. *In vivo* footprints (7) are underlined and cognate transcription factors previously shown to bind the *FMR1* promoter *in vitro* are listed underneath each site. Cytosines modified by *Sss* I methylase are marked with an asterisk. Cytosines methylated by *Bss* HII are marked with a circle. The major transcription start site is indicated with a bent arrow (57). The start of the luciferase reading frame is indicated by boxed 'ATG Luc'. The sequence of the mutated E-box used in transfection assays is shown in italics above the binding site.

bar 6 versus bar 2). However, when each was expressed relative to the respective methylated or unmethylated reporter alone (Fig. 2, bars 5 and 1), Nrf-1's stimulation of the methylated promoter was 2.5-fold less than that of the unmethylated reporter (6.8- versus 18-fold) due to the higher activity of the methylated reporter over the unmethylated reporter in the absence of transcription factors (Fig. 2, bar 5 versus bar 1). On an absolute basis, methylation had no effect on Nrf-1-dependent reporter activity (Fig. 2, bar 6 and 2). Sp1 was able to transactivate the *Bss*HII methylated DNA as well as the mock methylated DNA (bar 7 versus bar 3). These data suggest that there is a small effect upon Nrf-1 activation when its site is methylated and that Sp1-mediated activation is relatively refractory to methylation of neighboring cytosines. When Nrf-1 and Sp1 expression plasmids were co-transfected with the methylated reporter plasmid, a strong stimulation was again observed (bar 8 versus bar 4), hence methylation did not reduce the majority of transcription-factor stimulatory activity. Even though the absolute level of stimulation by both factors was comparable for the methylated or mock methylated promoter, the fold stimulation relative to the respective reporters alone (bar 8 versus bar 5 and bar 4 versus bar 1) was reduced by a factor of two (260- versus 116-fold) as a result of methylation. Therefore, both factors are quite active when the Nrf-1 site is methylated, but the synergistic component of stimulation appears to have been lost.

Dense methylation of the *FMR1* promoter results in loss of Nrf-1 stimulation but only partially reduces Sp1 activity

To determine how the transcription factors respond when the *FMR1* promoter is densely methylated, as seen in fragile X patients, we tested the activity of *Sss* I-methylated *FMR1* in SL2 cells. *Sss* I methylates cytosines in CpG dinucleotides; 388 such sites are found in this reporter plasmid. *Sss* I methylation eliminated Nrf-1-dependent stimulation (Fig. 2, bar 10 versus bars 6 and 2). As seen previously for the Sp1-dependent leukosialin promoter (25), Sp1 can still activate the *Sss* I-methylated *FMR1* promoter in SL2 cells (Fig. 2, bar 11 versus bars 7 and 3), albeit half as well as the unmethylated promoter. When dual Nrf-1/Sp1 activation was tested, a stimulation level lower than that of Sp1 alone was observed (bar 12 versus bar 11). This suggested that Nrf-1 was not contributing to Sp1 stimulation but was somewhat repressive under these conditions.

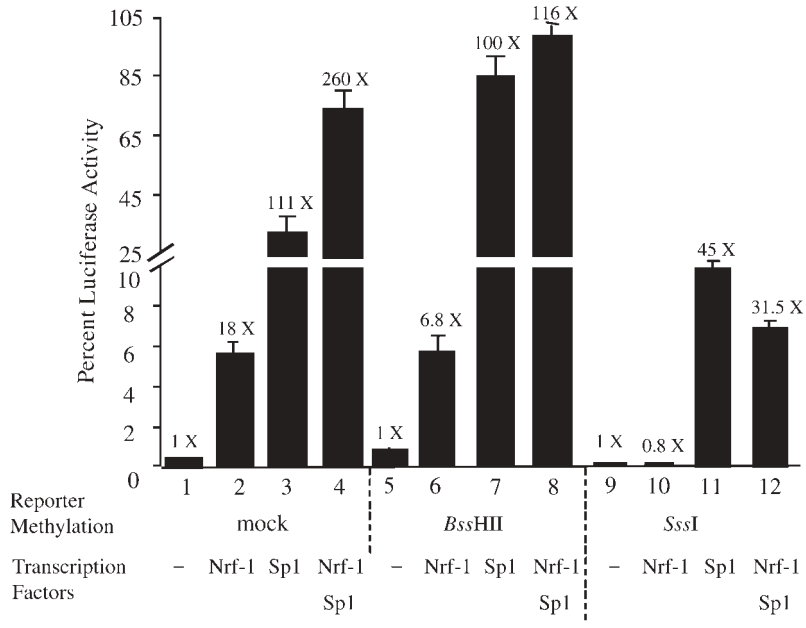


Figure 2. Transcription factor stimulation of *Bss* HII, *Sss* I-methylated and mock-methylated *FMR1* promoter activity in *Drosophila* SL2 cells. pFMR1-luc was mock-methylated with *Bss* HII methylase (bars 1–4), *in vivo* methylated with *Bss* HII methylase (bars 5–8) or *in vitro* methylated with *Sss* I methylase (bars 9–12). Sites of *Bss* HII and *Sss* I methylation are indicated in Figure 1. Either mock-methylated or methylated pFMR1-luc was co-transfected into SL2 cells with pActinFL-Nrf-1 and/or pPacSp1. The highest value (bar 8) was arbitrarily set to 100%. The fold-changes for bars 2–4 are relative to that of bar 1. The fold-changes for bars 6–8 are relative to bar 5 and those for bars 10–12 are relative to bar 9. Each bar represents the average of at least three transfections ±1 standard deviation.

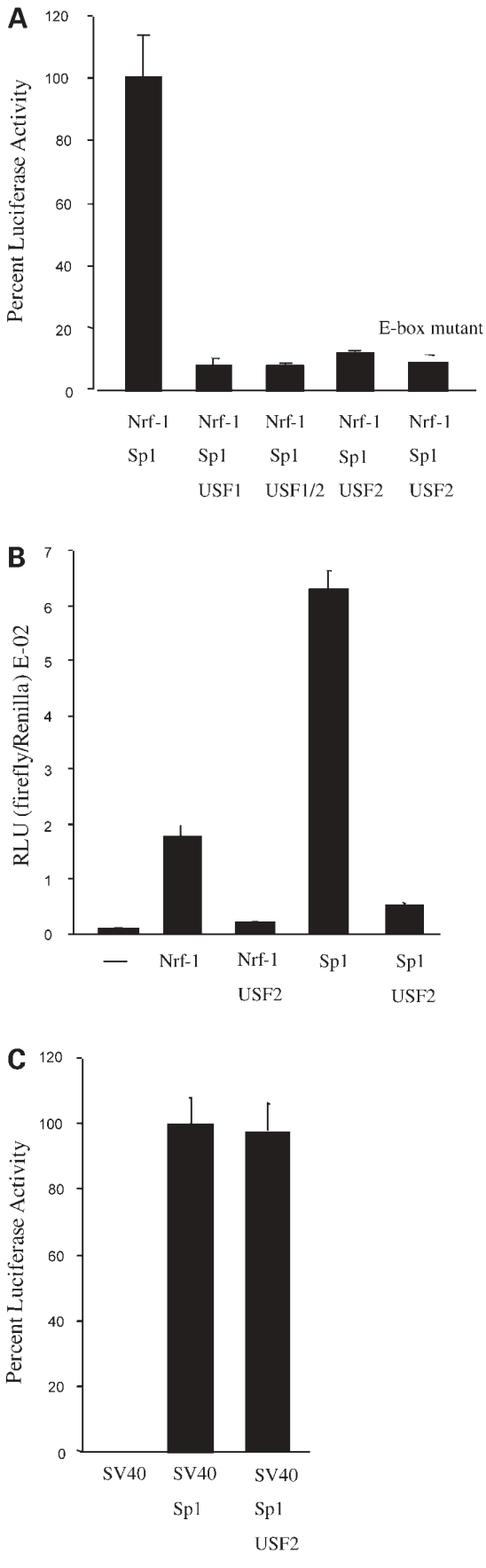
USF1/2 and Max repress *FMR1* transcription in *Drosophila* cells

The fourth occupied site on the *FMR1* promoter is an E-box, which is the recognition site for a number of factors including c-Myc, USF1/2 and Max (7,8). USF has been previously implicated in *FMR1* transactivation (9). We tested the ability of human USF1, USF2 and both together to activate transcription from the *FMR1* promoter. Neither USF1 nor USF2, nor both together activated the *FMR1* promoter (data not shown). Unexpectedly, when USFs were co-transfected with Nrf-1 and Sp1, *FMR1* transcription was much (~85%) lower than with Nrf-1 and Sp1 alone (Fig. 3A).

USF2 was also tested for the ability to repress transactivation by Sp1 and Nrf-1 separately. Nrf-1 activation was repressed by 4.5-fold and Sp1 activation was repressed 6-fold (Fig. 3B). Mutating the entire E-box, as shown in Figure 1, did not rescue the repression by USF2 (Fig. 3A), suggesting that USFs are not acting repressively by binding the E-box in our *FMR1*-reporter in SL2 cells. Although this repression was not mediated through the E-box, it was specific for *FMR1*, as USF2 did not repress Sp1-mediated activation of the SV40 promoter in these cells (Fig. 3C). Due to the unexpected repression by USFs, and to help clarify the role of E-box binding factors in *FMR1* transcription, we tested the role of an additional bHLH factor. Max was chosen because it had been previously reported to dimerize with α-Pal/Nrf-1 at the eIF2-α promoter (38). Since Nrf-1 is a potent activator of *FMR1*, we tested the effect of Max on Nrf-1 activation of *FMR1*. We used cDNAs encoding two transcript variants of Max. Max p22 is the longest form, while Max p21 has a short internal deletion; neither contains an activation domain (39). As expected, neither Max p22 nor p21 had an effect on *FMR1* transcription when expressed alone (Fig. 4, bars 2 and 3). Nrf-1 co-transfected with Max p22 led to activation of *FMR1*, but to a lesser degree than Nrf-1 alone (Fig. 4, compare bars 2, 4 and 5) suggesting that Max is inhibitory to Nrf-1 activation. Max p21 had previously been reported to bind DNA with lower affinity than full-length Max (39). When co-expressed with Nrf-1, Max p21 had a much weaker inhibitory effect on its activation, suggesting that DNA binding is important in Max's inhibition of Nrf-1 (Fig. 4, compare bars 3–6). Max p22 also had an inhibitory affect on Sp1 activation of *FMR1*, but to a lesser degree than Nrf-1; the shorter Max variant did not have a repressive effect on Sp1 activation (Fig. 4, bars 7–9). We conclude that Max has the potential to regulate the *FMR1* gene.

Sp1 and Nrf-1 bind to the *FMR1* promoter *in vivo*

Previous work has suggested that Nrf-1 and Sp1 are likely to act at *FMR1* (7–9,13). In addition, our data show a strong, synergistic activation of *FMR1* by co-expressing Sp1 and Nrf-1, further implicating these factors. To date, no one has shown that either transcription factor binds to the *FMR1* promoter *in vivo* in any cell type including the natural chromosomal DNA in human cells. To directly test this, we raised antisera specific to Nrf-1 (Fig. 5A), or used a commercially available Sp1 antibody and performed chromatin immunoprecipitation (ChIP). Indeed, both Nrf-1 and Sp1 (Fig. 5B)



occupy the *FMR1* promoter in lymphoblastoid cells that express *FMR1*. This was also true for the eIF2-β promoter (Fig. 5B). Neither factor binds to the *FMR1* promoter in fragile X cells that have an expanded methylated *FMR1* allele, but both remain associated with the eIF2-β promoter in the same cells (Fig. 5B).

The E-box binding proteins, USF1/2 and Max, but not c-Myc, bind to the *FMR1* promoter *in vivo*

Previous *in vitro* binding studies (9) implicated the E-box binding transcription factors USF1 and 2 in *FMR1* transcription. In addition, deletion or mutation of this site in reporter constructs leads to a decrease in activity in mammalian cells (9) (data not shown). Our results in SL2 cells unexpectedly suggested that USF1/2 are inhibitory at *FMR1*. To help clarify the role of USF1 and USF2, we employed ChIP on the endogenous *FMR1* promoter and determined that in fact, both USF1 and USF2 were bound to *FMR1* as well as the hTERT control promoter (40) in *FMR1*-expressing human cells (Fig. 5C). We reasoned that SL2 cells might lack another transcription factor that is necessary for proper USF1/2 function at *FMR1*. We therefore tested whether Max and c-Myc might associate with *FMR1*, since both are known to bind E-boxes. Interestingly, Max, but not c-Myc was bound to the *FMR1* promoter *in vivo* (Fig. 5C). This was not due to c-Myc's inability to be immunoprecipitated, as the endogenous cyclin D2 promoter, a previously identified Myc/Max target (41), was isolated with antibodies to both c-Myc and Max.

A short-interfering RNA against Nrf-1 lowers FMR1-luc expression in HeLa cells

Previous studies have stressed the importance of the Nrf-1 transcription factor at *FMR1*. Deletion of nine bases in the Nrf-1 site in reporter constructs lowers activity to ~25% of total expression in PC12 cells (9). To address the relative contribution of Nrf-1 at *FMR1* using a different approach, we targeted the endogenous Nrf-1 mRNA using RNA interference in HeLa cells. To determine that our knock-down was not a general effect of expressing short interfering RNAs into HeLa cells, we transfected a vector expressing a short interfering RNA against an irrelevant protein (Arl2) as a control. As a positive control, we expressed a short interfering RNA against

Figure 3. USFs repress the *FMR1* promoter in Schneider 2 cells. (**A**) Plasmids expressing either human USF1, USF2 or both were co-transfected with pACTINFL-Nrf-1, pPacSP1 and the wild-type pFMR1-luc or pFMR1-luc with a mutated E-box. Triplicate luciferase values were averaged for each (±1 standard deviation) and expressed as a percentage of the sample lacking USF, which was set to 100%. (**B**) Wild-type pFMR1-luc was co-transfected with either an empty expression vector or vectors encoding either Nrf-1 or Sp1, or USF2 and Nrf-1, or USF2 and Sp1. The data represent averages of triplicate experiments (±1 standard deviation) and are expressed as relative light units (RLU). (**C**) A luciferase vector driven by the SV40 promoter was transfected into SL2 cells with either an empty expression vector, pPacSp1, or pPacSp1 and pACTIN-USF2. Triplicate luciferase values were averaged for each (±1 standard deviation) and expressed as a percentage of the sample with Sp1 alone, which was set to 100%. The result from the SV40 transfection without any co-expressed transcription factors is not visible when graphed on this scale; the actual value is 0.16 ± 0.008%.

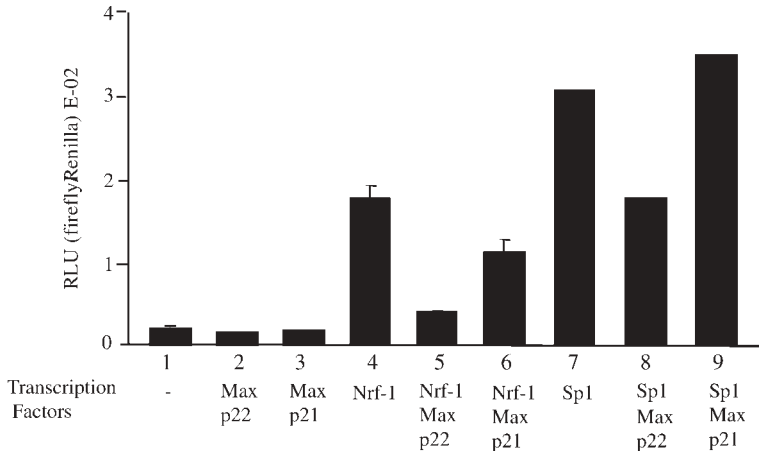


Figure 4. Max inhibits Nrf-1 and Sp1 activation of *FMR1*. pFMR1-luc was co-transfected into SL2 cells with either an empty expression plasmid (bar 1) or human Max expression plasmids: pACTIN-Maxp22 (bars 2, 5 and 8), pACTIN-Maxp21 (bars 3, 6 and 9) and either pACTINFL-Nrf-1 (bars 4–6) or pPacSp1 (bars 7–9). Bars 1–6 represent the averages of experiments done in triplicate (±1 standard deviation). Bars 7–9 were done in duplicate and the averages are plotted. Data is plotted in relative light units (RLU).

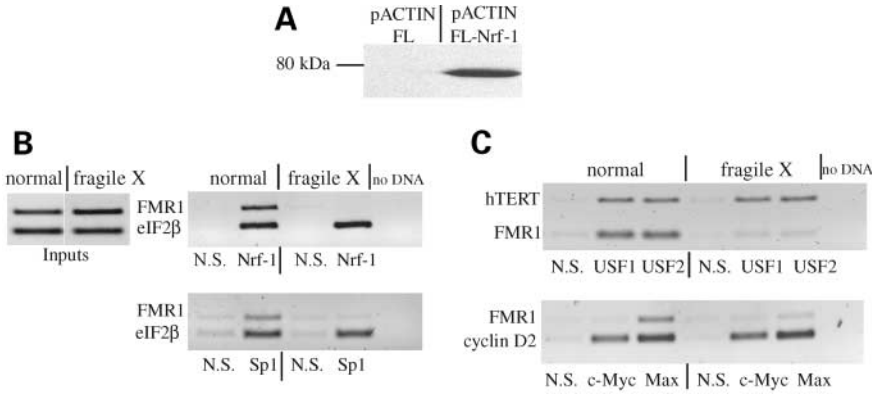


Figure 5. *In vivo* binding of transcription factors to the *FMR1* promoter in lymphoblastoid cells. (**A**) Western blot with anti-Nrf-1 antiserum on SL2 cell lysates expressing no transcription factor (left lane) or expressing human Nrf-1 (right lane). (**B**) Chromatin immunoprecipitation was carried out with either an anti-Nrf-1 IgG (top panel), an anti-Sp1 antibody (bottom panel), or non-specific rabbit IgG (NS in both panels). PCR was performed to amplify the *FMR1* promoter and eIF2-β promoter in normal and fragile X cells. (**C**) ChIP was performed using antibodies to human USF 1 or 2 (top panel) or c-Myc or Max (bottom panel) or non-specific rabbit IgG (NS in both panels) in both normal and fragile X cells. Control promoters were amplified in duplex PCR reactions with *FMR1*.

the firefly luciferase gene, which lowered luciferase activity from pFMR1-luc to ∼20% of the control (Fig. 6). Expression of a short interfering RNA against Nrf-1 in HeLa cells, lowered pFMR1-luc expression by ∼55% (Fig. 6). This result suggests that Nrf-1 is important for at least half of *FMR1* promoter activity and agrees with assays in which mutation of the Nrf-1 binding site in human and COS-7 cells impairs promoter function (9) (data not shown).

DISCUSSION

We have demonstrated the direct *in vivo* binding of several transcription factors at the clinically important *FMR1* promoter, including Nrf-1, Sp1, USF1/2 and Max; these interactions are disrupted at silenced, expanded *FMR1*. We also present *in vivo* evidence that Nrf-1 and Sp1 are potent and synergistic activators of the *FMR1* promoter. These findings provide the first direct *in vivo* evidence of specific transcription factors regulating *FMR1* and are the first to test and show the transactivation ability at *FMR1* of the two major positively acting factors, Sp1 and Nrf-1. Our *in vivo* studies verify and extend many of the previous element-mapping assays, *in vivo* footprinting, and *in vitro* DNA binding analyses (7–10). We did, however, discover that Max resides at the endogenous *FMR1* promoter *in vivo*, although Max did not bind an *FMR1* promoter fragment in previous *in vitro* analyses (9). The *in vivo* binding of Max is an interesting finding and important to consider for understanding the regulation of *FMR1*. Max has previously been reported to dimerize with several basic helix–loop–helix (bHLH) proteins, including c-Myc, several Mad proteins, and itself (39). The only known bHLH dimerization partner of Max that leads to transactivation is c-Myc (39). Since c-Myc was not bound to the

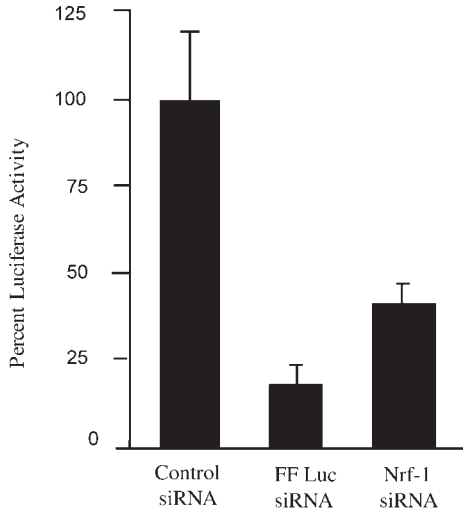


Figure 6. Expression of a short-interfering RNA against Nrf-1 reduces *FMR1* reporter activity. HeLa cells were transfected with the wild-type pFMR1-luc and either a vector expressing an siRNA against an irrelevant control (Arl2), firefly luciferase or Nrf-1. HeLa cells were lysed four days after transfections. Each bar represents the average of six transfections (± 1 standard deviation). Data are expressed as a percentage of the experiment containing the irrelevant siRNA vector, which was arbitrarily set to 100%.

endogenous promoter, we propose that Max either works with a yet to be identified positively acting factor, or as a repressor by controlling Nrf-1 and Sp1 activation at *FMR1*.

In contrast to previous suggestions that USF1/2 stimulates *FMR1*, we were unable to find an activator role for the USF1/2 proteins (9). On the contrary, USF1 and USF2 failed to activate *FMR1* alone and were strong repressors of Sp1 and Nrf-1 stimulated transcription in SL2 cells. Mutating the entire E-box did not have an affect on USF2's repressive activity, thereby suggesting that in SL2 cells, USFs do not bind the E-box. However, to rule out a squelching effect of the expressed human USFs in SL2 cells, we have shown that this repressive effect is specific for *FMR1*, as USF2 did not repress Sp1-mediated activation of the SV40 promoter. Since USF proteins have also been reported to bind to initiator (Inr) elements (42,43) it is possible that our results in SL2 cells reflect differential usage of the Inr elements and the E-box by USF proteins, depending upon the availability of other transcription factors.

The transcription factor Nrf-1 is essential. Homozygous disruption of the Nrf-1 gene in mice results in embryonic lethality (44,45). We have shown that Nrf-1, as well as Sp1 bind to the *FMR1* promoter *in vivo.* To our knowledge, this is the first direct demonstration that Nrf-1 binds to a specific promoter *in vivo.* Nrf-1 has been shown to be important for transcription of the eukaryotic translation initiation factors eIF2α and β, and an Sp1 site is also present in the eIF2β promoter (38,46). Therefore, we tested eIF2β for binding of Nrf-1 and Sp1 factors in our ChIP assays and have successfully shown that both factors do also bind this promoter *in vivo.* It is interesting that both factors reside on at least two genes involved in translation; eIF2β and *FMR1* and, most likely, also at hnRNP-A2 (8,46). The *FMR1* protein, FMRP, binds RNA in neurons and is involved in regulating translation of some of its targets (1,47–49) thereby functionally linking Nrf-1 and Sp1 to regulation of multiple genes involved in translation.

These studies also raise the question of what are the cellular signals that direct these transcription factors to *FMR1.* Nrf-1 and Sp1, in addition to being important for upregulation of translation factors, are involved in transcription of nuclear encoded mitochondrial genes (reviewed in 50). This raises the possibility that the signals that turn on Nrf-1 to upregulate mitochondrial biogenesis may also have an affect on *FMR1* transcription. Conversely, Nrf-1 may function differentially at mitochondrial-related genes than at *FMR1* or other target genes. It will be of interest to begin working out the regulatory signals for these transcription factors at *FMR1* and to see if the signaling pathways converge with other known regulatory circuits.

Since DNA methylation is a necessary step for transcriptional silencing of expanded *FMR1* (51), we examined the effect that DNA methylation has on Nrf-1 and Sp1 transactivation of *FMR1.* Methylation of the Nrf-1 site in the *FMR1* promoter was previously shown to decrease reporter activity by 50% in human cells (9) and it was suggested that this is part of the mechanistic basis of methylation-mediated silencing of *FMR1* in fragile X patients. It has also recently been suggested that Nrf-1's function is reduced when the Tfam promoter is methylated (52). To directly test whether Nrf-1 was methylation sensitive *in vivo*, we utilized *Drosophila* SL2 cells to see if the repressive effect occurred in the absence of MBD proteins. We found that Nrf-1's substantial (18-fold) transactivating activity was only slightly inhibited (<3-fold), when three cytosines in its binding site were methylated. The overall level of *FMR1* transcription by Sp1 and Nrf-1 was not reduced by methylation of the Nrf-1 site. At most, the effect on transcription of methylating the Nrf-1 site is limited to the loss of synergy between Nrf-1 and Sp1. This is consistent with bisulfite sequencing studies of human *FMR1* alleles upon de-methylation and reactivation (23) in which the α-Pal/Nrf-1 site often stayed methylated, suggesting that transcription can occur in the presence of Nrf-1 site methylation.

As observed by others, Sp1 is relatively immune to the effects of DNA methylation (14,25). While dense methylation of the type seen in fragile X patients (6,53), prevents Nrf-1 transactivation in SL2 cells, it cannot completely quench Sp1 activity. Yet, we found that Sp1 binding is abolished *in vivo* at silenced expanded *FMR1.* Prior evidence shows that the chromatin of *FMR1* in fragile X cells is heterochromatin-like (11,20). Therefore, we suggest that it is the binding of methyl-CpG binding proteins other repressive heterochromatin proteins or chromatin compaction itself that prevents Sp1 activity and binding at expanded methylated *FMR1*, thereby leading to complete silencing of *FMR1.*

Active alleles of *FMR1* in human cells show robust histone H3 and H4 acetylation and chromatin is largely in an open conformation (11). It will be of interest to determine which proteins are recruited by the *FMR1* transcription factors to modify the histones and facilitate in opening the chromatin at *FMR1.* Sp1 has been shown to interact with p300 acetyltransferase and stimulate the histone acetyltransferase activity of CBP (54–56), while Nrf-1 has been associated with the p300/CBP associated cofactor (P/CAF) (57).

We have successfully used chromatin immunoprecipitation at *FMR1* to show that Nrf-1, Sp1, USF1/2 and Max bind *in vivo*. These experiments can be extended to pre-mutation alleles of *FMR1*, which are known to have higher levels of *FMR1* transcription (58,59). At pre-mutation alleles, transcription was shown to increase in frequency at upstream start sites, as well as maintain the use of the downstream predominant start site (60). It is possible that the occupancy of one or more *FMR1* transcription factor(s) may be altered following CGG repeat expansion before extensive methylation occurs (60). Alternatively, the recruitment of additional factors at *FMR1* such as the CGG repeat binding protein may also occur upon repeat expansion (61,62).

MATERIALS AND METHODS

Plasmid constructions

pPacSp1 was a gift from Dr Jerry Boss (30). The control vector, pPac, was constructed by digestion of pPacSp1 with *Xho*I to remove Sp1 coding sequences and religation of the empty vector. pGL3con (SV40 promoter) and pGL3basic (no promoter) were purchased from Promega. To construct pFMR1-luc, the *Pst*I/*Xho*I fragment (−272 to +291 of the human *FMR1* promoter) from the clone pE5.1 was inserted into the multiple cloning site of pGL3basic (Fig. 1) (4). The E-box mutant was constructed by site-directed mutagenesis using the following oligonucleotide 5′-ACACTGAAACCAC GTAAGCTTATCAACGCTGTT-3′. To construct pACTIN-FL, the FLAG tag and part of the multiple cloning site were PCR-amplified from pCMV-tag2b (Promega) using 5′-CGGTTAACGCCACCATGGATTACAAGGATGACG-3′ and 5′-CCGTCTAGACTTGATATCGAATTCCTGCAGCCC -3′ and inserted into the pACTIN vector (63), a gift from Dr Paul Wade, using the *Hpa*I and *Xba*I sites. To construct pACTINFL-Nrf-1, Nrf-1 was PCR-amplified from a pC MVtag2b vector containing Nrf-1 inserted at the *Bam*HI and *Hind*III sites (pCMV-Nrf-1) using 5′ -TCTGGATCCATGG-AGGAACACGGAGTG ACCCAAACCGAA-3′ and 5′-C GACTCGAGTCACTGTTCCAATGTCACCACCTCC-3′. The Nrf-1 PCR product was cloned into pACTIN-FL at the *Bam*HI/ *Xho*I sites. To construct pRL-dA5C, the *Renilla* luciferase gene was cut from pRLCMV (Promega) with *Nhe*I and *Xba*I and ligated into pGL3basic vector cut with the same enzymes. This plasmid was cut with *Kpn*I and *Sac*I to insert the *Drosophila* actin 5C promoter amplified with 5′-GGG GTACCCCGCGCGTTGGCCGATTCATTAATGC-3′ and 5′-CGGAGCTCTGGATTAGACCACTGCTGGCTGATGG-3′ from pACTIN. To construct pACTIN-USF1, pACTIN-USF2, pACTIN-Maxp22 and pACTIN-Maxp21, a cDNA library from J-1 lymphoblastoid RNA was amplified using 5′-G CTCTAGAATGAAGGGGCAGCAGAAAACAGC-3′ and 5′-CGCTCGAGTTAGTTGCTGTCATTCTTGATGAC-3′ for USF1; 5′-GCTCTAGAATGGACATGCTGGACCCGGGTCT GGATC-3′ and 5′-CGCTCGAGTCACTGCCGGGTGCCCT CGCCCACCATC-3′ for USF2 and 5′-GCTCTAGAATGAG CGATAACGATGACATCGAGG-3′ and 5′-CGCTCGAGTT AGCTGGCCTCCATCCGGAGC-3′ for both Max forms. PCR-products were gel purified and cloned into pACTIN using the *Xba*I and *Xho*I sites. All constructs were confirmed by sequencing.

Reporter plasmid methylation

A plasmid expressing *Bss*HII methylase from *Bacillus stearothermophilus* H3 (pLGBssHII) or the backbone vector without the *Bss*HII gene were independently co-transfected with pFMR1-luc into *E. coli* K12 (ER1821) (New England Biolabs) (64). Plasmids were recovered and methylation of pFMR1-luc was confirmed by its resistance to digestion with *Bss*HII restriction enzyme (NEB). Alternatively, pFMR1-luc was incubated with *Sss*I methylase (NEB) according to the supplier's recommendations. Mock methylations were performed in reactions lacking enzyme. Complete methylation was confirmed by the plasmid's resistance to digestion with the methylation-sensitive enzymes, *Bst*UI, *Hha*I and *Hpa*II (NEB).

Culture and transfections of Schneider 2 cells

SL2 cells (a gift from Dr J. Lucchesi, Emory University) were cultured in serum-free insect cell media (HYCLONE) with 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (Cellgro) and grown at 25°C. The day before transfection, SL2 cells were plated in 6-well cell-culture dishes at a density of 4×10^5 cells/well with 2 ml of media per well. For transfections, 1.2 μg of total plasmid DNA were added (500 ng of firefly luciferase reporter, 100 ng of *Renilla* luciferase reporter as an internal transfection control and 200 ng of each of three additional expression plasmids). When the protein-expressing plasmids were omitted, 200 ng of their respective empty parent vector were added as a control. Cells were transfected using Effectene kit reagents (QIAGEN) 24 h after plating. Forty-six to 48 h after transfection, the media was removed and cells were harvested with 500 μl 1× Passive Lysis Buffer (Promega) by shaking at room temperature until lysed (typically 1 h). Lysates were collected in 1.5 ml tubes and spun at 14 000*g* for 1 min to remove cell debris. The supernatant was stored at −80°C until assayed.

Culture of lymphoblastoid and HeLa cells

The EBV-transformed lymphoblastoid cell lines J-1 and GM3200A (Coriell Cell Repositories), carry an unmethylated 30 CGG repeat allele and a methylated 530 CGG repeat allele, respectively. Cells were cultured at 37°C with 5% CO_2 in RPMI 1640 media containing 300 μg/ml L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. HeLa cells were cultured under the above conditions in DMEM with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Transfection of HeLa cells

The day before transfection, HeLa cells were plated in 6-well cell-culture dishes at a density of 1×10^5 cells/well with 2 ml of media per well. For transfections, 450 ng of total plasmid DNA were added (200 ng of firefly luciferase reporter, 50 ng

of the *Renilla* luciferase reporter pRLCMV as an internal transfection control and 200 ng of the pSUPER-siRNA constructs). The optimal amount of the siRNA construct used (200 ng) is the lowest amount that elicited maximum knock-down in previous titration experiments (data not shown). Cells were transfected 24 h after plating using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Four days after transfection, which we determined to be optimal for maximum knock-down (data not shown), cells were harvested with 500 μl 1× Passive Lysis Buffer (Promega) by shaking at room temperature until lysed (typically 30 min). Lysates were collected in 1.5 ml tubes and spun at 14 000*g* for 1 min to remove cell debris. The supernatant was stored at −80°C until assayed.

Luciferase assays and calculations

The protein concentration of each extract was determined by the Bradford assay (65). The dual luciferase assay kit (Promega) was used to determine firefly luciferase and *Renilla* luciferase activities. One-half or 1 μg of total protein was added to each reaction and measured using a 10 s read on a manual-load luminometer. Firefly luciferase values were divided by *Renilla* luciferase values for each transfection. Data from at least three transfections were averaged and the standard deviation was calculated.

Chromatin immunoprecipitation (ChIP)

Nrf-1 anti-sera was produced from immunization of New Zealand White Rabbits (Pacific Immunology Corporation, Ramona, CA) with a peptide corresponding to the C-terminal 14 amino acids of Nrf-1 (57). Antibodies for Sp1 (PEP-2), c-Myc (N-262), Max (C-17), USF1 (C-20) and USF2 (C-20) were purchased from Santa Cruz Biotechnology. ChIP was performed as described by Upstate Biotechnology Inc., with the exceptions that antibodies were pre-bound to blocked protein A-Sepharose by incubating overnight at 4°C in binding buffer (5 mM Tris pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.05% NP-40). Nuclei were isolated from formaldehyde cross-linked J-1 or GM3200A lymphoblastoid cells as described by Dignam *et al.* (66). Briefly, cells were washed twice with 1× phosphate buffered saline (138 mM NaCl, 2.7 mM KCl, 8.7 mM Na_2HPO_4, 1.8 mM KH_2PO_4) including 1 mg/ml $MgCl_2$ and resuspended in Buffer H (10 mM Tris-HCl pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and protease inhibitors) to an approximate concentration of 1.5×10^7 cells/ml. Nuclei were isolated by Dounce homogenization with 85 strokes of a type B Dounce homogenizer, washed once with buffer H and were lysed in SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0 and protease inhibitors). Sonicated chromatin from $\sim 3 \times 10^6$ cells was used for each immunoprecipitation. All immunoprecipitated samples were digested with *Pst*I to detach the CGG repeats from the upstream *FMR1* priming sites before PCR. Duplex PCR was performed with the *FMR1* promoter and a control promoter. For Nrf-1, Sp1, c-Myc and Max ChIPs, primer sequences for *FMR1* were 5′-CGACTCAATCCATGTCCC TTAAAGG-3′ and 5′-CCAGTTCGGCCTCTCTGGGATTCC -3′ and amplified from −629 to −322 in the promoter. For Sp1 and Nrf-1 ChIPs, the *FMR1* promoter was amplified with the eIF2-β promoter using 5′-GGACTACAAGTCCC GGCATGC-3′ and 5′-GCACTAGGCTCTTGCATCAGC G-3′, which span from −142 in the promoter to +39 after the transcription start site (46). Alternatively, for c-Myc and Max ChIPs, the *FMR1* promoter was amplified in the same reaction with the Cyclin D2 promoter using 5′-CCTGGAGT GAAATACACCAAAGGGC-3′ and 5′-CCTCACTCTGCC AGGCTTTCTCC-3′ which span from −1550 to −1354 with respect to the start codon (41,67). For USF1 and USF2 ChIPs, *FMR1* was amplified from −535 to −415 in the promoter using 5′-CTACGGGTCACAAAAGCCTGGGTCACC -3′ and 5′-GCAGTCTGACTGAGCGGGAGGTGGAG-3′ in the same reaction with the hTERT promoter (from −402 to −108) using 5′-CCTGTTCCCAGGGCCTCCACATC-3′ and 5′-GAGCTGGAAGGTGAAGGGGCAGG-3′ (40,68).

RNA interference

Oligos used to produce siRNAs directed against Nrf-1 were 5′-GATCCCCCATATGGCTACCATAGAAGTTCAAGAGA CTTCTATGGTAGCCATATGTTTTTGGAAA-3′ and 5′-A GCTTTTCCAAAAACATATGGCTACCATAGAAGTCTC-TTGAACTTCTATGGTAGCCATATGGGG-3′. Oligos to target the firefly luciferase gene were 5′-GATCCCCTGAA CGTGAATTGCTCAACTTCAAGAGAGTTGAGCAATTCA CGTTCATTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAA TGAACGTGAATTGCTCAACAGCTTTTCCAAAAAGTTGA GCAATTCACGTTCAGGG-3′. Twenty-five nanograms of each oligo were heated to 95°C and slowly cooled overnight to room temperature and directly ligated into pSUPER (a gift from Dr Reuven Agami, The Netherlands Cancer Institute) using the *Bgl*II/*Hind*III sites (69). As a control, a vector (PYW145) encoding an siRNA against human Arl2 (a gift from Dr Richard Kahn) was used and contained the following oligos: 5′-GATCCCGACCCTGGAGCACCGAGGATTCAA GAGATCCTCGGTGCTCCAGGGTCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGACCCTGGAGCACCGAGGAA GCTTTTCCAAAAATCCTCGGTGCTCCAGGGTC-3′.

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