Research article

Fragile X $(CGG)_n$ repeats induce a transcriptional repression *in cis* upon a linked promoter: Evidence for a chromatin mediated effect

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Abstract

Background: Expansion of an unstable $(CGG)_n$ repeat to over 200 triplets within the promoter region of the human FMR1 gene leads to extensive local methylation and transcription silencing, resulting in the loss of FMRP protein and the development of the clinical features of fragile X syndrome. The causative link between $(CGG)_n$ expansion, methylation and gene silencing is unknown, although gene silencing is associated with extensive changes to local chromatin architecture.

Results: In order to determine the direct effects of increased repeat length on gene transcription in a chromatin context, we have examined the influence of FMR1 $(CGG)_n$ repeats upon transcription from the HSV thymidine kinase promoter in the *Xenopus laevis* oocyte. We observe a reduction in mRNA production directly associated with increasing repeat length, with a 90% reduction in mRNA production from arrays over 100 repeats in length. Using a kinetic approach, we show that this transcriptional repression is concomitant with chromatin maturation and, using *in vitro* transcription, we show that chromatin formation is a fundamental part of the repressive pathway mediated by $(CGG)_n$ repeats. Using Trichostatin A, a histone deacetylase inhibitor, we show reactivation of the silenced promoter.

Conclusions: Thus, isolated fragile X associated $(CGG)_n$ repeat arrays can exert a modifying and transcriptionally repressive influence over adjacent promoters and this repressive phenomenon is, in part, mediated by histone deacetylation.

Background

Expansion of a genetically unstable $(CGG)_n$ triplet repeat was first observed within the promoter region of the human FMR1 gene in fragile X families (reviewed in refs [1,2]). In the normal population, the repeat is genetically stable and ranges from 6 to 54 triplets in length whereas in fragile X families it ranges from 55 to over several thousands of triplets in length [3]. Expansion to over 200 repeats is associated with loss of FMR1 gene transcription [4], hypermethylation [5–7], late replication [8] and the expression of a fragile site on the X chromosome. Loss of the encoded protein, FMRP, results in altered synaptic maturation (reviewed in ref [9] which leads to development of the fragile X syndrome. Similar expansions of $(CGG)_n$ have also been identified at other rare, folate sensitive fragile sites, including those at FRAXE [10], FRAXF

[11,12], FRA11B [13] and FRA16A [14]. In all cases, $(CGG)_n$ expansion is associated with a length dependent localised hypermethylation and loss of flanking gene expression.

An important regulatory mechanism for transcription in eukaryotes is chromatin architecture (reviewed in [15,16]). Numerous studies have shown the intimate and fundamental role of nucleosomal positioning and associated localised compaction of DNA as a repressive structure often refractory to transcription factor binding in yeast, animals and plants (reviewed in [17–19]). In some instances, nucleosomes have been shown to have a role in transcriptional activation, bringing disparate DNA sites into juxtaposition and allowing concomitant binding of activation functions. Chromatin architecture across the fragile X expansion is consistent with a transcriptionally silenced state, with the region becoming late replicating and extensively hypermethylated [5,6,20,21]. *In vitro* methylation of the FMR1 promoter region is known to induce transcriptional repression in transient transfection assays [22,23]. In earlier studies using cloned FMR1 arrays, it was shown that short methylated CGG repeats have a high affinity for the histone octamer and a highly positioned nucleosome assembles with the repeat sequences positioned at the nucleosomal dyad [24]. This suggests that the aberrant methylation found at expanded $(CGG)_n$ arrays might target a repressive chromatin structure to the FMR1 promoter region. Studies on the FMR1 promoter in human cell lines have shown that the repressive phenomenon is linked to hypoacetylated chromatin [24] and attempts to reactivate silenced FMR1 genes have had most success with a combination of agents targeting both methylation and histone acetylation [25].

Nucleosomal architecture is known to be modified through the action of remodelling machinery; complexes which are targeted through the modification of select histone tails (reviewed in [26,27]) and specific factor binding [17]. These can lead to alterations in the functionality of transcription from a particular promoter. DNA methylation and hypoacetylation of the local chromatin environment have been shown to be linked through the action of MeCP2 an epigenetic transcriptional repressor with affinity for methylated DNA [28]. This protein recruits deacetylases through Sin3A to methylated genomic regions [29,30]. No study to date has examined the influence of the $(CGG)_n$ array in isolation upon transcription.

In this investigation we have studied the effect of increasing $(CGG)_n$ repeat length on transcription from a viral promoter reporter construct after micro-injection into *Xenopus* oocytes in order to analyse the contribution of chromatin to any *cis* acting effect of the repeats upon transcription. We show that the RNA polymerase II regulated promoter from the Herpes simplex virus thymidine kinase gene (HSVtk) becomes transcriptionally repressed when increasing lengths of $(CGG)_n$ are linked in *cis*. Furthermore, this repression phenomenon is time dependent and correlates with the formation of chromatin. We show that the addition of Trichostatin A (TSA) can relieve the repeat induced repression. This suggests that the fragile X $(CGG)_n$ associated triplet repeat can direct transcriptionally repressive chromatin to adjacent promoters and that local deacetylation of nucleosomes is a major determinant in the repressive pathway.

Results
Reporter plasmid construction
We have developed a model system in which to examine the transcriptional impact of increasing lengths of fragile X associated $(CGG)_n$ repeats which allows us to investigate the role of chromatin in FMR1 transcriptional repression. Preliminary experiments with the native human FMR1 promoter failed to produce sufficient transcriptional activity in the *Xenopus* system (Chandler, Hirst and Wolffe, data not shown). We therefore utilised a stronger promoter, the constitutive HSVtk promoter, for subsequent studies. A short DNA fragment from the chloramphenicol acetyl transferase gene (CAT) was ligated downstream of the HSVtk promoter to serve as a primer binding site for primer extension studies. Transcripts detected with this primer represent extended mRNA initiated at the HSVtk transcription start site but which have not necessarily been extended through the $(CGG)_n$ array. Downstream of the CAT primer binding site we inserted increasing lengths of $(CGG)_n$ repeats. These are positioned approximately 140 bp downstream from the HSVtk transcription start site, a position similar to that found in the native human FMR1 promoter [22,42]. All constructs prepared were identical apart from the length of CGG repeat and all were validated to ensure the full length of the arrays were present. This was done because we are aware that deletions in these repetitive sequences can occur and lead to experimental artifacts. Figure 1a shows a schematic representation of the promoter region in our reporter construct illustrating the cloning site of the $(CGG)_n$ array. *Hind*III restriction analysis of reporter constructs containing 27, 70 and 105 repeats is shown in Figure 1b and illustrates the stability of these arrays within this reporter plasmid.

Increasing $(CGG)_n$ repeat length correlates with decreased detectable transcript
pHSVtk-CAT plasmid DNA containing increasing numbers of $(CGG)_n$ repeats was introduced by microinjection into stage 6 Xenopus laevis oocytes. As shown in Figure 2a, primer extension analysis of 18 hour mRNA pools shows that increasing repeat length leads to a gradual decrease in detectable transcripts. In all cases, we were
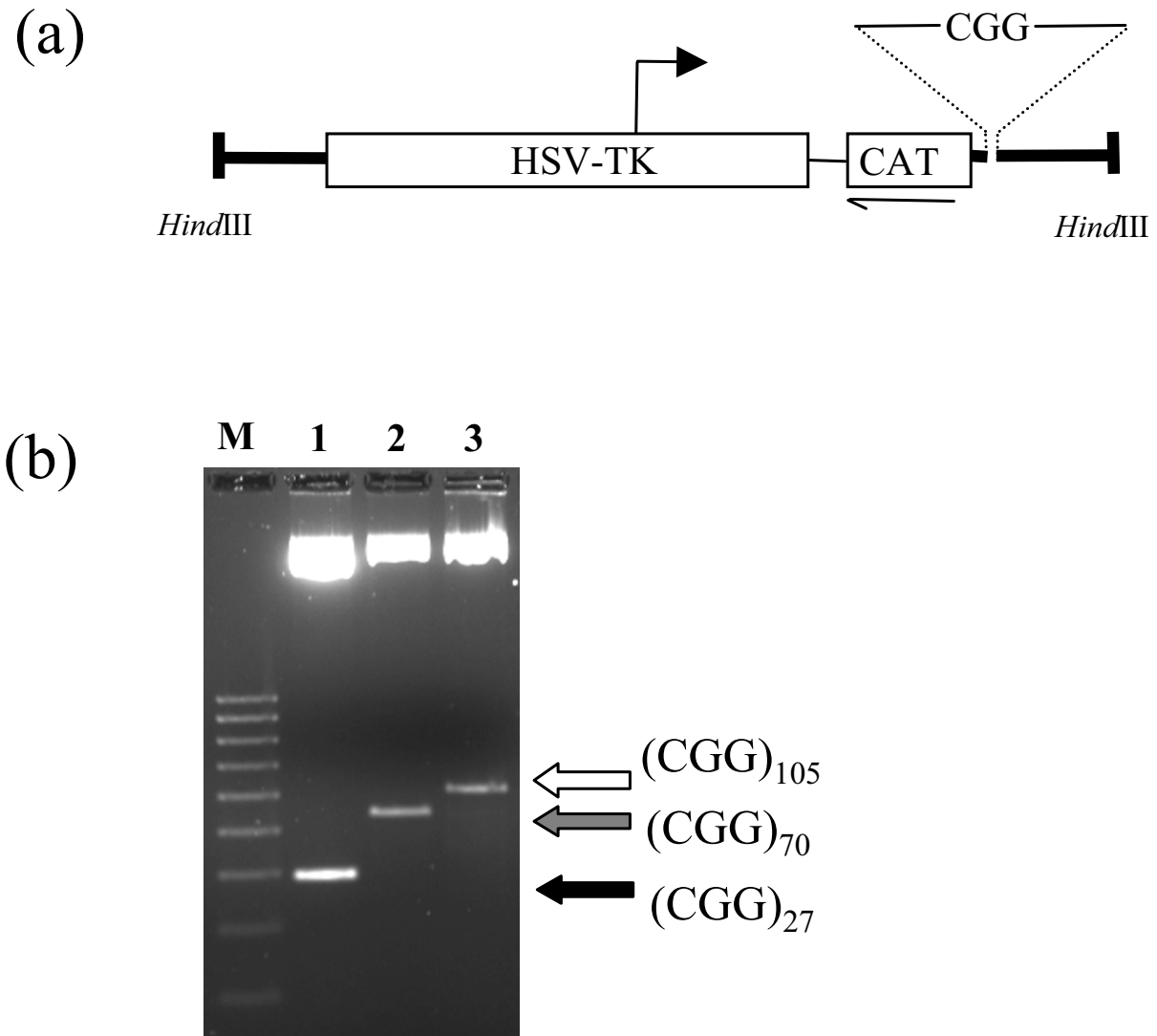
Figure 1
FMR1 (CGG)$_n$ TK-CAT constructs. (a) A schematic representation of the constitutive HSVtk-CAT promoter showing the position of the (CGG)$_n$ repeat cloning site. The distance from the start site of transcription to the repeats, in base pairs, is similar as that found in the native FMR1 promoter. The arrow represents the position of the primer used for the analysis of transcripts by primer extension and the thickened line represents pBR322 vector-derived DNA. (b) An agarose gel showing reporter plasmid digests in which *Hind*III treatment releases the (CGG)$_n$ and flanking sequences. Lanes 1, 2 and 3 are digested pBR TK-CAT plasmids containing (CGG)$_{27}$, (CGG)$_{70}$ and (CGG)$_{105}$ repeat lengths respectively. The released fragments are shown by the arrows corresponding to (CGG)$_{105}$, (CGG)$_{70}$ and (CGG)$_{27}$ respectively. The marker lane contains a 1 kb ladder
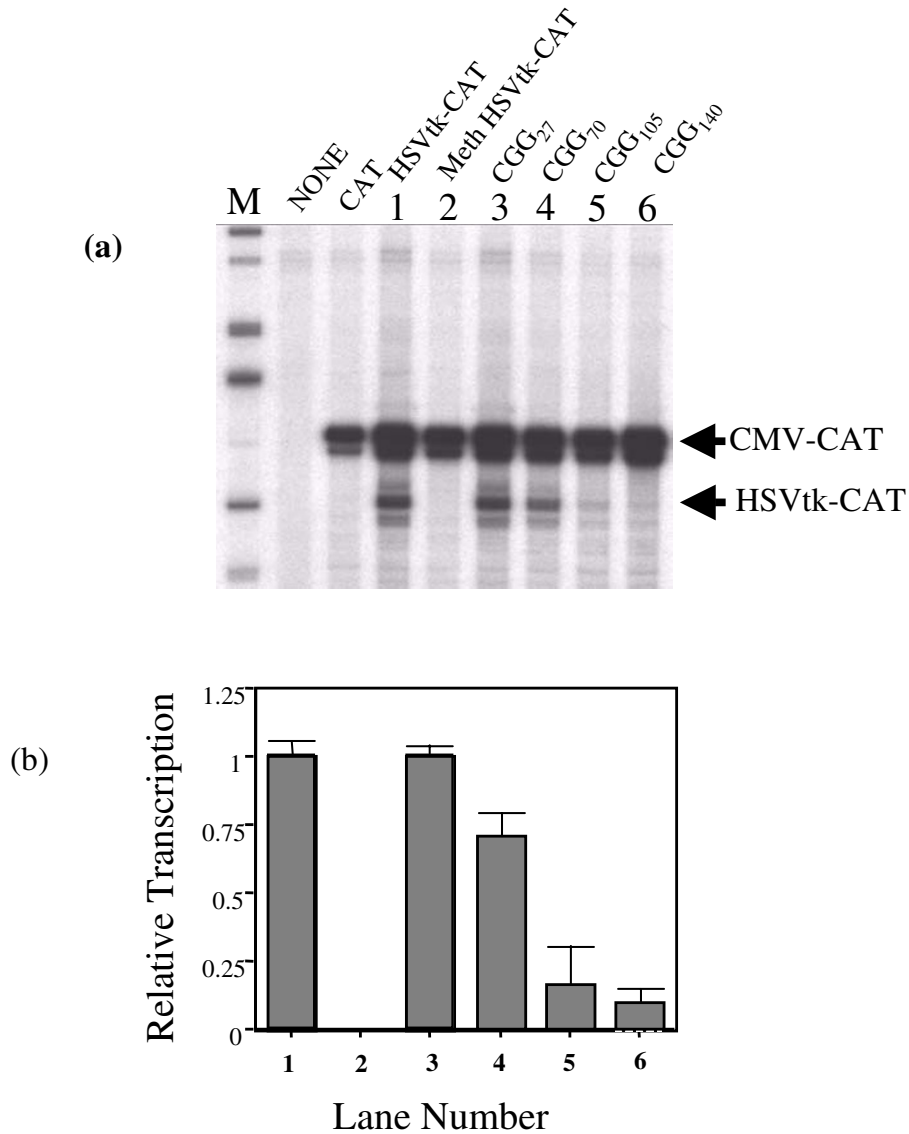
(a)

(b)

Figure 2
Increased Transcriptional Repression Correlates with Repeat Length. (a) Primer extension analysis of transcripts from the HSV-TK promoter adjacent to increased lengths of $(CGG)_n$ triplet repeat. None: no injected DNA, CAT: 0.3 ng pCMV-CAT alone. All other lanes represent co-injections of 3 ng of reporter DNA and 0.3 ng of control (pCMV-CAT) DNA per ooctye. Lane 1: pHSVtk-CAT vector; Lane 2: methylated pHSVtk-CAT vector. Lanes 3 to 6: pHSVtk-CAT containing 27, 70, 105 and 140 (CGG) triplet repeats, respectively (un-methylated). Post injection, oocytes were incubated for 18 hours at 18°C. Each extension reaction used RNA from 7 oocytes. (b) A graphical representation of the relative transcription from the HSV-TK promoter after normalisation to the co-injected pCMV-CAT control and for background signal. Lane numbers correspond to those in panel A.

microinjecting equivalent amounts of reporter DNA and the resultant mRNA levels are standardised against transcripts originating from co-injected pCMV-CAT DNA. Transcripts from this control plasmid are detected using the same CAT primer and are distinguished as larger extension products as seen in Figure 2a. In Figure 2b, it can be seen that pHSVtk-CAT constructs carrying zero or $(CGG)_{27}$ give similar levels of mRNA, suggesting that this wild-type number of repeats has little effect on transcription. As the repeat number is increased to 70 triplets, however, we see a 25% decrease in transcript levels (Figure 2a and 2b, lane 4) and upon further increases to 105 and 140 repeats we observe transcript levels drop to less than 10% of the levels of the wild-type $(CGG)_{27}$ plasmid (Figure 2a and 2b, lanes 5 and 6). This modulation of detectable mRNA levels is not a function of microinjection efficiency, as Southern blot analysis of co-isolated DNA from injected oocytes show no appreciable differences in injected DNA levels (data not shown). This strongly suggests that the $(CGG)_n$ repeats themselves are responsible for the decreased transcription from the HSVtk promoter.

We also note that a reporter containing no repeats and methylated at all CpG sites was transcriptionally silent in this assay. This is in agreement with previous studies on the methylated HSVtk promoter [45]. Hence, a possible repressive factor in repeat induced silencing might be a *de novo* methylation of the reporter construct mediated by the presence of longer repeats. However a comparative *HpaII*/*MspI* digestion of rescued reporters showed no methylation at least at HpaII sites (data not shown).

Repression of transcription is concurrent with chromatin maturation

Another potential mediator of transcriptional repression in the *Xenopus* oocyte is chromatin. To examine the contribution of chromatin to the $(CGG)_n$ linked transcriptional repression, we therefore performed a time course study where pools of injected oocytes were isolated at various time points after co-injection, up to 18 hours, the time at which we observed transcriptional silencing earlier. Results from this study are shown in Figure 3. As shown in Figure 3a, control injections with pHSVtk-CAT containing zero repeats shows that mRNA increases throughout the 18 hour incubation. To standardise for mRNA production we used a CMV-CAT co-injected control and, as can be see in Figure 3a, the amount of mRNA from this control gradually increases during the 18 hour incubation. We performed the same study with pHSVtk-CAT-$(CGG)_{70}$ as this construct induces transcriptional repression but, as shown earlier, generates a detectable level of mRNA, even after 18 hours incubation, so allowing us to quantify transcription levels throughout the time-course of the experiment. The transcriptional activity of pHSVtk-CAT-$(CGG)_{70}$ over this time course can be see in Figure 3a, and is shown graphi-

cally after standardisation to co-injected control DNA in Figure 3b. As is shown, up to 4 hours post injection, the two promoters transcribe equivalent amounts of detectable mRNA. However, after 4 hours there was no further detectable increase in the amount of mRNA from the $(CGG)_{70}$ containing construct. This suggests that by 8 hours, transcriptional repression mediated by the $(CGG)_n$ array has become established.

As we suspected that chromatin assembly was playing a role in this transcriptional repression, DNA isolated from the same oocytes injected with pHSVtk-CAT-$(CGG)_{70}$ and studied by primer extension above was examined on a gel containing chloroquine. As one positive supercoil is added per nucleosome assembled on the reporter DNA [40], direct visualisation of the supercoiling status of the injected plasmid DNA can serve as a direct measure of chromatin formation upon injected DNA. As can be seen in Figure 3c, a Southern blot of the chloroquine-containing gel, it is clear that by 8 hours after injection, chromatin formation is complete, as judged by the stabilisation of the nucleosomal ladder. This mature chromatin formation is concomitant with full $(CGG)_n$ mediated transcriptional repression of the HSVtk promoter as shown in figure 3b. This strongly suggests that the repression effect associated with increasing repeat length is causally related to the extent of chromatin formation upon the reporter. Another possibility to explain the loss of detectable transcript over time, is that the mRNA produced from reporters with longer repeats might have an inherent instability giving rise to a shorter half life. This seems unlikely as other studies on native FMR1 transcripts noted no appreciable difference in mRNA stability over the repeat lengths used in this study [43].

Transcriptional repression does not occur in the absence of chromatin formation

In order to confirm that we were observing a chromatin mediated effect and to exclude any direct effect of the $(CGG)_n$ repeats upon RNA polymerase II transcription, we performed an *in vitro* "run off" transcription reaction in Hela cell extracts, using primer extension to quantify the mRNA levels. Although these extracts contain the necessary components for mature chromatin formation, they are unable to chromatinise the templates during the short time course of this experiment. Hence any contribution of chromatin to transcriptional repression is removed. Figure 4a shows the primer extension products from such an experiment visualised after separation on a denaturing polyacrylamide gel. In this experiment, the control CMV-CAT reporter construct gives a much greater signal compared to the experimental pHSVtk-CAT DNAs. Relative transcriptional activity of each of the linearised $(CGG)_n$ containing reporter DNA's is shown in Figure 4a and shown graphically in Figure 4b. As can be seen, equivalent
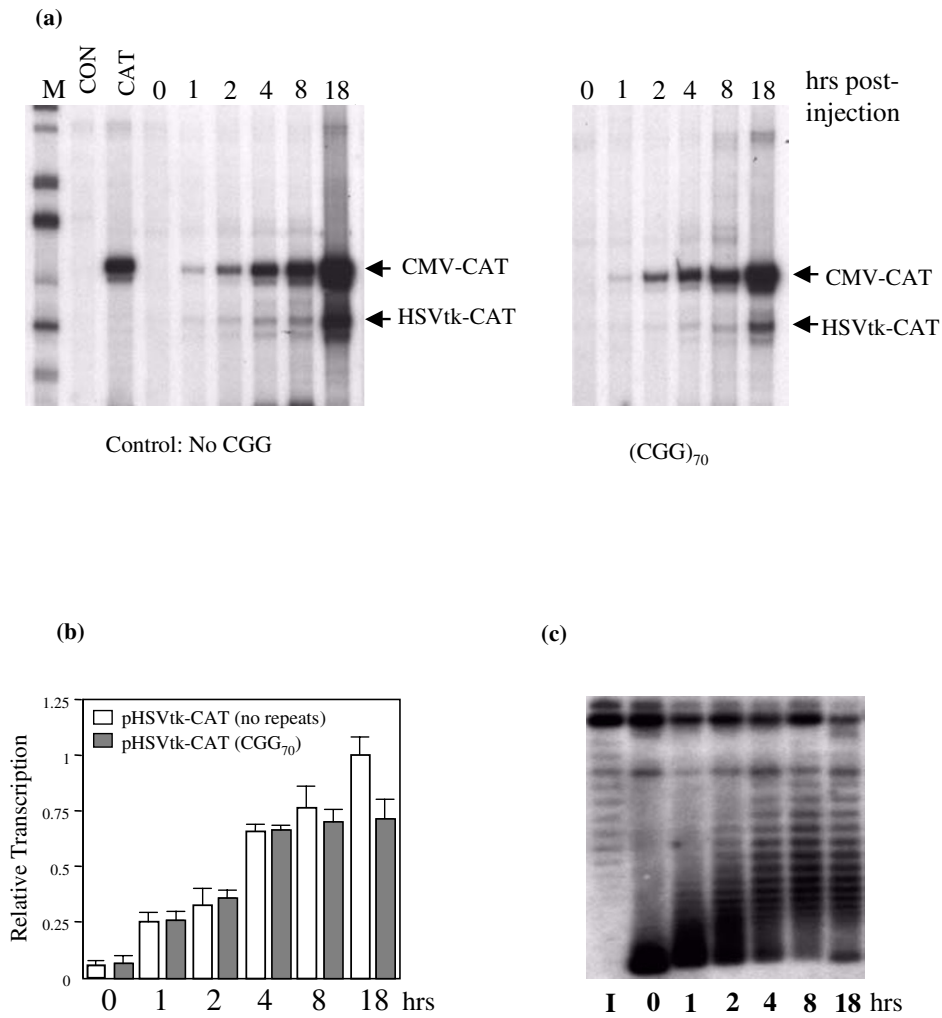
(a)

Control: No CGG

(CGG)$_{70}$

(b)

(c)

Figure 3
Repeat-Induced Transcriptional Repression is Time Dependent. (a) Primer extension products are shown from mRNA pools taken from oocytes injected with 5 ng of pHSVtk-CAT (no repeats), or pHSVtk-CAT (CGG$_{70}$) and with 0.3 ng pCMV-CAT as a control for the transcriptional competence of the oocytes. Lane numbers refer to time in hours post-injection. CON represents no oocyte injection and CAT refers to an injection of 0.3 ng pCMV-CAT alone. Primer extension products corresponding to, CMV-CAT and HSVtk-CAT mRNAs are shown. (b) A graphical representation of levels of transcription from the HSV-TK promoter based upon data shown in panel A. Transcription levels are normalised to the co-injected pCMV-CAT control and for background signal. (c) A Southern blot of a chloroquine supercoiling assay of injected pHSVtk-CAT (CGG)$_{70}$ DNA. Time points of injection mirror those of panel (b). Lane I represents input DNA, other lane numbers refer to time in hours post-injection.
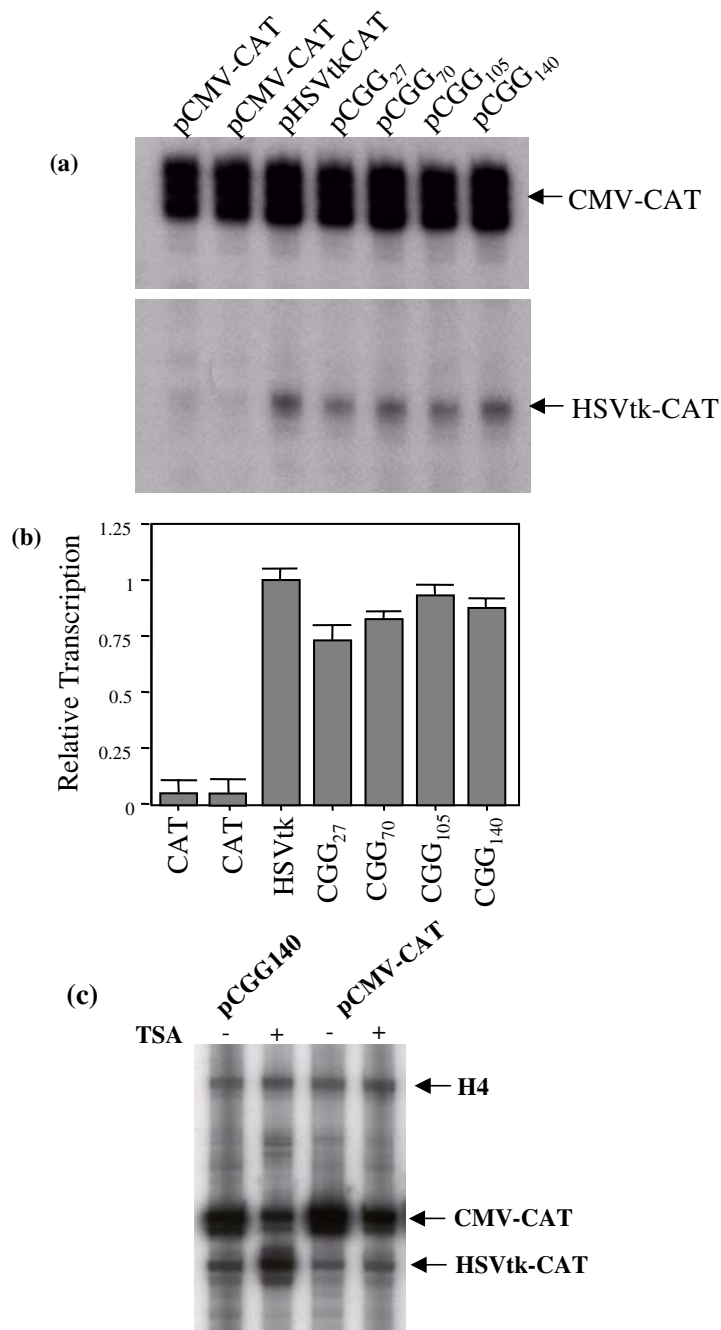
Figure 4
The Role of Chromatin in Repeat Induced Transcriptional Repression (a) Primer extension products are shown from run-off RNA transcripts performed *in vitro* using a Hela cell nuclear extract incubated with pHSVtkCAT, *in vitro* CpG methylated pHSVtkCAT, pHSVtkCAT-$(CGG)_{27}$, pHSVtkCAT-$(CGG)_{70}$ and pHSVtkCAT-$(CGG)_{105}$ DNAs. All reactions included control of pCMV-CAT DNA. (b) A graphical representation of the detectable transcript in the Helascribe reaction. The transcription was normalised to CMV-CAT as an internal control. (c) Primer extension analysis of the pHSVtkCAT-$(CGG)_{140}$ reporter and pCMV-CAT injection control. Injections were of 5 ng reporter and 0.3 ng injection control per oocyte. The injected oocyte populations were incubated for 18 hours in the presence of 30 nM TSA. The primer extension products are shown with arrows and include the endogenous H4 message as an mRNA preparation control.

transcriptional activity is exhibited by all reporter constructs, regardless of repeat number. Thus, in the absence of chromatin formation, we see no $(CGG)_n$ induced transcriptional repression. This confirms that we only see transcriptional repression after chromatin formation.

Repeat induced transcriptional repression is dependant on histone deactylases

To examine the nature of the chromatin – mediated repression, we analysed the effect of the global deacetylase inhibitor TSA on the transcriptional activity of the CGG_{140} containing reporter. As can be seen in figure 4c, in the absence of TSA, the CGG_{140} mRNA is undetectable, the band in the correct location on the gel is also present in the CMV-CAT control lanes, and hence is not reporter specific. The addition of 30 nM TSA leads to an almost complete reactivation of the CGG_{140} reporter to transcription levels approaching or surpassing that seen with the wild type CGG_{27}construct (Fig 2a). These results suggest an integral role for deacetylation of nucleosomes in the repressive mechanism mediated by isolated CGG repeats.

Discussion

We have shown that $(CGG)_n$ lengths in excess of 70 repeats can repress transcription from the HSVtk promoter in *Xenopus laevis* oocytes. They appear to do so in a length dependent manner, with arrays over $(CGG)_{105}$ leading to over 90% transcriptional repression. We have shown that this transcriptional repression occurs in a time dependent manner and is established by eight hours after injection. Analysis of chromatin formation indicates a correlation between maximal transcriptional repression and full chromatinisation of the injected DNA. Using a HeLa nuclear extract we show that $(CGG)_n$ arrays ranging from 27 to 140 triplets in length do not directly interfere with RNA polymerase II transcription. This strongly suggests that repression is mediated by the assembly of transcriptionally repressive chromatin over the HSVtk promoter and that this is directed by the $(CGG)_n$ array, in a length dependent manner. We show that TSA can relieve the repression associated with longer repeat length suggesting an integral role for histone deacteylation in the repressive pathway.

The results of this study are in contrast to the status of FMR1 gene transcription in fragile X carrier individuals with $(CGG)_n$ arrays between 55 and 200 repeats. In these individuals, the FMR1 gene is not only transcriptionally active, but appears to be producing elevated levels of mRNA, up to five times the wild type levels of mRNA for the longer arrays [43,44]. There are several possible explanations for this disparity.

Firstly, it may simply reflect an intrinsic feature of the experimental system we are using, in that the HSVtk promoter within our construct and within the components present in the *Xenopus* oocyte is more sensitive to the repressive effects of the $(CGG)_n$ array. As we observe a reduction of transcriptional competence of over 90%, this suggests that the repression mechanism mediated by the $(CGG)_n$ repeat sequence is extremely efficient, capable of virtually silencing a promoter linked to $(CGG)_{105}$. The fact that the HSVtk promoter is highly active compared to the wild type FMR1 promoter *in vivo* suggests that this repeat induced repressive mechanism would, most likely, result in complete silencing of the less active FMR1 promoter. Clearly, the ancillary factors required to establish transcriptional repression are not limiting in the stage six oocyte nucleus.

A second possibility is the transcriptional repression we observe might mask any transitory activation. This repressive response may be related to the quantity of injected DNA used in this study. Although the injection mass is a typical quantity used, it is the equivalent of a thousand copies of an identical DNA element. This amount of input DNA could induce transgene silencing mechanisms which would mask transient increases in transcript levels, leading to the establishment of a repressive chromatin environment. We do not believe that this type of transgene silencing is occurring for several reasons. We observe no repression of either the co-injected control reporter (CMV-CAT) or with identical amounts of injected pHS-Vtk-CAT carrying zero or 27 repeats. Previous studies have shown that methylation effects a time dependent repression of the HSVtk promoter by inhibition of transcriptional initiation [45]. If our observations were due to methylation, we might expect that a concomitant effect of this process would be *de novo* CpG methylation of the injected DNA. This does not appear to occur with our injected $(CGG)_n$ constructs, as assayed by comparison between *Hpa*II/*Msp*I restriction digestions on DNA samples after microinjection (data not shown). However, we cannot exclude methylation at a small number of perhaps crucial CpG sites within the HSVtk promoter not detectable by methylation sensitive enzyme cleavage reactions we performed.

We believe that the most likely explanation for the difference between our observations and those of the intrinsic FMR1 promoter lies in the *isolation* of the $(CGG)_n$ array away from its native promoter context. We suggest that the level of transcriptional activity of the FMR1 promoter is determined by a balance between the binding of *cis*-acting positive transcription factors and the *cis*-acting repressive effect of the $(CGG)_n$ array. Thus, in our experimental system, by isolating the $(CGG)_n$ repeat from the normal FMR1 chromosomal milieu, we may have removed regions of the promoter which serve to counteract the establishment of a repressive chromatin environment. The FMR1 promoter contains many such transcription factor

binding sites, including Sp1, a-Pal/NRF1, AP2, Myc, Zeste, USF1, USF2 and UBP1 [42,46] whereas the $(CGG)_n$ array itself is not essential for transcription [47].

This transcriptional repression effect of the $(CGG)_n$ may in itself serve as a functional cue for the *de novo* methylation across the repeats and the adjacent promoter. In figure 2, we show that the HSVtk promoter is sensitive to methylation at CpG sites. Several of the transcription factors which bind to the FMR1 promoter, including AP2 and α-Pal/Nrf-1, are known to be directly inhibited by methylation of cytosine residues within their recognition sites [50,51] and in the fully methylated state, these proteins are absent from the promoter [42,52]. Therefore, methylation of CpG residues within the FMR1 promoter would be expected to lead to the loss of transcriptional activation. Methylation is known to play an important regulatory role of the FMR1 promoter and differing degrees of promoter and repeat methylation are clearly associated with correspondingly amounts of FMR1 mRNA in development [4] and in cases of variability of clinical disease [53–55]. In addition to the direct effect upon transcription activator binding, the experiments of Godde *et al* (1996) showed that methylated $(CGG)_n$ arrays have a higher affinity for nucleosomes, indicating that methylation would also exert a direct effect upon local chromatin architecture [24]. Experiments are now underway to analyse the promoter region of injected DNA in more detail using bisulphite sequencing to determine if the formation of repressive chromatin can induce local DNA methylation.

We show an activation of transcription from the silenced CGG_{140} reporter by TSA, a broad spectrum deacetylase inhibitor. This ability of TSA to relieve repression has also been shown for the endogenous FMR1 promoter [25,63] albeit only partially, requiring interference with DNA methyltransferase for a full effect. We observe a complete derepression with TSA alone suggesting that upon our reporter constructs, which initially carry no methylation, the major determinant of the repressive effect we observe is histone deacetylation. The effect of TSA on viral reporter constructs is well documented [64,65], leading to a multifold activation in all cases. The important result here is that a previously silenced construct can express when deacetylase inhibitor is present. This would suggest that the first stage in FMR1 promoter silencing is mediated through histone deacetylation, with full and stable repression being 'locked – in' by methylation at a later stage.

In our suggestion that $(CGG)_n$ arrays can act as focus of repressive chromatin structures, is there any evidence for cellular factors which might mediate such a response? One potential mediator of a transcriptional effect may be the protein p20 which has been shown to bind the un-

methylated (but not methylated) FMR1 $(CGG)_n$ repeat array [56,57]. In transfection studies, expression of this protein leads to diminished expression from an FMR1 promoter carrying a $(CGG)_{16}$ repeat array [58]. This, or a similar protein in *Xenopus*, might bind to the injected $(CGG)_n$ containing transgenes and mediate or modulate the assembly of chromatin. In the case of the fragile X syndrome, we suggest that the balance between positively acting promoter factors and the repressive $(CGG)_n$ effect becomes altered when the arrays reaches over 200 repeats. This could be due to developmentally linked decreases in the level of transcriptional activators or could be related to the genetic instability of the array itself. It is possible that replication or transcription through the array could induce unusual structures within the $(CGG)_n$ array which act as substrates for methyltransferases with associated deacetylase activity [59,60] or direct altered chromatin components, such as histone H2AX, as part of the repair process [61,62]. At this point, the balance between the two regulatory forces would be then tipped in favour of the repressive chromatin effect. We have seen that $(CGG)_n$ transcriptional repression can act upon the strong HSVtk promoter to abolish 90% of transcription, even at 105 repeats. Thus, one might predict that in arrays of over 200 repeats the silencing effect would be absolute, the inhibition of transcription becoming stabilised through rapid alterations in local chromatin architecture.

Conclusions

Here we have shown that the role of chromatin is fundamental to the repression mediated by expanded fragile X repeats. We have devised an animal system in which we have isolated the transcriptionally repressive nature of the $(CGG)_n$ array, have shown that chromatin components are crucial to the process and that deacetylation has a major role in the repressive effect. The loss of the balance between this transcriptional repression and normal FMR1 promoter action most likely underlies the gene silencing seen in fragile X syndrome. This animal model will, therefore, prove a valuable tool in the analysis of therapies directed at reactivation of the silenced FMR1 gene. With this in mind, therapeutic avenues might in future explore the targeting and modification of the chromatin architecture in an effort to reactivate the silenced allele in the disease state before the establishment of a locked-down methylated FMR1 promoter.

Methods
Reporter plasmid construction
A reporter plasmid, pHSVtk-CAT, was constructed to allow stable propagation of human FMR1 $(CGG)_n$ arrays by the introduction of a 330 bp fragment from pBS-TK-CAT into the *Eco*RI site of pBR322. This fragment contains the 150 bp *BamH*I/*Bgl*II fragment from the HSV thymidine kinase gene promoter described by McKnight *et al* [31], a

short section of pBS derived polylinker and the 5' 30 bp from the CAT open reading from (ORF). The orientation of this fragment in pBR322 was determined by the observation that $(CGG)_n$ arrays can be stably propagated only in one replicative orientation [32]. Human FMR1 $(CGG)_n$ arrays of varying lengths were subsequently introduced immediately 3' to the CAT ORF by insertion of *Hin*pI fragments into the pBR322 the *Cla*I site. *Hin*pI digestion removed all flanking human FMR1 genomic DNA. FMR1 $(CGG)_{27}$ and $(CGG)_{70}$ *Hin*pI cassettes were isolated directly from the cloned arrays described by Hirst and White [32]. The FMR1 $(CGG)_{105}$ cassette was isolated by plasmid rescue from an *in vivo* expansion event in *S. cerevisiae* described in [33]. FMR1 $(CGG)_{140}$ was made by the concatenation of two $(CGG)_{70}$ *Hin*pI arrays. All constructs were validated using sequencing and diagnostic restriction analysis as described [33,34] and only plasmid DNA carrying full length arrays was utilised for further studies. Plasmid DNA was isolated using Qiagen reagents and reprecipitated before injection.

The methylated pHSVtk-CAT construct was prepared using *Sss*I CpG methylase (New England Biolabs) using the manufacturers protocol. The extent of methylation was tested using HpaII/MspI comparative digestion.

Preparation, microinjection, and maintenance of oocytes

Stage VI *X. laevis* oocytes were prepared as described previously [35]. For each experimental sample, 25–30 healthy oocytes were with 0.3–0.5 ng DNA reporter construct (see figure legend for specific amount) in 13.8 nl of double distilled water using a Nanoject II apparatus (Drummond Scientific, PA) and cultured in MBSH buffer [36] for 0–18 hrs. at 18°C. Specific details of each experiment can be found in the relevant figure legend.

Preparation of mRNA and Primer Extension Analysis

Total RNA was extracted from 7 oocytes per experimental group as previously described [37], with modifications as previously outlined [38]. Briefly, oocyte pools were homogenised in 70 μl 0.25 M Tris-HCl pH7.5 and subsequently mixed with 500 μl RNAzol B (Tel-Test, Friendswood, TX). After the addition of 50-μl chloroform, a 15 minute incubation on ice and centrifugation at 14,000 rpm for 15 minutes, the aqueous phase was removed and the RNA precipitated with an equal volume of isopropanol and microcentrifugation. The resultant pellet was resuspended in 100 μl DEPC water and the RNA reprecipitated with LiCl. The resultant pellet was resuspended in 20 μl of DEPC water. 10 μL of this was used for primer extension analysis.

0.2 pmol of labelled primer specific for the 5' region of the CAT gene in the reporter constructs was mixed with the total RNA preparation and annealing performed in 0.6 ×

first strand buffer (Life Technologies, Gaithersberg, MD). The annealing protocol was: 65°C for 10 minutes, 55°C for 20 minutes, 37°C for 30 minutes and room temperature for 5 minutes. 20 U of Superscript II reverse transcriptase were added in conjunction with 2.5 mM dNTP mixture and 2 U RNase inhibitor (Life Technologies, Gaithersberg, MD). The reaction was allowed to proceed for 1 hour at 42°C. The products of the primer extension were run on an 8% denaturing polyacrylamide gel and visualised using storage phosphor technology (Molecular Dynamics). The primer extension experiments in presence of TSA were performed as above except that the oocytes were incubated in the presence of 30 nM TSA for 18 hours prior to harvesting.

All primer extension experiments were performed in triplicate to ensure validity and statistical significance.

Analysis of the extent of chromatin formation

30 μl homogenate samples from above, corresponding to three oocyte equivalents, were mixed with an equal volume of Proteinase K buffer (30 mM EDTA pH 8.0, 20 mM Tris-HCl pH 7.5, 1% SDS). 3 μl of 10 mg/ml Proteinase K was added and the samples incubated for 1 hour at 42°C. Sample volumes equivalent to 0.5 oocytes were fractionated on a 1% agarose gel in 1 × TPE buffer containing 70 μgm/ml chloroquine as previously described [39,40]. Southern blots of these gels were performed using Biodyne B membrane (Pall, UK) and downward capillary blotting performed [41]. Probing was carried out exactly as per manufacturers instructions with Rapidhyb solution (Amersham Pharmacia Biotech, Little Chalfont, UK) and high stringency washes.

In Vitro Run Off Transcription analysis

In vitro transcription reactions were performed with 0.5 μg linearised reporter plasmid DNAs linearised at a single *Pvu*I site (within the pBR322 vector backbone) and purified by electrophoresis through 1% agarose. The corresponding bands were excised and electroeluted as previously described [28]. Transcription reactions were performed in Helascribe extracts as per manufacturers recommendations (Promega, UK). The resultant mRNA samples were analysed for transcript levels using primer extension as described above.

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