Altered Replication in Human Cells Promotes DMPK (CTG)_n · (CAG)_n Repeat Instability

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Myotonic dystrophy type 1 (DM1) is associated with expansion of (CTG)_n · (CAG)_n trinucleotide repeats (TNRs) in the 3' untranslated region (UTR) of the DMPK gene. Replication origins are *cis*-acting elements that potentiate TNR instability; therefore, we mapped replication initiation sites and prereplication complex protein binding within the ~10-kb DMPK/SIX5 locus in non-DM1 and DM1 cells. Two origins, IS_{DMPK} and IS_{SIX5}, flanked the (CTG)_n · (CAG)_n TNRs in control cells and in DM1 cells. Orc2 and Mcm4 bound near each of the replication initiation sites, but a dramatic change in (CTG)_n · (CAG)_n replication polarity was not correlated with TNR expansion. To test whether (CTG)_n · (CAG)_n TNRs are *cis*-acting elements of instability in human cells, model cell lines were created by integration of cassettes containing the c-myc replication origin and (CTG)_n · (CAG)_n TNRs in HeLa cells. Replication forks were slowed by (CTG)_n · (CAG)_n TNRs in a length-dependent manner independent of replication polarity, implying that expanded (CTG)_n · (CAG)_n TNRs lead to replication stress. Consistent with this prediction, TNR instability increased in the HeLa model cells and DM1 cells upon small interfering RNA (siRNA) knockdown of the fork stabilization protein Claspin, Timeless, or Tipin. These results suggest that aberrant DNA replication and TNR instability are linked in DM1 cells.

Myotonic dystrophy type I (DM1) is a human autosomal dominant neuromuscular disorder caused by the expansion of an unstable (CTG)_n · (CAG)_n trinucleotide repeat (TNR) tract mapped to human chromosome 19q13.3 in the 3' untranslated region (UTR) of the dystrophin myotonia protein kinase (DMPK) gene (62). Transgenic and knock-in mouse models that recapitulate the expansion of the DMPK TNRs showed that the integration site and the amount of flanking human genomic sequence affect TNR instability (19, 28, 35, 77, 79). These findings suggest that *cis*-acting elements may play a crucial role in determining TNR stability during normal differentiation or pathological states. Among the potential *cis*-acting factors, DNA replication origin activity has drawn increased attention since it was found that the DNA replication polarity and origin location strongly influenced the amount and type (contraction or expansion) of TNR instability in *Escherichia coli* plasmids (43, 47, 81, 87), *Saccharomyces cerevisiae* (31, 63, 64), and cultured human cells (17, 18, 56).

The initiation of DNA replication is regulated by the interaction of *cis*-acting DNA sequences, replication origins, with *trans*-acting protein factors in order to initiate DNA replication at the correct place and time once per cell division cycle (61). Recently, several DNA replication origins have been mapped close to the genomic sites of expandable microsatellite repeats at the FMR1 (9, 37), HD, SCA7, SBMA (69), FMR2 (11), SCA10 (55), and DMPK (19) loci, which may reflect evolutionary selection for the position of DNA replication origins relative to microsatellite repeat tracts. At three disease loci, SCA7, HD, and SBMA, the results of origin mapping suggested that the unexpanded (CAG) · (CTG) sequence is replicated from a downstream initiation region (relative to the direction of transcription) at the wild-type SCA7 locus, from an upstream initiation region at the wild-type HD locus, and from either upstream or downstream initiation regions at the wild-type SBMA locus, leading to the conclusion that a simple relationship does not exist between (CAG)_n · (CTG)_n replication polarity and TNR instability when these loci are unaffected (69). In contrast,

the instability of (CAG)_n · (CTG)_n repeats in the disease state may be related to their late-S-phase replication, which is characteristic of chromosomal fragile sites (11, 53), or the replication of the (CAG)_n sequence as the lagging-strand template at the human expanded HD locus (69).

Relevant to studies of the relationship between the DNA replication and (CAG)_n · (CTG)_n stability is a comparison of origin activity at the DMPK locus in human control and DM1 fetal fibroblasts and transgenic mice (19). In this work, two origins were identified, upstream and downstream of the DMPK (CAG)_n · (CTG)_n repeats, in control and DM1 human fibroblasts. Transgenic mice bearing a single copy of an ~45-kb genomic region of the expanded DM1 locus containing (CTG)_{>300} · (CAG)_{>300} repeats showed high levels of intergenerational and somatic repeat instability (76). Unlike in humans, however, when origin activity was quantitated over the ~45-kb human DM1 transgene from pancreatic cells of mice bearing either >300 (DM328) or 20 (DM20) (CTG)_n · (CAG)_n repeats (79), neither the upstream nor the downstream origin was active in the DM20 control mice, and the upstream origin was inactive in DM328 mice (19). Thus, the nuclear environment or human/mouse sequences flanking the transgene may also modulate its replication origin activity and any downstream effects on TNR stability.

Studies of the human DMPK locus indicate that the TNR and the adjacent binding sites of CTCF, a zinc finger DNA binding factor, form a CTCF-dependent chromatin insulator element (13,

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27). Correlated with $(CTG)_n \cdot (CAG)_n$ expansion from normal-size ($n = 5$ to 37 repeats) to disease-associated ($n > 80$ repeats) lengths are changes in the local chromatin structure, including DNA methylation, induction of heterochromatin, alteration of nucleosome positioning, decreased nuclease hypersensitivity, and decreased gene expression of the DMPK gene and the neighboring SIX5 gene (13). Suggesting a role for the perturbation of replication fork dynamics in DMPK $(CTG)_n \cdot (CAG)_n$ instability, it has been reported that DNA replication inhibitors and DNA-damaging chemotherapeutic drugs can modulate instability at the DMPK locus in DM1 patient-derived cells (41, 94) and transgenic-mouse cells (36).

In the current work, we investigated the DNA replication origin activity at the DMPK/SIX5 region using non-DM1 and patient-derived DM1 cells. Our results using a quantitative real-time PCR-based nascent-DNA abundance assay (NDAA) (34, 55, 57) demonstrate that two DNA replication initiation sites, IS_{DMPK} and IS_{SIX5} , are present in the DMPK/SIX5 region. Furthermore, pre-replication complex (pre-RC) binding sites in the DMPK/SIX 5 region have been identified by chromatin immunoprecipitation (ChIP). In non-DM1 cell populations, DNA replication initiation sites are located on either side of the DMPK TNRs, indicating that the $(CAG)_n$ repeats may be used as either a lagging-strand template, favoring TNR expansion, or a leading-strand template, favoring TNR contraction, during DNA replication (67). The presence of two replication origins in the DMPK locus was supported by the detection of the binding sites of the prereplication complex, which revealed that Orc2 and Mcm4 were recruited to two adjacent initiation sites near IS_{DMPK} and IS_{SIX5} . However, TNR expansion was not correlated with a change in origin activity or pre-RC protein binding at either origin, which differed in a cell-type-specific manner. Our mapping of replication origins upstream and downstream of the DMPK TNRs confirms, in part, and extends previous identification of two replication origins in the DMPK/SIX5 locus in human DM1 and control fetal fibroblasts (19).

These results suggest that the alteration of chromatin structure associated with DMPK $(CTG)_n \cdot (CAG)_n$ expansion does not stably change the relative activities of the upstream and downstream origins between DM1 and non-DM1 cell populations. In *Saccharomyces cerevisiae*, TNRs have been shown to increase the occurrence of stalled replication forks (49, 72, 75), and this form of replication fork stress has been shown to promote DNA breakage and genome instability (3, 10, 20, 21, 29, 56). To test whether the $(CTG)_n \cdot (CAG)_n$ TNRs themselves could play a role in instability in human cells and to characterize the relationship between DNA replication origin activity and TNR instability, apart from the effects of diverse genetic backgrounds, we constructed a series of isogenic cell lines in which the well-defined c-myc replication origin was fused to $(CTG)_n \cdot (CAG)_n$ TNRs, generating chimeric c-myc replicator-TNR structures, each of which was integrated at the same ectopic chromosomal site in HeLa cells (56). Previous results demonstrated that DNA hairpins form in a replication-dependent manner at the ectopic TNRs and that DNA replication origin activity increased the instability of the adjacent hairpin-prone TNRs *in vivo* (56). Since expanded $(CTG)_n \cdot (CAG)_n$ TNRs are sites of replication fork stalling and genome instability in yeast (30, 73, 89, 90), we tested the effect of $(CTG)_n \cdot (CAG)_n$ TNRs on replication fork progress and the possible requirement for stabilization of TNR replication by the fork protection proteins Timeless, Tipin, and Claspin. We found that replication forks were

stalled by flanking expanded TNRs and that TNR stability was dramatically decreased after knockdown of the replication-fork-stabilizing proteins. These results are consistent with earlier results showing that the formation of alternative DNA secondary structures (hairpins) by the TNRs is promoted by DNA replication and that the instability of the expanded TNRs is increased by DNA replication inhibitors (56). Taken together, the current data suggest that the perturbation of replication activity in the DMPK/SIX5 region can contribute to DMPK $(CTG)_n \cdot (CAG)_n$ TNR instability.

MATERIALS AND METHODS

Cell culture. HeLa derivative cell lines containing a Flp recombinase target site (HeLa/406) were grown and transfected as described previously (55, 57). MM (SCA10) lymphoblastoid cells were a generous gift from Tetsuo Ashizawa. 428-12D cells are lymphoblastoid DM1 cells (41) derived from 428 cells by chemotherapeutic treatment. IMR90 fibroblasts were a gift from Steven Berberich (Wright State University). GM04033 and GM03987 DM1 cells and non-DM1 GM01653 cells (age, tissue, and sex matched to GM04033) were purchased from the Coriell Institute for Medical Research. The cells were grown at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) (HeLa), RPMI 1640 (lymphoblasts), or minimal essential medium (MEM) (IMR 90, GM04033, GM03987, and GM01653) supplemented with 10% fetal bovine serum.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed as described previously (33). In brief, formaldehyde cross-linked chromatin was sonicated and subjected to micrococcal nuclease (MNase) digestion. Chromatin immunoprecipitation was performed by using either rabbit preimmune IgG or specific rabbit polyclonal antibodies against human Orc2 (Santa Cruz SC-2874) or human Mcm4 (Santa Cruz SC-22779). ChIP DNA (~0.5 to 1.0 kb) was purified with a DNA purification kit (Qiagen) and quantitated by SYBR green (Invitrogen) real-time PCR on the ABI 7000 (Applied Biosystems).

siRNA treatment. $(CTG)_{102} \cdot (CAG)_{102}$ cells (30% confluent) were transfected with 50 nM (final concentration) small interfering RNA (siRNA) targeting Claspin, Timeless, or Tipin in six-well plates every 48 h for a total of three transfections. DM1 GM04033 fibroblasts were transfected twice, at 48-h intervals, and harvested 48 h after the second transfection. Control experiments were carried out using nontargeting siRNA. The sequences of the Timeless siRNA oligonucleotides are GUAGCU UAGUCCUUUCAAAdTdT and UUUGAAAGGACUAAGCUACdTdT, those of the Tipin siRNA oligonucleotides are UGAUUGACCUACCAG AUUAdTdT and UAAUCUGGUAGGUCAAUCAdTdT, and those of the Claspin siRNA oligonucleotides are GACUCAGCUCUAAGCAAGdTdT and CUUGCUUAGAGCUGAGUCdTdT (Dharmacon Research, Inc.).

Western blotting. Whole-cell lysates were prepared from treated or untreated HeLa cells or DM1 cells and probed after SDS-PAGE using rabbit antibodies against human Claspin (Santa Cruz SC-48771), human Timeless (Bethyl Laboratories A301474A), human Tipin (a gift from Michael Kemp and Aziz Sancar, University of North Carolina), or human β -actin (Santa Cruz SC-7210).

DNA purification and PCR analysis. Isolation of short (0.5- to 2-kb) nascent DNA by single-step denaturing gel electrophoresis, isolation of genomic DNA, standard PCR, small-pool PCR (spPCR), and quantitative real-time PCR have been described previously (55–57). The PCR primers used in this work are provided in Table 1. For quantitative-PCR (qPCR) quantitation of relative nascent-DNA abundance, the data were normalized against the signal from the low nascent-strand abundance site sequence-tagged site 54.8 (STS-54.8) in the human β -globin locus (42). For qPCR quantitation of ChIP DNA, the relative enrichment at each STS is the copy number of the DNA precipitated with the specific antibody divided by the copy number of the DNA at the same STS precipitated by preimmune serum. The error bars indicate the standard deviations (SD) of two biological replicates (independent nascent DNA or ChIP isolations), each quantitated in triplicate.

TABLE 1 PCR primers

Primer	Sequence	
	Forward primer	Reverse primer
qPCR		
A	GACGACTGCTGCTCACATCTG	GCGCACCTTCCCGAATG
B	CAGGATTGAACCCATCCTCTATTAG	AACACGGCTCAGGACCTAGCT
C	CGTGCAAGCGCCAG	CTCCACCAACTTACTGTTTCATCCT
D	TTTTGTCGTGAGGGCATTAGG	GGTGCTCAAACTAACAACACCT
E	CAACACAGGCCGCTAGGAA	ACTCGCACGCTCGAATC
F	GCGTCTGGCGCGATCT	CCGAAAAAGCGGGTTTGG
G	CAGAGGCGGAGGAAAACA	CGGGTCTCTGTCCCCTTGT
H	CTTTGTTGTGAAACGTGAACCTTT	GGTGGGAGGAGAAGGGTTG
I	AGCCATTTCTCCGGCCA	AGGACTTCGGCTGGACTCG
J	AGCTGCACAGTCTCCACTTCTCT	GGTTGGCCAGGAGGAAGTTT
A'	CCCGCCCTGACGACTGCTGCTCACATCTG	
E'		ATTGGCTGCTTCTAGCGGCTGTGTTGATTG
A-10	ATGTGATTTCGGGGACCTTTTACGG	GAGAGGACGAAGAGCCAGGCCCTGCATGA
A-30	GCCAAAAGATTCTGTCTAATGT	TCTTAAAGAAATGTAGCTCCGT
A-50	GAGTCCCCTTAGCAACACT	GTTCACTTCTCGCTTTCTCACTGCGACCTT
A-75	CCTCCCACTCTCTTATTCTCCTTGCTCTCA	AAGGACTAATGCCCTCATAGCTC
A-100	ATTGCCCAAAACAGTAAGGTTCCAAGACTGA	TGGGTCCACCTGCCTTTGTGGGCTACTCCT
pVU	CCAAAGCGGTGCGACAGT	GCGCTATATGCGTTGATGCA
pVD	TCCCTGGCTCCCCTCCT	CATAAGTGCGGCGACGATA
Diagnostic PCR		
pDMPK	TTGTAGCCGGGAATGCTGC	
pSIX5		GGTCTGTGATCCCCCAG
spPCR		
pU	GGAAGGAGCTGACTGGGTTGAAGGCTCTCA	
pL		CTGGACGGCTGAGGACCCCCGAGCTGTGCT

RESULTS

Characterization of the DMPK TNRs. Several features associated with the DMPK (CTG)_n · (CAG)_n TNR region are diagrammed in Fig. 1a, including the nuclease-hypersensitive element (HSE) at the SIX5 promoter (48) and binding sites of the chromatin insulator transcription factor CTCF (14, 27, 68). To determine TNR length and DNA replication activity in the DMPK/SIX5 locus, we selected seven cell types, including three DM1 cell types, GM04033 and GM03987 fibroblasts and 428-12D lymphoblastoid cells, and four non-DM1 cell types, HeLa (epithelial), MM (lymphoblastoid cells), IMR90 (fibroblasts), and GM01653 (age, tissue, and sex matched to GM04033). Using standard PCR, primers pDMPK and pSIX5, flanking the DMPK TNR (Fig. 1a), amplified the unexpanded TNRs, about 12 to 22 repeats, in non-DM1 cells, HeLa, MM, and IMR90 (Fig. 1b, lanes 1 to 3). In contrast, in DM1 cells, GM04033, GM03987, and 428-12D, the same primer pair amplified multiple expanded bands (~300 to 2,000 bp in size), in addition to the unexpanded TNRs (Fig. 1b, lanes 4 to 6), indicating expansions of different sizes at the DMPK TNRs. Multiple expansion bands are evident, presumably because the cell populations are not clonal but represent mixtures of cells with different-length TNRs. The expansion of the DMPK TNRs was reconfirmed by PCR using another primer pair, pE_f/pSIX5_L (Fig. 1a and b, lanes 7 to 12). Since primer pE_f is about 560 bp upstream of pDMPK, PCR products amplified by pE_f/pSIX5_L are larger in Fig. 1b (lanes 7 to 12) for all cell types. Unexpectedly, the nonexpanded PCR product was not detected in the 428-12D cells (Fig. 1b, lane 12), suggesting that the deletion of a fragment upstream of

the DMPK TNR had occurred on the nonexpanded chromosome in 428-12D cells. This was further confirmed by quantitative PCR, which demonstrated that the qPCR signal in 428-12D cells at STS-A, -C, and -E was ~50% of that in the non-DM1 or other DM1 cells using genomic DNA as a template (each normalized to the STS-G [Fig. 2] signal from their own genomic DNA) (Fig. 1c). The nonexpanded (CTG)_n · (CAG)_n TNR is amplified by the pDMPK/pSIX5 primer pair, but not by the pE_f/pSIX5 primer pair, placing the 3' end of the deletion in 428-12D cells in the ~560-bp region between pE_f and pDMPK. To confirm that STS-A, -C, and -E were present on the same chromosome, the 4.3-kb PCR product amplified by primers A' and E', overlapping STS-A_f and STS-E_r (the reverse complement of pE_f), respectively, was amplified from genomic DNA of 428-12D cells (Fig. 1d). These results (Fig. 1b to d) show that STS-A and -E are linked on the expanded chromosome and therefore deleted from the nonexpanded chromosome.

qPCR was also performed using primers approximately 10 kb, 30 kb, 50 kb, 75 kb, and 100 kb 5' to STS-A. The results shown in Fig. 1c suggest that sites up to 100 kb 5' of STS-A are hemizygous in 428-12D cells. The simplest explanation of these results is that a large deletion (>100 kb) occurred on the nonexpanded chromosome during the chemotherapeutic treatment of 428-1 cells (41), although we have not formally disproven that sites A to E are deleted on the nonexpanded chromosome while sites up to ~100 kb upstream of STS-A are deleted on the expanded chromosome. Irrespective of the complexity of the deletions, these results show

FIG 1 TNR instability at the DMPK locus. (a) Structural features and map of the DMPK/SIX5 region. The bent arrows indicate the 3' end of the DMPK gene and the transcriptional start site of the SIX5 gene (nucleotide positions are based on GenBank accession number NT_011109). The primers used for the PCR shown in panel b, P_{Ef} , P_{DMPK} , and P_{SIX5} , are indicated by three short horizontal arrows. DMPK, dystrophin myotonic protein kinase gene; SIX5, sine oculis homeobox homolog 5 gene; DMPK-AT, DMPK antisense transcript attenuated by CTCF binding (14). (b) Agarose gel electrophoresis of PCR products amplified by upper primer P_{Ef} or P_{DMPK} and lower primer P_{SIX5} using genomic DNA from six cell types as templates. PCR bands amplified from unexpanded $(CTG)_n \cdot (CAG)_n$ ($n = 8$ to 27) and bands from expanded $(CTG)_n \cdot (CAG)_n$ ($n = 80$ to 780) are indicated by arrows. Note that the binding site for P_{Ef} has been deleted in the hemizygous cell line 428-12D (lane 12). M, molecular size markers. (c) Primer sites A, C, and E are present as single copies in 428-12D cells. Quantitative PCR was used to measure the copy number of STS-A, -C, and -E sequences in genomic DNAs. A-10, A-30, A-50, A-75, and A-100 indicate quantitation of sites ~10, 30, 50, 75, and 100 kb upstream of STS-A in 428-12D cell genomic DNA. (d) Primer sites A, C, and E are present on the same chromosome.

FIG 2 Replication origin activity in the DMPK/SIX5 region in non-DM1 cells. (a) Structural features and map of the DMPK/SIX5 locus (see the legend to Fig. 1a). A to J denote the 5' nucleotide coordinates of STSs used for real-time qPCR. (b) DNA replication initiation sites mapped by 0.5- to 2-kb nascent-DNA abundance assay at sequence-tagged sites A to J normalized against an internal-control low NDAA STS-54.8 (β -globin locus [42]). The error bars indicate SD.

that sites STS-A to STS-E are present on the expanded chromosome.

DNA replication initiates from two sites flanking the DMPK TNRs in non-DM1 cells. The observation that DNA replication can drive TNR instability in prokaryotic and eukaryotic cells (66, 70) prompted us to analyze the replication origin activity in the DMPK/SIX5 region. In the NDAA, origins are identified by the enrichment of sequence-tagged sites in short (0.5- to 2.0-kb) nascent DNA isolated by denaturing gel electrophoresis (15, 42, 46, 57, 86). Short nascent DNA strands were isolated from asynchronous cultures of HeLa (transformed epithelial cells), IMR90 (non-transformed fetal lung cells), MM (adult transformed SCA10 lymphoblastoid cells), and GM01653 (nontransformed adult skin fibroblasts) and measured by real-time quantitative PCR (15, 55, 57) at 10 sequence-tagged sites (STS-A to -J) in the DMPK/SIX5 region (~10 kb), including the 1.5-kb intergenic region between the DMPK and SIX5 genes (Fig. 2a). As indicated by this NDAA, in the non-DM1 cells, two DNA replication initiation sites (near STS-C and STS-G) were identified flanking the TNRs (Fig. 2b). We called the upstream initiation site near STS-C, inside the DMPK gene 3.8 kb upstream of the DMPK TNRs, IS_{DMPK} . The second initiation site near STS-G, termed IS_{SIX5} , is located in the intergenic region between the DMPK and SIX5 genes, about 1.7 kb downstream of DMPK TNRs. IS_{SIX5} falls into the region with a nonnucleosomal nuclease, HSE; the SIX5 enhancer; and a transcription initiation site. These results imply that there are two potential replication initiation sites in the DMPK/SIX5 locus in non-DM1 cells, but they do not indicate whether both sites are active in individual cells.

Pre-RC assembly near replication initiation sites. To confirm the presence of origins in the DMPK/SIX5 region, pre-RC assembly was examined by ChIP using antibodies against human Orc2 and Mcm4. As shown in Fig. 3a, in three non-DM1 cell types, the most pronounced enrichment of Orc2 was near STS-D and STS-F. ChIP assay also revealed that Mcm4 was prominent and distributed approximately symmetrically near IS_{DMPK} at STS-B and near STS-G, overlapping the second DNA replication initiation site, IS_{SIX5} (Fig. 3b). STS-B is located inside the DMPK gene, ~650 bp upstream of IS_{DMPK} and 1.1 kb upstream of the Orc2 binding site at STS-D. A similar separation of the binding sites of Orc2 and Mcm4 has been reported by others (see Discussion) and was also found at the human c-myc and SCA10 replication origins (15, 33, 55).

DMPK replication origin activity in DM1 cells. Using NDAA in GM04033 and GM03987 adult skin fibroblasts from DM1 patients, we found that nascent-DNA abundance at replication initiation site IS_{DMPK} (STS-C) was reduced by about half and at IS_{SIX5} (STS-G) was reduced to a lesser extent, by about one-fourth (Fig. 4), compared to origin activity in transformed or fetal cells but similar to the nascent-strand abundance at IS_{DMPK} and IS_{SIX5} in control, matched GM01653 adult fibroblasts. To test whether IS_{DMPK} and IS_{SIX5} were similarly suppressed on the wild-type and (CTG)_n · (CAG)_n expanded copies of chromosome 19, we examined the nascent-strand abundance in the 428-12D (adult transformed lymphoblastoid) DM1 cells, which have the 3' segment of the DMPK locus (including IS_{DMPK}) on the nonexpanded chromosome deleted (Fig. 1). NDAA demonstrated the complete loss of replication initiation activity at the haploid site IS_{DMPK} on the

FIG 3 Assembly of pre-RC on the DMPK/SIX5 region in non-DM1 cells. (a) Orc2 binding at the DMPK/SIX5 region. Shown is immunoprecipitation of cross-linked chromatin from asynchronous IMR90, MM, and HeLa/406 cells with antibodies against human Orc2. The immunoprecipitated DNA was used for qPCR amplification by primers at STS-A to -J. The relative enrichment (ordinate) at each STS is the copy number of the DNA precipitated with the specific antibody divided by the copy number of the DNA at the same STS precipitated by preimmune serum. (b) Binding of Mcm4 at the DMPK/SIX5 region assayed by immunoprecipitation of cross-linked chromatin, as in panel a, with antibody against human Mcm4. The immunoprecipitated DNA was used for qPCR amplified by primers at STS-A to -J, as in panel a. The error bars indicate SD.

expanded chromosome in 428-12D cells but replication origin activity at the diploid initiation site IS_{SIX5} comparable to that in adult GM04033 DM1 cells, GM03987 DM1 cells, and GM01653 control adult fibroblasts (Fig. 4). The differences in origin activity between the three non-DM1 cell types (HeLa, IMR90, and MM) and the three DM1 cell types (GM04033, GM03987, and 428-12D), therefore, may not be due to (CTG)_n · (CAG)_n TNR expansion but may be tissue, age, or transformation related, as previously reported (19, 24).

Gross chromosomal deletions have occurred at the DMPK locus on one or both chromosomes in 428-12D cells, which were derived by chemotherapeutic treatment of DM1 lymphoblasts

(41). Therefore, it is not possible to ascribe the loss of origin activity at the hemizygous IS_{DMPK} on the expanded chromosome to (CTG)_n · (CAG)_n expansion. Despite the loss of origin activity at IS_{DMPK} in 428-12D cells, it is notable that origin activity at IS_{SIX5} is the same in 428-12D cells as in the GM04033, GM03987, and GM01653 cultures. This suggests that the suppression of origin activity at IS_{DMPK} on the expanded chromosome of 428-12D cells does not reduce the activity at IS_{SIX5} to levels lower than that in other adult cell types, and hence, we conclude that the IS_{DMPK} and IS_{SIX5} origins are independently regulated in 428-12D cells.

Decreased origin activity correlates with decreased binding of pre-RC components. Diminished recruitment of Orc2 was ob-

FIG 4 Replication origin activity at the DMPK/SIX5 region in DM1 cell lines. DNA replication activity (nascent-strand abundance) was measured as in Fig. 2. GM04033 and GM03987 are DM1 patient fibroblasts with 1.5- to 2.0-kb TNR expansions (Fig. 1b) on one allele. 428-12D is a lymphoblastoid cell line with ~1- to 3.5-kb TNR expansions on the expanded chromosome and a large deletion of IS_{DMPK} upstream of the DMPK TNR on the unexpanded chromosome (Fig. 1b). HeLa cell data from Fig. 2 are included for comparison. The error bars indicate SD.

served at the STS-D and STS-F sites in GM04033 and GM03987 cells, consistent with the decreased replication origin activity at IS_{DMPK} and IS_{SIX5} (Fig. 5). In 428-12D cells, only background Orc2 binding was detected at the hemizygous IS_{DMPK} on the expanded chromosome, mimicking the decrease in nascent-strand abundance. In addition, decreased binding of Mcm4 (Fig. 5b) at STS-B and STS-G was observed in the GM04033 and GM03987 cells. These results suggest that the cell-type-dependent levels of origin activity at IS_{DMPK} and IS_{SIX5} are correlated with the levels of pre-RC formation at IS_{DMPK} and IS_{SIX5}. Once again, as assayed by ChIP, pre-RC formation at the upstream origin, IS_{DMPK}, and the downstream origin, IS_{SIX5}, are differentially affected in 428-12D cells, reflecting the respective changes in origin activity independently observed by quantitation of nascent strands.

Replication fork stalling by the expanded DMPK TNRs. The above-mentioned results suggest that DMPK origin activity differed modestly between DM1 and non-DM1 cells (excluding IS_{DMPK} in 428-12D cells) but that stable loss of IS_{DMPK} or IS_{SIX5} origin activity in DM1 cells could not account for the instability of (CTG)_n · (CAG)_n TNRs. If the polarity of TNR replication is not

significantly changed in non-DM1 versus DM1 cells, might the difference in length of the (CTG)_n · (CAG)_n TNRs contribute to the decrease in stability in this replication environment? Indeed, trinucleotide repeat sequences are themselves length-dependent *cis*-acting hot spots of chromosome instability that destabilize replication forks and elicit DNA damage responses in yeast cells (49, 65, 80, 84, 89). To test whether the size of the (CTG)_n · (CAG)_n TNRs affected replication fork progress in human cells, we constructed HeLa cell lines in which (CTG)_n · (CAG)_n TNRs were fused in either orientation to the 2.4-kb core *c-myc* replicator and integrated at an ectopic chromosomal site. The *c-myc*-(CTG)_n · (CAG)_n cells were otherwise isogenic. The 2.4-kb *c-myc* core replicator at this ectopic site displays the replication timing, chromatin structure, and replication protein (ORC, Cdc6, MCM, DUE-B, and Cdc45) binding of the endogenous *c-myc* origin (15, 33, 46). In these cell lines, replication-dependent hairpin formation and time-, length-, replication polarity-, and origin proximity-dependent patterns of instability were observed at the (CTG)_n · (CAG)_n TNRs (56).

When DNA replication starts from the *c-myc* replicator, forks

FIG 5 Binding of Orc2 and Mcm4 on the DMPK/SIX5 region in DM1 cells. Chromatin immunoprecipitation was performed as in Fig. 3 to assay Orc2 binding (a) and Mcm4 binding (b) in GM04033, GM03987, and 428-12D fibroblasts. HeLa cell data from Fig. 3 are included for comparison. The error bars indicate SD.

pass through DMPK TNRs using (CTG)_n as the lagging-strand template in (CTG)_n · (CAG)_n cell lines or (CAG)_n as the lagging-strand template in (CAG)_n · (CTG)_n cell lines (Fig. 6a). Two STSs, STS-pVU and -pVD, were chosen for qPCR quantitation of the abundance of bidirectionally synthesized nascent DNA strands upstream and downstream, respectively, of the TNRs. The nascent-DNA abundance upstream of the origin, at STS-pVU, remained at similar levels in all five cell lines, c-myc [without (CTG)_n · (CAG)_n repeats], (CTG)₁₂ · (CAG)₁₂, (CAG)₁₂ · (CTG)₁₂, (CTG)₁₀₂ · (CAG)₁₀₂, and (CAG)₁₀₂ · (CAG)₁₀₂, indicat-

ing that fork movement upstream from the replicator and origin activity at the c-myc replicator *per se* were unaffected by the (CTG)_n · (CAG)_n or (CAG)_n · (CTG)_n TNRs. In contrast, the nascent-DNA abundance at STS-pVD in both (CTG)₁₀₂ · (CAG)₁₀₂ and (CAG)₁₀₂ · (CTG)₁₀₂ cell lines was reduced to about 70% of the nascent-DNA abundance in the cells containing the wild-type c-myc replicator (without repeats) or in cell lines harboring nonexpanded TNRs, (CTG)₁₂ · (CAG)₁₂ and (CAG)₁₂ · (CTG)₁₂. While the overall c-myc replication origin activity is not suppressed by the TNRs, the asymmetry of nascent-strand abun-

FIG 6 Fork stalling at an ectopic chromosomal site is TNR repeat length dependent and TNR orientation independent. (a) Diagram of HeLa/406 cell-derived isogenic cell lines created by Flp recombinase-mediated site-specific integration (56, 57). A 2.4-kb c-myc replicator (gray bar) was connected to a (CTG)_n · (CAG)_n ($n = 12, 102$ repeats) repeat tract (open bar). The positions of sequence-tagged sites STS-pVU and STS-pVD used in qPCR, in flanking vector sequences upstream and downstream of the origin, respectively, are also indicated. (b) Nascent-DNA (0.5 to 2.0 kb) abundance measured by qPCR at STS-pVU and STS-pVD in HeLa Flp recombinase-treated cell lines harboring (CTG)_n · (CAG)_n ($n = 12, 102$) sequences in either orientation relative to the c-myc replicator or the wild-type c-myc replicator (c-myc; $n = 0$). The error bars indicate SD.

dance upstream and downstream of the origin indicates that the expanded TNRs in (CTG)₁₀₂ · (CAG)₁₀₂ and (CAG)₁₀₂ · (CTG)₁₀₂ cells hinder the progress of the replication forks moving in one direction, downstream through the TNRs (Fig. 6b). Notably, the decreased nascent-strand abundance at STS-pVD is not distinguishable between two orientations of the DMPK TNRs, (CTG)₁₀₂ · (CAG)₁₀₂ and (CAG)₁₀₂ · (CTG)₁₀₂ (Fig. 6b), suggesting that the ability of the DMPK TNR to impede the replication fork is independent of repeat orientation relative to the adjacent DNA replication origin. This observation is consistent with the finding, using a two-dimensional gel assay, that modest fork stalling (~12% of forks stalled) occurred at the (CTG)₈₀-(CAG)₈₀ repeat tract independent of replication polarity in yeast plasmids (72).

Accurate replication of an expanded (CTG)_n · (CAG)_n TNR requires the replication fork protection complex. Trinucleotide repeat sequences impose stress on the replication fork machinery, as evidenced by polymerase stalling and checkpoint activation (30, 51, 73, 83, 84, 88). Thus, proteins that stabilize the replication fork are required to protect against TNR instability (5, 8, 16, 22, 25, 73, 89, 91). Our previous data show that hairpins form frequently in a replication-dependent manner at (CTG)_n · (CAG)_n repeats, although instability is rarely observed (56), consistent with the notion that fork stabilization or repair mechanisms work efficiently to prevent hairpin-induced TNR instability.

In *S. cerevisiae*, the replication fork protection complex proteins Tof1, Csm3, and Mrc1 prevent (CTG)_n · (CAG)_n TNR instability and chromosome breakage (30, 73). The orthologous metazoan protein complex comprising Tipin, Timeless, and Claspin is believed to stabilize stalled replication forks and protect replisomes from dissociation (26, 45). To test directly whether the human orthologs of the yeast replication fork protection complex

proteins are required to maintain the integrity of the DMPK (CTG)_n · (CAG)_n TNRs during replication from the ectopic c-myc origin, these proteins were knocked down by RNA interference (RNAi) in the c-myc-(CTG)₁₀₂ · (CAG)₁₀₂ and c-myc-(CAG)₁₀₂ · (CTG)₁₀₂ cell lines.

Claspin, the human ortholog of Mrc 1, travels with the replication fork and is required for checkpoint signaling by interaction with ATR and Chk1 (12, 50). As shown in Fig. 7, Claspin siRNA effectively reduced the Claspin protein level in both c-myc-(CTG)₁₀₂ · (CAG)₁₀₂ and c-myc-(CAG)₁₀₂ · (CTG)₁₀₂ cells. Small-pool PCR across the (CTG)_n · (CAG)_n TNR (Fig. 7b) showed that Claspin siRNA treatment led to marked instability of the (CTG)_n · (CAG)_n TNRs. These results suggest that replisome stability plays an essential role in faithful TNR replication in human cells. To preclude off-target effects and confirm that the expansion or contraction at the (CTG)₁₀₂ · (CAG)₁₀₂ TNRs is related to suppression of the replication fork protection complex, cells were treated with siRNA to knock down two other components of this complex, Tipin (Fig. 7c) and Timeless (Fig. 7d). After knockdown of Tipin or Timeless by siRNAs, spPCR detected both TNR expansion and contraction. The similarity of the spPCR patterns after Claspin, Timeless, or Tipin knockdown is consistent with these proteins acting in unison to stabilize the (CTG)₁₀₂ · (CAG)₁₀₂ TNRs during replication. The spPCR data show that instability appears more often and in more diverse patterns in the template strands, resulting in contractions, than in nascent strands, resulting in expansions. Although this result may possibly be biased by the preferential amplification of short PCR templates, an expanded band ~180 bp larger than the progenitor band similarly appears following knockdown of Claspin, Timeless, or Tipin. These data suggest that one or more preferred structures totaling approximately 180

FIG 7 The fork protection complex is essential for (CTG)₁₀₂ · (CAG)₁₀₂ stability. (a) Diagram of the ectopic c-myc-(CTG) · (CAG) integration site. pU and pL are small-pool PCR primers used to detect TNR length. (b) (Left) Immunoblot confirmation of siRNA knockdown of Claspin. Actin, loading control. (Right) spPCR of DNA from c-myc-(CAG)₁₀₂ · (CTG)₁₀₂ [(CAG)₁₀₂] or c-myc-(CTG)₁₀₂ · (CAG)₁₀₂ [(CTG)₁₀₂] cells treated with control siRNA or Claspin siRNA analyzed by PAGE. Similar results were obtained with other control siRNAs. M, molecular size markers. (c) (Left) Immunoblot confirmation of siRNA knockdown of Tipin. (Right) spPCR of DNA from c-myc-(CAG)₁₀₂ · (CTG)₁₀₂ or c-myc-(CTG)₁₀₂ · (CAG)₁₀₂ cells treated with control siRNA or Tipin siRNA. (d) (Left) Immunoblot confirmation of siRNA knockdown of Timeless. (Right) spPCR of DNA from c-myc-(CAG)₁₀₂ · (CTG)₁₀₂ or c-myc-(CTG)₁₀₂ · (CAG)₁₀₂ cells treated with control siRNA or Timeless siRNA. Cells were treated twice, 48 h apart, with siRNA and analyzed 48 h later. The low-mobility shadow bands observed above the ~450-bp amplification product of the (CTG)₁₀₂ · (CAG)₁₀₂ progenitor sequence in control cell DNA and the corresponding bands in siRNA-treated cell DNA are slipped-strand products of reannealing during the PCR (71), as demonstrated by reamplification of the purified linear ~450-bp product band (56).

bp form as expansion intermediates in nascent DNA strands in the absence of the fork protection complex.

The c-myc-(CTG)_n · (CAG)_n system permits instability analysis of various length TNRs in a single replication orientation and

genetic background at a common, unique ectopic chromosomal site. However, HeLa cells are defective for the major DNA damage signaling protein p53 due to its interaction with the HPV16 protein. Although (CTG)_n · (CAG)_n instability has been reported to

FIG 8 The fork protection complex is essential for DMPK $(CTG)_n \cdot (CAG)_n$ stability in DM1 cells. (a) Immunoblot (WB) confirmation of siRNA knockdown of Timeless, Tipin, or Claspin in DM1 cells (GM04033). Actin, loading control. (b, c, d, and e) spPCR of DNA from cells treated as in Fig. 7 with control siRNA (lanes 1 to 8), Claspin siRNA (lanes 9 to 17), Timeless siRNA (lanes 18 to 24), or Tipin siRNA (lanes 25 to 31) analyzed by PAGE. The spPCR primers were pDMPK/pSIX5 (Fig. 1). The same molecular size markers (M) were used in panels b, c, d, and e. (a) Linear PCR products of the $(CTG)_{13} \cdot (CAG)_{13}$, $(CTG)_{\sim 400} \cdot (CAG)_{\sim 400}$ (minor), and $(CTG)_{\sim 1,000} \cdot (CAG)_{\sim 1,000}$ alleles (dark arrowheads) and slipped-strand structures (light arrowheads) formed *in vitro* during PCR (56) are indicated. The PCR product of the $(CTG)_{\sim 400} \cdot (CAG)_{\sim 400}$ allele (cf. Figure 1b agarose gels) is seen faintly in this experiment.

be independent of p53 function (32), we also tested whether knockdown of the fork stabilization proteins would produce TNR instability in DM1 cells. GM04033 fibroblast cultures contain both nonexpanded and expanded $(CTG)_n \cdot (CAG)_n$ alleles (Fig. 1). The major nonexpanded allele is $(CTG)_{13} \cdot (CAG)_{13}$, and the major expanded alleles are $(CTG)_{\sim 400} \cdot (CAG)_{\sim 400}$ and $(CTG)_{\sim 1,000} \cdot (CAG)_{\sim 1,000}$, although additional expanded alleles are faintly visible in the population. As shown in Fig. 8 (lanes 9 to 31), following siRNA treatment, multiple bands smaller than the 344-bp marker are clustered around the progenitor PCR products of the nonexpanded $(CTG)_{13} \cdot (CAG)_{13}$ TNRs. Based on the size and distribution of these bands, it is likely that the unexpanded TNRs can expand or contract as a consequence of polymerase slippage and hairpin formation in the absence of the fork protection complex (40, 82). In addition, PCR products more than 10 times the size of the unexpanded $(CTG)_{13} \cdot (CAG)_{13}$ TNRs (>517 bp, which includes ~ 50 bp of flanking DNA) and several high-molecular-weight bands ($>3,054$ bp) are observed in multiple samples when the fork protection complex proteins are knocked down. The large changes in allele size over the course of a small number (4 or 5) of population doublings during siRNA treatment suggest that the bands in the range of 500 to 1,200 bp and $>3,054$ bp arise, respec-

tively, from contraction or expansion of the expanded DM1 $(CTG)_n \cdot (CAG)_n$ alleles. Taken together, these results indicate that the replication fork protection complex is essential for accurate replication of the DMPK microsatellite at its endogenous chromosomal location in DM1 cells and at the ectopic c-myc replicator site in HeLa c-myc- $(CTG)_n \cdot (CAG)_n$ and c-myc- $(CAG)_n \cdot (CTG)_n$ cells.

DISCUSSION

Replication origin activity in the DMPK/SIX5 region. Two independent approaches were used for origin mapping in the ~ 10 -kb DMPK/SIX5 region. NDAA was employed to detect the start sites of DNA strand synthesis, while Orc2 and Mcm4 ChIP revealed the location of pre-RC assembly. Our results suggest that replication can initiate at or near either of two sites, upstream (IS_{DMPK}) or downstream (IS_{SIX5}) of the $(CTG)_n \cdot (CAG)_n$ TNRs in non-DM1 cells and in DM1 cells. In 428-12D DM1 lymphoblasts that have lost IS_{DMPK} on the nonexpanded chromosome, $(CTG)_n$ is copied as the leading-strand template from IS_{SIX5} , which is the orientation that preferentially gives rise to expansions (56, 67, 70). In the GM04033 and GM03987 DM1 cell populations, both IS_{DMPK} and IS_{SIX5} origins are active. It is possible that expansion occurs pref-

entially in those GM04033 and GM03987 cells in which (CTG)_n is replicated from IS_{SIX5} as the leading-strand template and lagging-strand nascent DNA, as in 428-12D cells, consistent with the *ori* switch (67), *ori* shift (67), and fork shift (18) models of TNR instability. Thus, the absence of a stable difference in replication origin usage between DM1 and control cells does not preclude a role for replication in DMPK (CTG)_n · (CAG)_n TNR instability.

Our results agree partially with the finding by Cleary et al. that there are two origins flanking the DMPK (CTG)_n · (CAG)_n TNRs in DM1 and non-DM1 cells (19). The location of the IS_{DMPK} replication origin in non-DM1 cells (IMR90 fibroblasts, HeLa cells, MM lymphoblasts, and GM01653 fibroblasts) and DM1 fibroblasts (GM04033 and GM03987) coincides with the upstream origin mapped by Cleary et al. in fetal control and DM1 fibroblasts (19). Orc2 was enriched at STS-D, about 450 bp downstream of IS_{DMPK}, while Mcm4 was enriched at STS-B, approximately 650 bp upstream. In comparison, Cleary et al. (19) mapped a second origin within the body of the SIX5 gene approximately 2.1 kb farther downstream than IS_{SIX5} (near STS-I) in non-DM1 and DM1 fetal fibroblasts and transgenic mouse tissues, while our results place the downstream origin, IS_{SIX5}, close to the SIX5 promoter (STS-G) in a total of seven DM1 and control cell lines, with Orc2 enriched approximately 930 bp upstream at STS-F and Mcm4 enriched at STS-G, coincident with IS_{SIX5}. The disparity in the locations of the downstream origin may be due to cell-type-specific differences in origin usage (19, 39, 52, 53) or to differences in the size fractionation of nascent DNAs under denaturing conditions (this work) versus nondenaturing conditions (19).

Transgenic mice bearing a single copy of the DMPK/SIX5 ~45-kb genomic region cloned from a DM1 patient show high levels of intergenerational (76) and somatic-repeat (54) instability that mirror the tissue-specific patterns of instability of DM1 patients (38, 76, 79). NDAA over the ~45-kb human DMPK/SIX5 transgene from the pancreases of mice bearing either >300 (DM328) or 20 (DM20) repeats showed that origin activity was lost at the upstream origin (IS_{DMPK}) but maintained at the downstream origin in DM328 transgenic mice (19). In one interpretation of these data, the loss of IS_{DMPK} origin activity in DM328 transgenic mice could be related to (CTG)_n · (CAG)_n expansion. However, in contrast to parallel experiments in human control and DM1 fetal fibroblasts, neither the upstream or the downstream origin was active in control DM20 mice carrying the unexpanded DMPK/SIX5 transgene (19). These observations suggest that in transgenic mice, as in other species, the chromosomal integration site (59) or elements outside the ~45-kb DMPK/SIX5 fragment control origin activity (2, 19, 59).

Protein binding in the DMPK/SIX5 region. Cho et al. found that a nucleosome is positioned at the nonexpanded DMPK TNR, and CTCF molecules bind on each side of the TNR, forming an insulator in the DMPK locus (14). Expansion of the DMPK (CTG)_n · (CAG)_n TNR can alter nucleosome positioning, abolish the binding of the CTCF chromatin insulator, and promote antisense transcription and heterochromatinization of the DMPK/SIX5 locus (14, 27). We note also that deletion of either of the CTCF binding sites that delimit the core c-myc replicator decreased ectopic origin activity by 50 to 80% (57). Nevertheless, despite the TNR expansion in GM04033 and GM03987 DM1 fibroblasts, we detected a level of IS_{DMPK} and IS_{SIX5} origin usage similar to that in GM01653 non-DM1 fibroblasts, consistent with previously reported results (19).

In non-DM1 cells, there are two binding regions of Orc2, STS-D and STS-F, with the latter representing the most enriched binding site. It is unclear whether these two proximal sites actually share the same ORC due to the juxtaposition of DNA sequences after forming higher-order chromatin structures. During DNA replication initiation, ORC recruits MCM and other factors to replication origins, forming pre-RC and preinitiation complex assemblies (6). Comparison of ChIP and NDAA signals suggests that ORC and MCM binding sites are not precisely the same as the DNA replication initiation sites by these assays. We are aware that the resolution of ChIP varies with the ability of the immunoprecipitated DNA to be sheared or digested with nuclease (44), that some sites of ORC or MCM binding may be resistant to cross-linking or immunoglobulin precipitation, and that the distribution of MCM proteins may differ from that of ORC in cells that are not synchronized. These issues notwithstanding, ORC and MCM have been colocalized at the human lamin B2 (1) and Top1 (44) origins, while other reports suggest that NDAA and ChIP signals need not coincide precisely. Thus, in microarray analysis of experimentally verified *S. cerevisiae* autonomously replicating sequence sites, 77% were sites at which ORC and MCM colocalized and 23% were MCM-only sites (92). Separation of ORC and MCM binding has also been observed at AT-rich sites of the fission yeast ARS2004 (85). Furthermore, ORC binds at multiple sites around replication origins in yeast, *Sciara*, and *Drosophila* Kc cells (7, 60). ChIP at the PRKDC/MCM4 origin in asynchronous HeLa cells showed that >60% of Mcm3 was found at Orc2-null sites (78). Using two independent approaches (nuclease digestion and ChIP), Ritzi et al. observed that ORC and MCM binding sites on chromatin are separated by at least 0.5 to 1.0 kb (74). In our laboratory, we have found that ORC binding is offset from MCM binding at the c-myc origin in HeLa cells (33) and at the ATXN10 locus in 428-12D cells and VM (SCA10) lymphoblasts (15).

Expanded DMPK TNRs impede replication fork progress. The construction of targeted integrant cell lines allowed us to investigate the effect of TNR on replication fork progression. When we connected the DMPK TNRs to the c-myc replicator, all cell lines were derived from the same HeLa-derived acceptor cell line with the same genetic background, except for the repeat number and orientation of the TNRs relative to the flanking c-myc origin. We found that the repeat number of the DMPK TNR, but not its replication polarity, determined the capability to pause a replication fork initiated from the c-myc replicator, suggesting that the expanded TNRs hindered the passage of replication forks regardless of the leading- or lagging-strand template sequence.

In the current experiments, short (0.5- to 2-kb) nascent DNAs were isolated by denaturing gel electrophoresis. The presence of a fork barrier within ~1 kb of the origin is shown by a decreased amount of single-stranded nascent DNA at a diagnostic STS (also within ~1 kb of the origin) distal to the barrier. Theoretically, forks that are stalled at the barrier grow unidirectionally in the opposite direction at one-half the bidirectional fork rate. Thus, in the case of an absolute barrier to fork progress, the unidirectional growth of the nascent DNAs beyond 2 kb would result in a loss of nascent-strand abundance downstream of the barrier and an approximate 2-fold increase in the lifetime of nascent strands stalled at the barrier. Why, then, is no effect of the (CTG)_n · (CAG)_n barrier (increased nascent-strand abundance) observed at STS-F from forks initiating at IS_{SIX5}? A plausible explanation is that rightward forks moving in the cotranscriptional direction from

IS_{SIX5} quickly enlarge the nascent strands beyond the 2-kb size cutoff, although alternative possibilities [e.g., the position and severity of stuttering of the leftward polymerase affects the size of the nascent single-stranded DNA (ssDNA); the endogenous (CTG)_n · (CAG)_n barrier is inefficient or does not affect forks from IS_{SIX5}; the barrier effect differs for convergent replication forks from IS_{SIX5} and IS_{DMPK} versus the proximal c-myc origin; or IS_{DMPK} fires earlier than IS_{SIX5}, and forks move through the barrier and fuse with forks from IS_{SIX5}] may also be relevant.

On native two-dimensional (2D) gels of replicating yeast plasmid double-stranded restriction fragments, stalling of forks at a trinucleotide repeat barrier results in a “bulge” on the arc of replication intermediates (49, 89, 90). In contrast to single-stranded nascent-DNA analysis, the intensity of a “bulge” is enhanced beyond 2-fold by restriction digestion that separates divergently moving forks. Several other factors may also affect the nascent-DNA signals near replication fork barriers, including barrier efficiency; relative rates of fork movement; the activation of dormant origins of replication; and the positions of the origin, barrier, and sequence-tagged sites used for PCR quantitation.

We found that fork stalling by the (CTG)₁₀₂ · (CAG)₁₀₂ TNR is independent of replication polarity. Replication-polarity-independent fork stalling has also been observed at certain TNRs in yeast plasmids (72, 90). This observation and the finding that the (CTG)_n · (CAG)_n microsatellite is sensitive to replication stress are both consistent with our previous report that hairpin formation occurs on both leading- and lagging-strand template DNAs and that the replication inhibitors Fen1 siRNA, emetine, and aphidicolin induced (CTG)₁₀₂ · (CAG)₁₀₂ TNR instability irrespective of replication polarity, although the patterns of TNR expansion or contraction differed qualitatively with replication orientation (56).

Knockdown of the replication fork protection complex enhances TNR instability. The siRNA knockdown of Claspin, Timeless, or Tipin markedly increased the occurrence of contractions and expansions at the c-myc-(CTG)₁₀₂ · (CAG)₁₀₂ TNR and at the endogenous DMPK locus. Moreover, knockdown of the fork protection complex proteins in DM1 cells appeared to stimulate instability even at the normal-length (CTG)₁₃ · (CAG)₁₃ allele. These results are consistent with genetic assays in yeast showing that mutation of Mrc1 increased instability due to long, pathological-length (CTG)_n · (CAG)_n TNRs (30), while mutation of Mrc1, Csm3, or Tof1 also strongly enhanced the instability of shorter (CTG)_n · (CAG)_n TNRs in the normal length range (73). To our knowledge, our results are the first to show directly that the related human proteins are essential to stabilize chromosomal TNRs.

In the HeLa c-myc-(CTG)₁₂ · (CAG)₁₂ model system, the normal-length TNR was stable to replication inhibition by aphidicolin, Fen1 siRNA, or emetine (56), whereas the similar-length (CTG)₁₃ · (CAG)₁₃ TNR in DM1 cells was destabilized by knockdown of the fork protection proteins. This may reveal a difference between the effects of inhibition of fork progress versus fork destabilization on TNR stability or between the HeLa c-myc-(CTG)_n · (CAG)_n model and DM1 cells. The similar patterns of expanded or contracted bands after siRNA knockdown of any of these three proteins may occur because Claspin, Tipin, and Timeless work together in the stabilization of the replication fork (4, 26, 45). Notably, these patterns of instability are different from those generated by Fen1 siRNA, aphidicolin, or emetine (56), suggesting

that the pattern of TNR instability reflects the *in vivo* biological activity of the inhibited replication fork component rather than cell type differences. Taken together, these results indicate that (CTG)_n · (CAG)_n TNRs are sensitive to replication stress.

In summary, our current data support the following model. At the nonexpanded allele in DM1 or non-DM1 cells, DNA replication can initiate from two nearby sites, IS_{DMPK} and IS_{SIX5}. An individual cell may use two initiation sites, and two replication forks may encounter one another at the region of the DMPK TNR or use one of the initiation sites in a specific cell cycle. In the cell populations studied here, the efficiency of initiation at IS_{DMPK} was slightly lower than at IS_{SIX5}. Using cell culture models, we have shown that DNA hairpins form *in vivo* at unstable (CTG)_n · (CAG)_n TNRs but that instability even of expanded (CTG)_n · (CAG)_n TNRs is infrequent in comparison to hairpin formation (56). Similar efficiencies of IS_{DMPK} and IS_{SIX5} may maintain a balance between two replication forks in the cell population, leading to occasional TNR expansion or contraction. Based on our previous data (56) and the findings in this work, we hypothesize that variations in the DNA replication initiation pattern, fork stability, and chromatin structure build on one another to enhance TNR hairpin formation, fork stalling, and instability.

The rule for DNA replication is to duplicate the genome once per cell division cycle in order to maintain genome stability and integrity, which is fulfilled by fine regulation of the activity of a dynamic group of replication origins genome-wide, following the view that “many are called, but few are chosen” (23). Consistent with the present data, it has been shown that other proteins involved in DNA replication can also modulate TNR instability (58, 93). In the DMPK/SIX5 locus, the presence of two potential replication origins presents an opportunity to screen for *trans*-acting second-site genes and therapeutic agents affecting chromatin structure, DNA replication, and TNR stability.

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