Activation of p53 or Loss of the Cockayne Syndrome Group B Repair Protein Causes Metaphase Fragility of Human U1, U2, and 5S Genes

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Summary

Infection by adenovirus 12, transfection with the Ad12 E1B 55 kDa gene, or activation of p53 cause metaphase fragility of four loci (RNU1, PSU1, RNU2, and RN5S) each containing tandemly repeated genes for an abundant small RNA (U1, U2, and 5S RNA). We now show that loss of the Cockayne syndrome group B protein (CSB) or overexpression of the p53 carboxy-terminal domain induces fragility of the same loci; moreover, p53 interacts with CSB in vivo and in vitro. We propose that CSB functions as an elongation factor for transcription of structured RNAs, including some mRNAs. Activation of p53 would inhibit CSB, stalling transcription complexes and locally blocking chromatin condensation. Impaired transcription elongation may also explain the diverse clinical features of Cockayne syndrome.

Introduction

Infection of human cells at low multiplicity by adenovirus serotype 12 (Ad12) causes four, and only four, chromosomal loci to exhibit metaphase fragility (zur Hausen, 1967). Surprisingly, each of these four loci contains a tandemly repeated array of short, powerful transcription units encoding a small, highly structured RNA: RNU1 spans 1.35 Mbp and contains 30 tandemly repeated U1 snRNA genes (Lindgren et al., 1985b; Dahlberg and Lund, 1988; van der Drift et al., 1995); RNU2 spans 30-150 kb and contains 5–25 tandemly repeated U2 snRNA genes (Hammarsstrom et al., 1985; Lindgren et al., 1985a; Pavelitz et al., 1995, 1999; Liao et al., 1997); RN5S spans about 400 kb and contains 200 tandemly repeated 5S rRNA genes (Sorensen et al., 1991); and the ancient PSU1 locus, which once encoded U1 snRNA, now consists entirely of U1 pseudogenes (Denison and Weiner, 1982; Lindgren et al., 1985b) and is much less fragile than the RNU2, RN5S, and RNU1 loci. We therefore proposed that Ad12 preferentially fragilizes chromosomal regions exhibiting unusually high levels of transcriptional activity (Lindgren et al., 1985a). Consistent with this hypothesis, U2 transcription is required for Ad12-induced fragility of the RNU2 locus (Bailey et al., 1995; Gargano et al., 1995) and U1 transcription for fragility of the RNU1 locus (Li et al., 1998b).

We subsequently found that transient expression of the Ad12 (but not Ad2 or Ad5) E1B 55 kDa protein can induce RNU2 fragility in the absence of all other viral functions (Liao et al., 1999). Although Ad12 E1B 55 kDa does not bind p53 (Zantema et al., 1985), the Ad2/5 E1B 55 kDa proteins bind p53 and block transcriptional activity (Lindgren et al., 1985a). Consistent with this hypothesis, U2 transcription is required for the cause of the neurological and developmental defects, as well as slow recovery of mRNA synthesis after UV irradiation, but does not result in a predisposition to skin cancer (van der Horst et al., 1997; Lindahl et al., 1997; Gargano et al., 1995); and U1 transcription complexes and locally blocking chromatin condensation. Impaired transcription elongation may also explain the diverse clinical features of Cockayne syndrome.

Remarkably, CSB protein can also interact with p53 in vitro (Wang et al., 1995). To explain why activation of p53 or loss of CSB function both cause fragility of the RNU1, RNU2, and RN5S loci, we propose that activated p53 sequesters, modulates, or inactivates scarce CSB protein, thus phenocopying a CSB mutation. Consistent with this hypothesis, we demonstrate that RNU2 fragility
Figure 1. Phenotype of the RNU1, RNU2, and RN5S Loci in Human Fibroblast Cell Lines

(A) Morphology of adenovirus 12-induced RNU2 fragility in the human fibrosarcoma cell line HT1080. Nearly all chromosomes exhibit splitting of the RNU2 signal, and many also exhibit dislocation of the chromosome axis.

(B) Phenotype of the RNU2, RNU1, and RN5S loci in several SV40-transformed human fibroblast lines. Cell lines examined are MRC5 from a healthy individual, TTD1BRSV (TTD-A) from a trichothiodystrophy (TTD) patient, CS3BE(SV/CS-A) and CS1AN(SV/CS-B) from CSA and CSB patients, respectively, and E61ANa and E61ANd. E61ANa and E61ANd are both stable transformants of the CS1AN(SV/CS-B) line expressing normal CSB cDNA (Troelstra et al., 1992). One of the two chromosomes 17 in the CSB line CS1AN(SV/CS-B) has undergone a translocation with an unidentified partner and appears longer than the normal counterpart; this translocation is preserved in the E61ANa and E61ANd lines (B). Spikes are seen with equal frequency for both RNU2 alleles, each of which contains 25-40 tandem U2 genes (Figure 2). The U2, U1, and 5S probes were labeled with biotin, detected with fluorescein-coupled avidin, and superimposed on the DAPI-stained chromosomes. Although the RNU1 locus at 1p36 predominates with the U1 probe, a faint subcentromeric PSU1 signal at 1q21 can occasionally be seen (Lindgren et al., 1985b).

can be induced by overexpression of the carboxy-terminal domain of p53 (CTD), but not by a CTD with mutations (K320A and K382A) that prevent acetylation by PCAF (Gu and Roeder, 1997) and p300 (Sakaguchi et al., 1998) in response to DNA damage. We further demonstrate that the p53 CTD (residues 319-393) is sufficient to interact with CSB. To explain why loss of functional CSB causes fragility of genes transcribed by pol II (U1 and U2 snRNA) and pol III (5S rRNA), we propose that CSB protein functions as an elongation factor for transcription of highly structured RNAs by both polymerases. In the absence of functional CSB, RNA polymerase would stall on the U1, U2, and 5S genes, locally blocking metaphase chromatin condensation and, thereby, causing metaphase fragility.

Results

Morphology of the RNU1, RNU2, and RN5S Loci in CSB Mutant Cells

As shown in Figure 1A, Ad12-induced metaphase fragility is manifested as splitting of the RNU2 signal, pronounced intensification of the signal, or a dislocation of the otherwise straight chromosomal axis (Schramayr et al., 1990; Li et al., 1993; Bailey et al., 1995; Gargano et al., 1995). Comparable Ad12-induced fragility can also be observed at the RNU1 locus (Bernstein et al., 1985) and the PSU1 locus (Lindgren et al., 1985b), but fragility of the RN5S locus is usually difficult to observe and may be more pronounced in primary cells (zur Hausen, 1967). In the CSB cell line CS1AN(SV/CS-B), however, the RNU2 and RN5S loci, and especially the RNU2 locus, appear as bright beads on an invisible string. Each bead is a cluster of locally packed U2 or 5S genes, and the invisible string between beads (accounting for roughly one-tenth the length of the entire 0.3 μm chromosome) could correspond to as few as 50 bp of naked duplex DNA or perhaps as much as 1 kb of nucleosomal chromatin (Figure 1B). In contrast, no spikes or beads are seen in any other SV40-transformed fibroblasts examined thus far: a normal human fibroblast line (MRC5), a trichothiodystrophy (TTD) line [TTD1BRSV(TTD-A)], or a CS complementation group A line CS3BE(SV/CS-A) (Figure 1). Slight splitting of the RNU2, RNU1, and RN5S signals was occasionally observed in these other cell lines, but at far lower frequency than in the CSB line. The failure of a CSA defect to cause constitutive fragility is particularly puzzling, because the Cockayne synd-
RNA (rRNA) genes of the RN5S absence 15% of the TTD1BRSV(TTD-A) metaphases. Fragility in the phases, 10% of the CS3BE(SV/CS-A) metaphases, and (Schramayr et al., 1990; Li et al., 1993; Bailey et al., 1995; expression vector and then, 10 hr later, infected with RNU2 40, 25, and 15 genes and three chromosomal loci: two apparently normal chromosomes 17 and one chromosome 17 derivative. Only the normal chromosomes 17 In an effort to understand how a CSB mutation could be... the one chromosome 17 derivative. Only the normal chromosomes 17 In an effort to understand how a CSB mutation could be... the one chromosome 17 derivative. Only the normal chromosomes 17 In an effort to understand how a CSB mutation could be...

*Figure 2. U2 Gene Copy Number in CSB and Normal Cell Lines*

Intact tandem arrays of U2 snRNA genes were excised from high molecular weight genomic DNA by digestion with three different "null cutters" that do not cut within the 6.1 kb U2 repeat unit: BamHI (left panel), EcoRI (center panel), and XbaI (right panel). The arrays were resolved by field inversion agarose gel electrophoresis (FAGE) and blotted for U2 genes as described (Pavelitz et al., 1995). The five cell lines represented in each panel are HT1080, MRC5, CS1AN(SV/CS-B), E61ANa, and E61ANd lines (as in Figure 1B). For convenience, CS1AN(SV/CS-B) is abbreviated as CS1ANcsb in the figure. HT1080 contains 22 U2 genes in arrays of 9 and 13 genes (Pavelitz et al., 1995); we estimate that MRC5 contains 40 U2 genes (arrays of 30 and 10), while the CS1AN(SV/CS-B), E61ANa, and E61ANd lines each contain 80 U2 genes (arrays of 40, 25, and 15). These estimates were confirmed by digesting the same DNAs to completion with the "one cutter" HindIII, followed by genomic blotting and normalization of the 6.1 kb U2 repeat fragment to the single copy 15 kb right junction fragment (Pavelitz et al., 1995, 1999). Note that the absolute size of each array in kb depends on the size of the junction fragments as well as on gene copy number. The parental CSB-derived cell line (and both derivatives) all have the same three U2 tandem arrays containing 40, 25, and 15 genes and three chromosomes with RNU2 loci: two apparently normal chromosomes 17 and one chromosome 17 derivative. Only the normal chromosomes 17 are shown in Figure 1.

CSB Expression Rescues Constitutive RNU1, RNU2, and RN5S Fragility

Untransformed primary CS1AN fibroblasts are a compound heterozygote: the 1493-residue CSB coding region is carboxy-terminally truncated after 336 residues in one allele and after 857 residues in the other (Troelstra et al., 1992); the allele encoding the longer protein was then coincidentally lost in the SV40-transformed derivative CS1AN(SV/CS-B) (Figure 6 of Troelstra et al., 1992). Nonetheless, it was possible that the constitutive RNU2 beads-on-a-string phenotype might be due to a second, unrelated mutation rather than to the CSB defect. A fortuitous mutation in the SV40-transformed line itself seems unlikely, as the constitutive RNU2 phenotype was not observed in control SV40-transformed fibroblasts (Figure 1B) or in any of numerous other cell lines examined (HeLa, HT1080, Saos-2, EBV-transformed lymphocytes of human and primate origin) that have been propagated for years. Strict proof that the CSB defect is responsible for the RNU2 phenotype was obtained by demonstrating that stable expression of normal CSB protein in two independent transformants of CS1AN(SV/CS-B) restored normal metaphase packing of both RNU2 alleles (Figure 1, E61ANa and E61ANd lines).

The p53 Carboxy-Terminal Domain Induces RNU2 Fragility in Saos-2 Cells Lacking p53

In an effort to understand how a CSB mutation could cause constitutive fragility of the same chromosomal loci as Ad12 infection, we recalled that p53 expression is required for induction of RNU2 fragility by Ad12 infection (Li et al., 1998a), Ad12 E1B 55 kDa expression (Liao et al., 1999), or treatment with araC (MacArthur et al., 1997) or actinomycin D (Yu et al., 1998). Moreover, CSB protein interacts in vitro with p53, and the carboxyl terminus of p53 is important for this interaction (Wang et al., 1995). Taken together, these observations suggested that the Ad12 E1B 55 kDa protein, actinomycin D, and araC all might activate p53, allowing it to interact with scarce CSB protein, thus phenocopying a CSB mutation. To test this hypothesis, we asked whether expression of various p53 constructs could induce RNU2 fragility in the absence of Ad12 infection or treatment with actinomycin D (Table 1).

In a complementation assay (Table 1, Experiment 1), Saos-2 cells lacking p53 were transfected with a p53 expression vector and then, 10 hr later, infected with
In Experiment 1, Saos-2 cells lacking p53 were transfected with the p53 expression construct, then infected 10 hr later with Ad12 at a multiplicity of 10. In Experiment 2, Saos-2 cells were transfected with p53 expression constructs and then treated with 50 ng/ml actinomycin D for 2 hr prior to colcemid arrest (Yu et al., 1998). In Experiments 3 and 4, Saos-2 cells were transfected with p53 expression constructs, but not infected by Ad12 or treated with actinomycin D. All p53 expression constructs were driven by the CMV promoter. At least 100 metaphases were examined for each data point. Fragility was scored as previously described (Bailey et al., 1998). The percentage of total RNU2 loci exhibiting fragility (Bailey et al., 1995). All data were normalized for transfection efficiency using a β-galactosidase color assay (Li et al., 1998a).

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In Experiment 1, Saos-2 cells lacking p53 were transfected with the p53 expression construct, then infected 10 hr later with Ad12 at a multiplicity of 10. In Experiment 2, Saos-2 cells were transfected with p53 expression constructs and then treated with 50 ng/ml actinomycin D for 2 hr prior to colcemid arrest (Yu et al., 1998). In Experiments 3 and 4, Saos-2 cells were transfected with p53 expression constructs, but not infected by Ad12 or treated with actinomycin D. All p53 expression constructs were driven by the CMV promoter. At least 100 metaphases were examined for each data point. Fragility was scored as previously described (Bailey et al., 1998). The percentage of total RNU2 loci exhibiting fragility (Bailey et al., 1995). All data were normalized for transfection efficiency using a β-galactosidase color assay (Li et al., 1998a).

Acetylation but not Phosphorylation of the p53 Carboxy-Terminal Domain May Be Required to Induce RNU2 Fragility

Activation of p53 by DNA damage can induce modification of the carboxy-terminal domain by acetylation of lysines 315, 320, and 382 and phosphorylation of serines 378 and 392 (Gu and Roeder, 1997; Sakaguchi et al., 1998) to ask whether phosphorylation or acetylation of these sites is required for induction of RNU2 fragility. In Experiments 1, 2, and 3, the four mutations were introduced into a larger carboxy-terminal fragment, CTD305-393, to ensure that any effects of the K320A mutation would not be compromised by truncation. Upon transfection into Saos-2 cells, the K320A and K382A mutants reduced the level of RNU2 fragility by 2- and 5-fold, respectively, relative to the CTD305-393 and CTD319-393 controls, whereas the S378A and S392A mutations had no effect (Table 1, Experiment 4). We conclude that CTD acetylation, but not phosphorylation, may be required to induce fragility.

Importantly, RNU2 fragility was not observed when the carboxy-terminal regulatory domain of p53 was deleted (Δ30) or when three point mutations (L323A, Y327A, L330A) that abolish tetramer formation (Sakamoto et al., 1994) were introduced into the tetramerization domain of m319-393 (m319-393AAA). Similar results were obtained when the effect of Ad12 infection was phenocopied by treatment with low doses of actinomycin D (Table 1, Experiment 2), which activate p53 by inducing DNA damage (Khan et al., 1997) without significantly inhibiting transcription (Yu et al., 1998). We conclude that the carboxy-terminal tetramerization and regulatory domain of p53 is required to support RNU2 fragility when p53 is activated by Ad12 infection (Li et al., 1998a) or by DNA damage (Yu et al., 1998).

Most remarkably, RNU2 fragility could be induced by the carboxy-terminal fragment of p53 (m319-393) without activation of p53 by Ad12 infection or actinomycin D-induced DNA damage (Table 1, Experiment 3). Specifically, RNU2 fragility was induced in uninfected Saos-2 cells by expression of a carboxy-terminal p53 fragment spanning the tetramerization and regulatory domains (m319-393), but not by expression of wild-type p53, a p53 mutant lacking the regulatory domain (Δ30), or a carboxy-terminal p53 fragment that cannot form tetramers (m319-393AAA). Since m319-393 induces RNU2 fragility in the absence of Ad12 infection or DNA damage but wild-type p53 cannot, these data suggest that the carboxy-terminal fragment of p53 (m319-393) spontaneously assumes an "activated" conformation that must normally be induced in response to DNA damage by posttranslational phosphorylation (Ko and Prives, 1996; Levine, 1997), acetylation (Gu and Roeder, 1997), reduction (Jayaraman et al., 1997), binding to single-stranded DNA (Jayaraman and Prives, 1995), or interaction with other proteins such as Ref-1 (Jayaraman et al., 1997) or c-abl (Nie et al., 2000).

p53 Interacts with CSB

To further test the hypothesis that RNU2 fragility is induced by a direct interaction between p53 and CSB, we asked whether CSB associates with p53 in vivo. Cell
lysates prepared from 293T cells transiently expressing HA-tagged CSB were immunoprecipitated with either anti-p53 or anti-HA antibodies; immunoprecipitated proteins were then resolved by SDS-PAGE and analyzed by Western blotting with a monoclonal anti-HA antibody. As shown in Figures 4A and 4B, either anti-HA or anti-p53 antibodies can coprecipitate overexpressed HA-CSB and endogenous p53. Thus, CSB binds p53 in vivo, supporting the hypothesis that p53 induces RNU2 fragility by interacting directly with CSB. Conversely, we found that overexpressed p53 and endogenous CSB can be coprecipitated by a monoclonal anti-p53 antibody (van Gool et al., 1997a). As CSB and p53 can coprecipitate when either protein is overexpressed, the in vivo interaction is most unlikely to reflect an overexpression artifact.

Since the carboxy-terminal fragment of p53 alone (residues 319–393) can induce RNU2 fragility, we next asked if the p53 CTD319–393 can interact with CSB in vivo; however, we were unable to detect such an interaction, perhaps because it is weak or requires other factors for stability. We therefore examined the interaction between the p53 CTD and CSB in vitro. Full-length p53 and p53 CTD319–393 were fused to GST and purified from bacteria. Lysates of 293T cells transiently expressing HA-CSB were incubated with GST-p53 fusion proteins immobilized on glutathione-agarose beads. HA-CSB bound to immobilized p53 and p53 CTD319–393, demonstrating that the carboxy-terminal fragment of p53 is sufficient to interact with CSB in vitro (Figure 4D). These data suggest that overexpression of the p53 CTD phenocopies a CSB mutation because the p53 CTD can interact directly with CSB and modulate its activity. The alternative hypothesis that overexpression of p53 or Ad12 E1B 55 kDa protein causes CSB degradation can be ruled out (Figure 4 and data not shown).

Discussion

Cockayne syndrome (CS) is a recessive genetic disorder that causes profound developmental defects, poor repair of oxidative DNA damage (Le Page et al., 2000), and slow recovery of mRNA synthesis following UV irradiation (for reviews, see Bootsma et al., 1997; van Gool et al., 1997a, 1997b). Two CS genes (CSA and CSB) have been identified by complementation, and the cDNAs have been cloned and sequenced (Troelstra et al., 1992; Henning et al., 1995). The precise roles of CSA and CSB in transcription-coupled repair (TCR) and recovery of mRNA synthesis remain elusive. CSA encodes a WD repeat protein that apparently does not interact or cofractionate with CSB protein (van Gool et al., 1997b) notwithstanding an earlier report (Henning et al., 1995). CSA (also known as ERCC6) encodes a protein that contains a SWI2/SNF2-like DNA-dependent ATPase domain (Troelstra et al., 1992; Selby and Sancar, 1997a, 1997b) and can interact with the XPB/ERCC3 (Selby and Sancar, 1997a) and p62 components of TFIIH (Tantin, 1998), RNA polymerase II (Tantin et al., 1997; van Gool et al., 1997b), the NER protein XPG (Iyer et al., 1996), and p53 (Wang et al., 1995). XPB/ERCC3 and p62 are two of the six core subunits of the dual function transcription/repair factor TFIIH that is shared between the nucleotide excision repair (NER) and pol II basal transcription apparatus (Marinoni et al., 1997; but see Araujo and Wood, 1999).

Taken together, these data suggest that CSB might be a chromatin-remodeling factor that enables the repair apparatus to gain access to RNA polymerase II complexes stalled at sites of DNA damage. Consistent with this view, CSB is required for TCR of 8-oxoguanine damage (Le Page et al., 2000); however, CSB does not appear to be required for repair of UV damage per se, but only for rapid resumption of RNA synthesis after repair is complete (van Oosterwijk et al., 1996; Selby and Sancar, 1997b). Thus, depending on the kind of damage, CSB could function before, during, or after repair (van Oosterwijk et al., 1996). Three possible roles for CSB can be imagined: chromatin remodeling; dissociation of the stalled transcription complex, although current evidence argues against this (Selby and Sancar, 1997a, 1997b); or ubiquitination of RNA polymerase if the first polymerase to encounter the damage is degraded rather than restarted (Bregman et al., 1996).

CS patients may display mild photosensitivity, but the primary clinical features are developmental: impaired growth and sexual development, and a variety of severe...
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Figure 4. p53 Interacts with CSB In Vivo and In Vitro

(A) Cell lysate from HA-CSB transfected 293T cells (lane 1) was mock immunoprecipitated (lane 2) or immunoprecipitated with polyclonal anti-p53 antibody (lane 3). Cell lysate and immunoprecipitates were resolved by SDS-PAGE, and coimmunoprecipitation of HA-CSB with endogenous p53 was detected by Western blotting with anti-HA monoclonal antibody HA.11. Input lysate ([A] and [D], lanes 1) corresponds to 13% of the experimental lanes. The smaller CSB bands are only seen for overexpressed CSB and most likely reflect degradation of excess CSB. Molecular weight standards are indicated at left in kDa.

(B) Cell lysate from HA-CSB transfected 293T cells (as in [A], lane 1) was mock immunoprecipitated (lane 1) or immunoprecipitated with the anti-HA antibody (lane 2). Immunoprecipitates were resolved by SDS-PAGE, and coimmunoprecipitation of endogenous p53 with HA-CSB was detected by Western blotting with rabbit anti-p53 polyclonal antibody.

(C) Cell lysate from p53-FL transfected 293T cells was immunoprecipitated with anti-p53 monoclonal antibody Do-1 (lane 4) or mock immunoprecipitated (lane 5). Immunoprecipitates were resolved by SDS-PAGE, and coimmunoprecipitation of endogenous CSB with p53 was detected by Western blotting with rabbit polyclonal anti-CSB antibody. Cell lysates from the MRC5, CS1AN/CSB, and E61ANd lines served as controls to demonstrate that the CSB antibody detects CSB protein in normal MRC5 (lane 1) and the E61ANd rescue line (lane 3) but not in the mutant CS1AN/CSB line (lane 2).

(D) Cell lysate from 293T cells expressing HA-CSB was mixed with GST protein, GST-p53 fusion protein [GST-p53 (1-393)], or GST-p53 CTD fusion protein [GST-p53 (319-393)] immobilized on glutathione-agarose beads. Lysate (lane 1) and bound proteins (lanes 2-4) were resolved by SDS-PAGE, and HA-tagged CSB protein visualized by Western blotting with anti-HA antibody.

neurological dysfunctions (Bootsma et al., 1997). The developmental nature of CS suggests that defects in CSA and CSB also affect transcriptional regulation of specific genes. Indeed, the ability of CSB protein to associate with RNA polymerase II (Tantin et al., 1997; van Goor et al., 1997b), and to enhance pol II elongation both in vitro (Selby and Sancar, 1997b) and in permeabilized cells (Balajee et al., 1997), argues that CS is a “transcriptional syndrome” in which CSB functions as a bona fide transcription factor as well as in TCR (Le Page et al., 2000) and recovery from UV irradiation (van Goor et al., 1997a).

Any model for virally induced locus-specific metaphase fragility of the RNU1, RNU2, RN5S, and PSU1 loci must account for the following observations.

First, several different regimes induce fragility of the same four chromosomal loci: Ad12 infection (Li et al., 1993; Bailey et al., 1995; Gargano et al., 1995); transient expression of the Ad12 E1B 55 kDa protein alone (Liao et al., 1999); mutations in CSB (Figure 1); and treatment with DNA-damaging reagents such as actinomycin D (Yu et al., 1998) and araC (MacArthur et al., 1997).

Second, transcription of the RNU2 locus (Bailey et al., 1995; Gargano et al., 1995) and p53 function (Li et al., 1998a, 1998b) are both required for fragility whether it is induced by Ad12 infection (Li et al., 1998a, 1998b), transient expression of Ad12 E1B 55 kDa protein (Liao et al., 1999), araC (MacArthur et al., 1997), or low doses of actinomycin D (Yu et al., 1998).

Third, U1 and U2 snRNA are transcribed by pol II (Kuhlman et al., 1999) and 5S rRNA by pol III, yet the same conditions render the RNU1, RNU2, and RN5S loci fragile.

Fourth, sequence-specific binding of p53 to DNA is not required, because induction of fragility is unaffected by severe mutations (Li et al., 1998a) or outright deletion (Table 1) of the duplex DNA binding domain of p53.

Fifth, the carboxy-terminal domain of p53, which we have shown to interact with CSB, suffices to induce RNU2 fragility in uninfected Saos-2 cells lacking p53 function (Table 1).

Our working model (Figure 5) readily accounts for these observations. We propose that CSB, in addition to playing a role in TCR, also functions as a transcription elongation factor for highly structured RNAs. It has been known for many years that prokaryotic and eukaryotic RNA polymerases occasionally stall after transcribing stable stem/loop structures, perhaps because formation of the duplex stem causes withdrawal of the 3' end of the nascent transcript from the active site, collapse of the transcription bubble, or a switch from the highly processive elongation mode to the abortive initiation mode (Palangat et al., 1998; Keene et al., 1999; Mooney and Landick, 1999). Thus, transcription of highly structured RNAs like U1 and U2 snRNA, and 5S rRNA, might be unusually dependent on an elongation factor to restart or abort RNA polymerases stalled by secondary structure in the nascent transcript. In the absence of CSB function, these stalled RNA polymerases would remain on the DNA at metaphase, blocking chromatin condensation and causing localized chromosome fragility.
Stalled RNA polymerase II or III could interfere with chromatin condensation because the active transcription complexes (1.5 MDa; Asturias et al., 1999) dwarf nucleosome octamer cores (110 kDa). Indeed, this could explain why cells go to great lengths to ensure mitotic shutdown of transcription: TBP (Leresche et al., 1996; Segil et al., 1996), RNA polymerase II transcription factors such as Oct-1 (Segil et al., 1991) and Sp1 (Martinez-Balbas et al., 1995), and TFIIIB (Gottesfeld et al., 1994; White et al., 1995) are all inactivated by phosphorylation as metaphase approaches (for review, see Stukenberg et al., 1997); some nascent transcripts are aborted (Shermoen and O’Farrell, 1991); and only a handful of transcription factors remain on the DNA as mitotic “bookmarks” presumably to identify active promoter regions (Michelotti et al., 1997).

In our model, the various regimes that induce fragility (Ad12 infection, transient expression of Ad12 E1B 55 kDa protein, treatment with araC or low doses of actinomycin D) would each activate p53 (Ko and Prives, 1996; Levine, 1997); the CTD of activated p53 would then interact with CSB and modulate CSB activity. Consistent with this model, we find that p53 associates with CSB in vivo (Figure 4A); the p53 CTD is sufficient for interaction with CSB in vitro (Figure 4B); and deletion of the p53 CTD prevents association of p53 with CSB in vivo (data not shown) as it does in vitro (Wang et al., 1995).

Equally important, deletion of the p53 CTD completely blocks virally induced RNU2 fragility (Table 1, Experiment 1), and overexpression of the p53 CTD induces RNU2 fragility even in the absence of viral infection (Figure 3 and Table 1). This latter observation also suggests that the isolated p53 CTD can assume an “activated conformation” when freed from the constraints of p53 tertiary and quaternary structure. The function of the interaction between p53 and CSB is still unknown (Wang et al., 1995), but at least two scenarios can be envisioned: CSB might recruit p53 to sites of transcription-coupled repair, or p53 might recruit CSB to sites of DNA damage, perhaps using the ability of the p53 CTD to recognize a DNA heteroduplex (Lee et al., 1995) or single-stranded DNA (Jayaraman and Prives, 1995). The function of the long, hypermethylated G that the isolated p53 CTD can assume an “activated conformation”. For example, CSB might recruit p53 to sites of transcription-coupled repair, or p53 might recruit CSB to sites of DNA damage, perhaps using the ability of the p53 CTD to recognize a DNA heteroduplex (Lee et al., 1995) or single-stranded DNA (Jayaraman and Prives, 1995).
Experimental Procedures

Cell Lines

The MRC5, C53B(EVS/C5-A), CS1AN(SV-CS-B), E61ANa, and E61Anid lines (Troelstra et al., 1992, and references therein) and the TTD1BR5V (TTD-A) line (Bootsma et al., 1997) have been described previously.

FISH

U1 and U2 probes were prepared as previously described (Yu et al., 1998). The SS probe was a 350 bp BamHI/Rsal fragment that includes the SS coding region but excludes an Alu element (Little and Braaten, 1989). Cell growth, viral infection, actinomycin D treatment, colcemid arrest, in situ hybridization, and image collection were as described previously (Bailey et al., 1995; Yu et al., 1998). When required, cells were infected with Ad12 (Li et al., 1998a) after 10 hr incubation with the calcium phosphate precipitate.

Immunoprecipitation and Western Blotting

82T3 cells were transfected with HA-CSB by the calcium phosphate method as described (Yu et al., 1998). Cells were collected by trypsinization 36 hr later, washed with PBS, and lysed at 4°C in lysis buffer (10 M NaCl, 140 mM KCl, 0.5 mM MgCl2, 20 mM HEPES [pH 7.1], 1% Triton X-100, 10% glycerol, 2 mM DTT) containing a cocktail of protease inhibitors (Set III, Novagen). After a clearing spin at 13,000 rpm for 10 min at 4°C, the supernatants were reacted for 1 hr on ice with polyclonal anti-p53 antibody (Santa Cruz Biotechnology). Protein G–agarose (Boehringer Mannheim) was then added, and the reactions rocked gently for 30 min at 4°C. Agarose beads bearing the immunocomplexes were collected and washed three times in lysis buffer. Western blotting and chemiluminescence detection were performed according to van Gool et al. (1997b).

p53 and CSB Expression Constructs

The p53 expression vectors pcCMV hp53wt, pRc/CMV hp53mA19, and pRc/CMV hp53mA22,23 (Lin et al., 1994) were kind gifts of Dr. J. Jaylin (University of Michigan). 330/pC contains a carboxy-terminally truncated human p53 coding region (lacking residues 364–393) in the expression vector pcDNA3.1 (Invitrogen) and was a kind gift of Dr. Carol Priess. The p53 construct m319–393 expresses a carboxy-terminal p53 fragment spanning residues 319–393 and was constructed by inserting a 228 bp PCR fragment between the BamHI and EcoRI sites of pcDNA3. The same PCR fragment was also ligated into pGEX-4T (Pharmacia) to generate GST-p53 (319–393); a PCR product encoding full-length p53 was ligated into pGEX-4T to generate GST-p53 (1–393). Constructs CTD305–393K320A, CTD305–393K382A, CTD305–393K378A, and CTD305–393K375A were derived from CTD305–393 by point mutations using the QuikChange method (Stratagene), m319–393AAA was generously communicated results in advance of publication

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References


Sorensen, P.D., Lomholt, B., Frederiksen, S., and Tommerup, N.
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