The ATTCT repeats of spinocerebellar ataxia type 10 display strong nucleosome assembly which is enhanced by repeat interruptions

Katharine A. Hagerman a,b,1, Haihe Ruan c,1, Kerrie Nichol Edamura b, Tohru Matsuura d, Christopher E. Pearson a,b,1, Yuh-Hwa Wang e,*

1 Both authors contributed equally.

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1. Introduction

The expansion of repeat tracts is associated with at least 42 human diseases (Cleary and Pearson, 2003; Pearson et al., 2005). Much attention has been paid to trinucleotide repeat diseases such as myotonic dystrophy (DM1) and fragile X syndrome (FRAXA) as at least 14 diseases are caused by the instability of these repeats. However, other non-trinucleotide repeat diseases have been discovered such as myoclonus epilepsy type 1, myotonic dystrophy type 2 (DM2) and spinocerebellar ataxia type 10 (SCA10). SCA10 is caused by a large ATTCT expansion in intron 9 of the ATXN10 (SCA10) gene on chromosome 22q13, and is the only known pentanucleotide expansion to cause human disease (Matsuura et al., 2000). SCA10 is characterized by progressive ataxia, seizures and anticipation (Grewal et al., 1998; Matsura et al., 1999; Rasmussen et al., 2001; Grewal et al., 2002). Unaffected individuals have between 10 and 29 ATTCT repeats, whereas affected individuals typically have between 800 and 4500 repeats (Matsuura et al., 2000; Matsura et al., 2004). As has been suggested for other repeat diseases such as DM1 and SCA7, large variations in repeat instability may be associated with a number of cis- and trans-factors at the ATXN10 locus (Pearson et al., 2005). Recent evidence has implicated the binding of the chromatin insulator protein CTCF adjacent to the SCA7 CAG tract as a regulator of CAG instability (Libby et al., 2008).

Many cis-elements have been implicated in contributing to repeat instability such as the length and purity of the repeat tract, sequences flanking the repeat, and the surrounding epigenetic environment including DNA methylation and chromatin structure (Cleary and Pearson, 2003; Libby et al., 2008). Matsura et al., (2006) examined the SCA10 repeat tract purity in detail, and found that the ATCTC expansions in the 5′ end and ATTCTAT interruptions at the 3′ end, and revealed interruptions in 71% of alleles sized 17–29 in the penultimate repeat. This suggested that interruptions may explain the complex relationship between repeat length and disease severity. Association of pure and interrupted repeats with variably expressed disease phenotypes also supported the belief that repeat purity may be a
ATXN10 DNA (Handa et al., 2005; Lin and Ashizawa, 2005; Wakamiya and histone acetylation status on nucleosome assembly. ATTTTCT and ATATTCT repeat interruptions found in SCA10 patients ability of the ATTCT repeat to form nucleosomes, and the effect of random sequence than pure repeats. In this paper, we examined the nucleosomes more strongly and weakly, respectively, than random sequence. Thus, interruptions in the ATTCT repeat may affect the overall DNA sequence purity in repeat diseases.

The molecular mechanism for the pathogenesis of SCA10 is unknown. Some suggest that the expanded AUUCU m RNA products may act as toxic-RNA species, or alter binding of neuronal proteins to the ATXN10 DNA (Handa et al., 2005; Lin and Ashizawa, 2005; Wakamiya et al., 2006; Waragai et al., 2006). Other data suggests altered chromatization of the expanded repeat may alter expression of adjacent genes, as occurs in facioscapulohumeral dystrophy (FSHD) and FRDA (Gabellini et al., 2002; Saveliev et al., 2003). Recent data suggest that the SCA10 repeat can act as a DNA unwinding element (DUE) (Potaman et al., 2003) which has been shown to be a critical element for the initiation of DNA replication (Kowalski et al., 1998). Given that the SCA10 expansion causes the region to become highly A+T-rich this may induce DNA unwinding, which may affect gene expression over long distances as well as DNA replication at the ATAX10 locus (Liu et al., 2007). The ATCTC and ATTCCTAT interruptions found in the SCA10 repeat would decrease and increase the A+T-content of the ATCT tract, respectively, which might affect the propensity to form slipped-DNA structures, as occurs for interrupted CAG and CGG repeats (Pearson et al., 1998), and its ability to readily unwind. The interruptions would also place the repeat out of phase with the DNA helical pitch thereby changing the face of DNA and possibly affect protein-DNA interactions.

These features could affect nucleosome formation over the ATCTC repeats. Thus, interruptions in the ATCTC repeat may affect the overall DNA structure, and further influence chromatization of the SCA10 region. However, there is no information about the ability of the ATCTC repeat to assemble into nucleosomes or the effect of sequence interruptions on the assembly.

We previously reported altered nucleosome formation on trinucleotide repeats depending on the repeat sequence, repeat purity, and the acetylation status of the histones (Mulvihill et al., 2005).

We found that CAG and CGG repeats formed hypoacetylated nucleosomes more strongly and weakly, respectively, than random sequences. Naturally occurring interruption patterns found in patients caused the nucleosome formation to change, and behave more like random sequence than pure repeats. In this paper, we examined the ability of the ATCTC repeat to form nucleosomes, and the effect of ATTTTCTC and ATATTCTC repeat interruptions found in SCA10 patients and histone acetylation status on nucleosome assembly.

2. Materials and methods

2.1. DNAs and proteins

Genomic clones (ATTCT)73p and (ATTCT)71i were obtained by amplifying regions of disease-associated ATCTC repeats from SCA10 patients. Repeat primed-PCR amplified the large repeat and determined that the (ATTCT)71i plasmid had an interruption pattern of 39-A-1-B-1-B-1-A-1-B-5-B-7 where the numbers represent the number of uninterrupted ATCTC repeats, and an “A” represents an ATTTTCTC interruption and a “B” represents an ATATTCTC interruption (Fig. 1) (Matsuura et al., 2006). Repeats containing fragments were isolated from the plasmids by EcoRI digestion, with the 104 bp of the endogenous ATAX10 locus 5' of the repeat as well as 9 bp and 34 bp of plasmid DNA 5' and 3' of the repeat, respectively. In total, the (ATTCT)73p fragment was 512 bp and (ATTCT)71i was 514 bp. A 488-bp pUC19 fragment was obtained by PCR amplification of pUC19 using primers from nt 137 to 161 and from nt 609 to 624. DNA fragments were purified by 4% polyacrylamide gel electrophoresis, and labeled with T4 polynucleotide kinase (NEB) in the presence of [γ-32P] ATP (Amersham Biosciences).

Hypoacetylated histone octamers were isolated from HeLa cells (Orphanides, 1998). Also, HeLa cells were treated with 10 mM sodium butyrate for 24 h to generate hyperacetylated histone octamers. Hyperacetylation of core histone proteins was about 33-fold higher compared to hypoacetylated octamers, as quantified by Western blot analysis using an antibody to acetylate H3 (Upstate Biotechnology). Recombinant histone octamers were purified from Escherichia coli which was expressing full-length Xenopus laevis histones (Luger et al., 1999). All three types of histones were tested to be fully active in nucleosome reconstitution (Trojer et al., 2007).

Recombinant Drosophila nucleosome assembly protein-1 (NAP-1) was expressed and purified from baculovirus-infected Sf9 cells (Pavri et al., 2006). Remodeling and spacing factor (RSF) was purified from the HeLa nuclear pellet fraction (Loyola et al., 2001).

2.2. Competitive nucleosome reconstitution

Competitive nucleosome reconstitution by a salt dilution method was carried out as previously described (Hsu and Wang, 2002; Trojer et al., 2007). Briefly, 50 ng of radioactively labeled DNA was mixed with 10 µg of unlabeled calf thymus DNA (Invitrogen) and 2.5 µg of histone octamers in a solution containing 2 M NaCl. NaCl was incrementally reduced to a final concentration of 0.1 M. The assembly mixtures were then electrophoresed directly on 4% polyacrylamide gels to separate free DNA from the nucleosome-assembled DNA. Results were visualized by autoradiography. The amount of DNA in each band was determined by PhosphorImager (Molecular Dynamics) scanning. A “no histones” assembly reaction was performed as a control, in which the reaction mixture did not contain histone octamers.

Competitive nucleosome reconstitution using the NAP-1 histone chaperone protein to more closely mimic physiological conditions was also carried out to examine the ability of the ATCTC repeats to form nucleosomes. 50 ng of radioactively labeled DNA fragment was mixed.

Fig. 1. SCA10 genomic fragments. The fragments used in nucleosome reconstitution were derived from clones of SCA10 patient cells (Matsuura et al., 2006). In the schematic, the ATCTC repeats are indicated by hollow circles and interruptions indicated by filled circles. The interruption sequence is indicated by an ‘A’ for ATTTTCTC and ‘B’ for ATATTCTC. The cloned repeats were isolated by EcoRI digestion, and 104 bp of endogenous ATAX10 flanking sequence is included 5’ of the repeat along with 9 bp and 34 bp of plasmid DNA upstream and downstream of the repeat, respectively.
with 10 μg of unlabeled calf thymus DNA, 2.5 μg of histone octamers, and 5 μg of recombinant NAP-1 in buffer (10 mM Hepes, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 0.5 mM EGTA, 0.1 mM EDTA, and 10% glycerol), and incubated at 30 °C for 4 h. The assembly mixtures were then electrophoresed on 4% polyacrylamide gels. Results were also visualized by autoradiography. The amount of DNA in each band was determined by PhosphorImager (Molecular Dynamics) scanning.

2.3. Assembly and analysis of nucleosome arrays

Assembly reactions were performed as described in Loyola et al. with 1 μg of plasmid DNA, 0.9 μg of hypoacetylated histones, 0.25 μg of RSF, 3.1 μg of NAP-1, 50 μg of bovine serum albumin (New England Biolabs), 3 mM ATP, 30 mM phosphocreatine, 0.2 μg of phosphocreatine kinase, 10 mM MgCl₂, 50 mM KCl, 10 mM Hepes, pH 7.6, 0.2 mM EDTA, and 5% glycerol at 30 °C for 16 h (Loyola et al., 2001). The reaction mixtures were then partially digested with 0.0008 or 0.0025 U of micrococcal nuclease (Sigma). The digestion patterns were analyzed by Southern blots probing with an oligonucleotide containing 5 repeats of ATTCT (Integrated DNA Technologies, Inc.).

3. Results

3.1. ATTCT repeat is a strong nucleosome positioning element

Towards determining the affinity of SCA10 ATTCT repeats to form nucleosomes, we isolated ATTCT repeats from cloned plasmids containing patient DNA sequences (Matsuura et al., 2006). The pure repeat clone (ATTCT)₇₃ had 73 ATTCT repeats whereas the interrupted clone (ATTCT)₇₁ had 71 interrupted ATTCT repeats (Fig. 1). The pattern of interruptions were those present in described SCA10 families (Matsuura et al., 2006).

Using hyperacetylated, hypoacetylated, or recombinant histone octamers, nucleosomes were assembled upon these repeat-containing DNA fragments as well as a size-matched pUC control DNA used as a baseline of nucleosome assembly, according to the previously described competitive nucleosome reconstitution assay by salt dilution (Mulvihill et al., 2005). Following gel electrophoresis, the nucleosome-associated radiolabeled DNAs appeared as a retarded band above the protein-free radiolabeled DNA. Nucleosome assembly was quantified as the ratio of the nucleosome-assembled DNA to free DNA and normalized to the unit ratio generated from the pUC nucleosome assembly. Overall, both pure and interrupted ATTCT repeats formed nucleosomes significantly more efficiently than random non-repetitive DNA, regardless of histone type. Nucleosome assembly was determined by PhosphorImager (Molecular Dynamics) scanning.

3.2. Repeat interruptions increase hyperacetylated nucleosome formation on the ATTCT repeat under physiological conditions with NAP-1

In the salt dilution method above, large variability between experimental replicates was observed. During salt dilution, ATTCT repeats may undergo structural changes that could affect the stability of nucleosome formation, and cause the variability. This could mask the differences in nucleosome assembly, between pure and interrupted ATTCT repeats. To further elucidate possible differences in nucleosome assembly of pure and interrupted ATTCT repeats, we performed competitive nucleosome reconstitution under physiological conditions using NAP-1.

The NAP-1 has been shown to chaperone histones to DNA, and prevent non-specific aggregation of histones (Fujii-Nakata et al., 1992; Ito et al., 1996; Gemmen et al., 2005). Using the same pure and interrupted ATTCT repeats, nucleosome assembly was performed with the addition of NAP-1. Hyperacetylated histones showed a greater than 3-fold increase in nucleosome assembly on interrupted ATTCT repeats over pure repeats (p = 0.03) (Fig. 3). There was no significant difference in nucleosome assembly between pure and interrupted ATTCT repeats with hypoacetylated and recombinant histones.
Therefore, under more physiological conditions, interrupted ATTCT repeats display a stronger assembly of hyperacetylated nucleosomes than pure repeats.

3.3. Both pure and interrupted repeats form evenly-distributed nucleosome arrays

Given that both pure and interrupted ATTCT repeats display a strong ability for nucleosome assembly, we next investigated whether both repeats formed well positioned nucleosomes in a nucleosome array setting. Nucleosome arrays were assembled onto plasmids with pure or interrupted ATTCT repeats, using NAP-1 and the RSF protein. The RSF protein spaces nucleosomes and remodels chromatin (Loyola et al., 2001). The pUC19 control plasmid was analyzed in parallel as well. All three plasmids were assembled with hypoacetylated histones, and the reaction mixtures were partially digested with micrococcal nuclease to examine the periodicity of nucleosome formation. In ethidium bromide stained gels (Fig. 4A), 180 bp nucleosomal ladders were observed in all three samples, indicating that all three plasmids form regularly spaced nucleosome arrays. In both pure and interrupted ATTCT repeats, the nucleosomal ladders were further confirmed by Southern blot with a (ATTCT)5 probe (Fig. 4). This suggests that well-spaced nucleosomes, rather than altered periodicities or aggregates, are formed over the ATTCT repeat regardless of the presence or absence of interruptions.

4. Discussion

In this study, we examined the ability of the ATTCT repeats to assemble nucleosomes, and the effects of disease-associated ATTCT repeat purity and histone acetylation on the assembly. We found that SCA10 ATTCT repeats are strong nucleosome assembling sequences compared to random sequences, with stronger formation on interrupted repeats with hyperacetylated histones at physiological conditions (with NAP-1), as well as formation of well-positioned nucleosomes, not aggregates, on the repeats. The differences observed in this study with over 70 ATTCT repeats may be considerably amplified in the genomic context where the SCA10 disease-causing expansions are typically more than 800 and up to 4500 ATTCT repeats (Matsuura et al., 2004). Such increased binding has been observed for the large CTG tracts of DM1 (Wang et al., 1994; Wang and Griffith, 1995). The increased nucleosome assembly on the SCA10 repeats was similar to that on the CAG repeats of the SCA1 and DM1 locus (Supplementary table 1, Mulvihill et al., 2005; Wang et al., 1994; Wang and Griffith, 1995), and therefore the assembly on the interrupted SCA10 repeat could be even greater.
Nucleosome formation on repeats has been studied for many years, with focus on diseases caused by trinucleotide repeat expansions (Wang, 2007). CAG repeats from diseases such as DM1 and SCA1 are the strongest nucleosome positioning elements (Wang and Griffith, 1995) whereas CGG repeats associated with FRAXA preferentially exclude nucleosomes (Godde et al., 1996; Wang et al., 1996; Wang and Griffith, 1996). The exclusion of nucleosome formation at CGG repeats may be associated with both fragile site formation (Wang et al., 1996) and altered transcription dependent upon repeat size (Tassone et al., 2000; Tassone et al., 2007).

Understanding the in vitro behavior of the ATTCT repeat may similarly lead to an understanding of its behavior in cells and downstream metabolic and pathogenic mechanisms. SCA10 repeats are known to form unwound DNA and the high A+T-content is attributed to its serving as a DUE (Kowalski et al., 1988) and facilitating the in vitro activation of replication origins (Kowalski et al., 1988; Potaman et al., 2003). In vivo, there is a natural origin of replication in the ATXN10 gene just 5′ of the ATTCT repeat which produces 5- to 10-fold more nascent DNA in SCA10 patient cells than controls (Liu et al., 2007). It is noteworthy that Kemp et al., (2005) found that alteration of chromatin packaging can dramatically affect the pattern of origin activation at various replication origins. Thus, the strong nucleosome positioning of the ATTCT sequences, and differences between pure and interrupted repeats may affect origin activity, transcription and DNA instability. The replication machinery must unwind and separate duplex DNA strands from each other and from histones, and in the process, replication fork progression may be stalled or slowed by the presence of hyper stable nucleosomes. This could be further exacerbated by the presence of interruptions. In addition, initiation of replication and transcription is controlled, at least in part, by the degree of local unwinding of nucleosomal DNA (Kowalski et al., 1988; Potaman et al., 2003). This unwinding can be regulated by histone acetylation; increased acetylation results in a more loosely wound structure with reduced thermal melting temperatures (Yau et al., 1982; Thomsen et al., 1991) allowing access of replication or transcription factors. Furthermore, acetylation of histones can effectively induce negative supercoiling (Norton et al. 1989) which may enhance DNA unwinding (Kowalski et al., 1988; Potaman et al., 2003). The DNA unwinding activity of the SCA10 repeat, coupled with our finding of its preferential binding to acetylated histones, along with their association with replication origins (Aggarwal and Calvi, 2004; Zhou et al., 2005), and the cell cycle regulation of histone hyperacetylation (Bradbury, 1992), prompts us to propose that histone acetylation at flexible repeat tracts may participate in replication initiation (Liu et al., 2007).

Repeat interruptions occur in various human diseases (DM2, FRAXA, FRDA, SCA1, SCA2, SCA3, SCA8, and SCA10), and only rarely detected in other diseases like DM1 (Leeflang and Arnhem, 1995). Unlike the reduced nucleosome formation caused by interruptions in CAG repeats, we show increased formation on interrupted ATTCT repeats with hyperacetylated histones under physiological conditions. This difference may be reflected upon in the stability of the locus, since interrupted trinucleotides are typically more stable (Eichler et al., 1994) whereas interrupted SCA10 pentanucleotides found in patients are unstable (Matsura et al., 2006). The strong nucleosome assembly by the ATTCT repeats is likely due to the abundance of flexible TA dinucleotides, which increase bending of the repeats to wrap DNA around histones (Shrader and Crother, 1990; Lowary and Widom, 1998; Packer et al., 2000; Tolstoshev et al., 2007). The interruption pattern described in this paper increases the number of flexible dinucleotides in the repeat tract, and may thus further increase the DNA bending ability, making nucleosome formation more favorable. Also, hyperacetylated histones are often associated with transcriptionally active genes; preferential formation of hyperacetylated nucleosomes onto the interrupted repeat may prime the interrupted alleles into a different transcription pattern from the pure alleles. Interestingly, the patient families that these pure and interrupted sequences were derived from have different disease symptoms, with the pure repeats found in family 1 having no seizures and the interrupted repeats found in family 2 with seizures (Matsura et al., 2006).

Most untranslated repeat disease pathogenic mechanisms are centralized around RNA expression, toxic RNAs (Ranum and Cooper, 2006), DNA repair (Slean et al., 2008) and gene chromatinization (Cho et al., 2005), therefore nucleosome formation of the repeats can be important in understanding the disease mechanism. The increased formation of nucleosomes on interrupted versus pure ATTCT repeats could add to our understanding of SCA10 pathogenesis and instability.

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Appendix A. Supplementary data


References


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