The DNA-binding domain of p53 contains
the four conserved regions and the
major mutation hot spots

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Mutations in the p53 tumor suppressor gene are the most commonly observed genetic alterations in human cancer. The majority of these mutations occur in the conserved central portion of the gene, but there has been little information about the function of this region. Using proteolytic digestion of the 393-amino-acid human p53 protein, we have identified a 191-amino-acid protease-resistant fragment (residues 102–292) that corresponds to the central portion of p53, and we show that this core fragment is the sequence-specific DNA-binding domain of the protein. DNA binding is inhibited by metal chelating agents, and we find that the core domain contains zinc. Proteolytic digests also reveal a 53-amino-acid carboxy-terminal domain which we show to be the tetramerization domain of p53.

[Key Words: p53, DNA-binding domain; tumor suppressor; tetramerization domain]

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The p53 tumor suppressor gene is the most commonly mutated gene identified in human cancers, and mounting evidence points to the inactivation of p53 as a critical step leading to neoplastic transformation (for review, see Hollstein et al. 1991; Levine et al. 1991; Vogelstein and Kinzler 1992). Loss of p53 function results in an enhanced frequency of genomic rearrangements (Livingstone et al. 1992; Yin et al. 1992) and eliminates the growth arrest response induced by DNA damage (Kastan et al. 1992; Kuerbitz et al. 1992). These studies suggest that p53 controls a cell cycle checkpoint that is important for maintaining the integrity of the genome (Hartwell 1992; Lane 1992).

The exact mechanisms through which p53 exerts its biological function are not known, but its ability to bind to specific DNA sequences (Bargonetti et al. 1991; Kern et al. 1991; El-Deiry et al. 1992; Funk et al. 1992) and activate transcription (Fields and Jang 1990; Raycroft et al. 1990; Farmer et al. 1992; Kern et al. 1992; Unger et al. 1992) and its interactions with several cellular proteins, such as the MDM-2 oncogene product (Momand et al. 1992; Oliner et al. 1992), seem to play important roles. For example, tumor-derived p53 mutants that have lost their ability to cause cell cycle arrest also are inactive in DNA binding and trans-activation (Kern et al. 1992; Unger et al. 1992). Furthermore, many of these mutants apparently promote neoplastic processes by forming hetero-oligomers with wild-type p53 and abrogating its activity in a dominant negative manner (Milner and Medcalf 1991; Shaulian et al. 1992). The transcriptional activation function of p53 has been mapped to residues 1–42 at the amino terminus (Fields and Jang 1990; Raycroft et al. 1990; Farmer et al. 1992; Kern et al. 1992; Unger et al. 1992), and the oligomerization activity has been roughly mapped to the carboxy-terminal portion (Shaulian et al. 1992; Stürzbecher et al. 1992). It has been proposed that the DNA-binding domain may also reside in the carboxy-terminal region of p53 (Foord et al. 1991), but no data have been presented to show that this region is involved in sequence-specific DNA binding.

In an attempt to locate the sequence-specific DNA-binding domain, we used proteolytic digestion to identify the major structural domains in the human p53 protein, and we then expressed these domains in Escherichia coli and characterized their function in vitro. The proteolytic digestion experiments show that the amino-terminal portion of p53 is highly susceptible to proteolytic digestion and suggest that it is solvent exposed and loosely folded. In contrast, the highly conserved middle portion of p53 is strikingly resistant to proteolytic digestion and thus seems to be an independently folded, compact structural domain. Finally, the pattern of cleavage sites in the carboxy-terminal portion of p53 reveals a second, smaller structural domain. In vitro experiments using the recombinant domains show that the central, core domain contains the sequence-specific DNA-binding activity of p53, and this activity appears to be dependent on zinc binding. The carboxy-terminal do-
main contains the tetramerization activity of p53. Contrary to a previous report (Stürzbecher et al. 1992), a 20-residue basic region does not seem to be necessary for tetramer formation.

Results

Proteolytic digestion of human p53 reveals two structural domains and a loosely folded amino-terminal region

The proteolytic digestion experiments were done with immunopurified human p53 (Bargonetti et al. 1992) and the protease subtilisin (subtilisin has relatively low sequence specificity, making it a useful probe for structural domains). The subtilisin concentration was varied over a wide range to determine which regions of the molecule are the most and the least susceptible to proteolysis. After subtilisin digestion, the proteolytic fragments were purified and analyzed by amino-terminal sequencing, mass spectroscopy, and amino acid analysis. The cutting points inferred from these analyses are presented in Figure 1A.

Figure 1B shows the pattern of fragments produced when human p53 is digested with increasing concentrations of subtilisin. Cutting occurs most readily at the extreme amino terminus (at residues 8, 18, 23) and at three clusters of sites in the carboxy-terminal portion of p53 (at residues 305 and 311; at residues 364, 366, and 368; and at residues 387 and 388). The digestion pattern in the carboxy-terminal region reveals the existence of a 53-amino-acid fragment (residues 311–363), which is relatively resistant to proteolytic digestion, and suggests that this fragment is an independently folded structural domain (Fig. 1B, lanes 2,3). This carboxy-terminal domain is very readily cleaved from p53, and we presume that it is connected to the rest of the protein by a flexible linker. Immediately after this domain, and separated from it by a protease-sensitive linker, there is a ~20-amino-acid region rich in basic amino acids (residues 368–387). It appears that this basic region has considerable secondary structure, as subtilisin does not readily cut internal to it, even though it cuts the flanking regions with ease.

Digestion with higher subtilisin concentrations gives additional cuts in the amino-terminal region, which is cleaved progressively at residues 39, 40, 44, 54, and 68 and eventually at residues 94, 96, and 102. The observed digestion pattern indicates that the region containing residues 1–67 is readily accessible to subtilisin, suggesting that it is generally solvent exposed and loosely folded. The transcriptional activation function of p53 has been attributed to residues 1–42 in this region (Unger et al. 1992), and the accessibility or flexibility of this region may be important for the protein–protein interactions involved in trans-activation. Interestingly, the amino-terminal portion of p53 also contains the binding site for the MDM2 oncoprotein (residues 1–118, Oliner et al. 1993).

At high concentrations, subtilisin also cuts the region between residues 293 and 306, giving a 191-amino-acid core fragment (residues 102–292) that is strikingly resistant to digestion and persists even at a fivefold excess (wt/wt) of subtilisin (Fig. 1B, lane 6). This clearly shows that the core fragment is an independently folded, compact structural domain. Consistent with this evidence for a tightly folded structure, the core domain contains the evolutionarily highly conserved regions of p53. These include residues 117–142, 171–181, 234–258, and 270–286. (The one conserved region of p53 that is not in the core domain is found near the amino terminus, at residues 13–19). The core domain also contains the majority of the mutation sites identified in tumors that are concentrated in the conserved regions in the central portion of p53 (Hollstein et al. 1991).
The core domain contains the sequence-specific DNA-binding activity of p53

Using the gel mobility shift assay, we tested the products of the subtilisin digestion reaction for DNA binding and found that the fragments retained significant activity (data not shown). Binding to a p53 site could be observed even when the digestion mixture contained no detectable intact p53 and consisted predominantly of the core domain. The shifted DNA band had a mobility faster than that of the intact p53–DNA complex, suggesting that a proteolytic fragment of p53, presumably the core domain, was responsible for the DNA-binding activity. To test this more carefully (the subtilisin digests contained small amounts of other fragments as well), we expressed the core domain (residues 102–292) in E. coli, purified it to near homogeneity, and then performed gel mobility-shift experiments with a p53-binding site from the ribosomal gene cluster promoter (RGC site; Kern et al. 1991).

Figure 2A shows that the recombinant core domain can form a complex with the RGC site and gives a shifted band with a mobility faster than that of the intact p53–RGC site complex. (In the presence of 175 mM NaCl and 28 μg/ml of nonspecific competitor DNA, 50% binding is obtained with 0.2 μM intact p53 or with 1.1 μM core domain.) To determine whether the core domain binds specifically to the RGC site, we challenged the complex with either the wild-type RGC site or a mutant site. Figure 2A demonstrates that, like intact p53 [lanes 8–11], the core domain strongly prefers the wild-type RGC site over the mutant site [lanes 3–6].

We also compared the DNA sequence specificities of the core domain and of intact p53 by means of a methylation interference assay. Kern et al. (1991) reported that the RGC site contains several guanines which, when methylated by dimethylsulfate (DMS), interfere with the binding of p53. The methylation interference pattern that we observe with the core domain–RGC complex (Fig. 2B) reveals that this complex also is sensitive to guanine methylation. When this pattern is compared with the pattern observed with intact p53 (as reported by Kern et al. 1991) it appears that the methylation-sensitive guanines of the core domain–RGC complex form a subset of those found with the intact p53–RGC complex. The methylated guanines that interfere most strongly with the binding of intact p53 also interfere with the binding of the core domain (these are guanines 25 on the upper strand and guanines 20′, 21′, and 25′ on the lower strand; Fig. 2B). On the other hand, the methylated guanines that only partially interfere with the binding of intact p53 [Kern et al. 1991] show little or no interference with the binding of the core domain (these are guanines 15, 19, and 20 on the upper strand and guanines 30′ and 31′ on the lower strand; Fig. 2B). These results demonstrate that the core domain can make many of the key DNA contacts made by the intact p53 protein.

The methylation interference patterns of the core domain and intact p53 can also be interpreted by referring to the pentamer consensus sequence Pu-Pu-Pu-C-[A/T]. The majority of the known p53-binding sites contain four repeats of this pentamer motif (although some of the repeats only partially match the consensus). The RGC site contains three repeats with a perfect match to the consensus and one repeat with a partial match (Fig. 2B). In the case of intact p53, the methylated guanines that interfere most strongly with binding occur in only two of these repeats; the third repeat shows only weak interference, and the fourth (imperfect) repeat shows no interference at all. As mentioned above, the core domain contacts mimic the strongest contacts seen with the intact protein. The methylated guanines that interfere strongly also occur in the first two repeats, whereas the third and fourth repeats do not show any significant interference. In summary, methylation interference shows that the core domain interacts preferentially with two of the pentamer motifs of the RGC site, and these are the same regions where the intact p53 makes its strongest contacts.

Two pentamer sequences are required for the binding of the core domain

p53 has been shown to form tetramers (Stenger et al. 1992; Friedman et al. 1993), and since binding sites typically contain four pentamer sequence motifs it seems possible that each p53 molecule interacts with a single pentamer motif. This model would predict that the isolated core domain, which is missing the oligomerization domain and appears to be a monomer in solution (gel filtration data, not shown), might bind to a single pentamer sequence. However, methylation interference experiments suggest that the situation is more complicated, and we did additional experiments to determine how many pentamers are required for tight binding by the core domain. We used competition experiments similar to those of Figure 2A, and we challenged the core domain–RGC complex with sites that contained one-, two-, or four-pentamer motifs. Figure 3A shows that the most effective competitors are the RGC site and a four-pentamer site. A site with two pentamers is also a good competitor, whereas a site with a single pentamer is a poor competitor (comparable to the mutant RGC site or to a site with no pentamer sequences). When we compare the amount of competitors required to give 50% reduction in binding (Fig. 3B), it becomes apparent that the core domain requires at least two pentamer sequences for specific binding. This finding suggests that two core domain molecules may interact with two pentamers in a cooperative fashion. Interestingly, when the amount of competitor is normalized for the pentamer content (Fig. 3B), the two-pentamer site seems to have the same affinity for the core domain as the four-pentamer site. Although cooperative binding (to form a dimer on the DNA) seems to be the most plausible explanation for our methylation interference and competition data, we cannot rule out the alternative possibility that one core domain molecule may interact simultaneously with two adjacent pentamers.
Zinc is required for the DNA-binding activity

The p53 protein contains 10 cysteines, all of them in the core domain, and this raises the possibility that p53 binds a metal ion. To address this possibility, we tested the effect of metal-chelating agents on DNA binding. Figure 4 shows that the metal-chelating agent 1,10-phenanthroline abolishes DNA binding by both the core domain and intact p53. Similar results were obtained with EDTA [data not shown]. The metal seems to be tightly bound to p53, because relatively high concentrations of metal-chelating reagents are needed to abolish DNA binding (~5–10 mM of 1,10-phenanthroline or EDTA).

To analyze the metal content of the core domain, we performed plasma emission spectroscopy (this analysis can detect 31 different elements, including Ca, Co, Cu, Fe, Mg, Mn, and Zn), and found that the only metal that the core domain contains is zinc. This result, taken together with our finding that metal-chelating agents abolish DNA binding, suggests that p53 is a zinc metalloprotein. Our conclusion is consistent with a recent report [Hainaut et al. 1993] showing that metal-chelating agents induce p53 to adopt a conformation that is recognized by monoclonal antibodies specific for the mutant form of the protein.

The plasma emission analysis was repeated several times, and the zinc content consistently corresponded to a ratio of ~ 0.5 mole of zinc per mole of core domain. Although this stoichiometry could in principle result...
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Figure 4. Gel mobility-shift assay showing that DNA-binding by the core domain and by intact p53 requires metal binding. Binding reactions, similar to those of Fig. 2A, with either the core domain [lanes 1–4] or with intact p53 [lanes 5–8] contained the following amounts of 1,10-phenanthroline: (lanes 1, 5) no 1,10-phenanthroline; (lanes 2, 6) 0.1 mM; (lanes 3, 7) 1 mM; (lanes 4, 8) 10 mM.

Figure 3. Competition experiments showing that the core domain requires two pentamer motifs for sequence-specific binding. (A) Binding reactions, similar to those of Fig. 2A, contained the core domain peptide (1.2 μM) and labeled RGC site (1 ng). The core domain–RGC complex was challenged with increasing amounts [10, 33, 100, 333, and 1000 ng] of the unlabeled competitor fragments [top]. [Lanes 1–5] A site with no pentamer motifs [but containing the same flanking sequences as the sites with pentamers]; [lanes 6–10] a site with a single pentamer, [lanes 11–15] a site with two pentamers, [lanes 16–20] a site with four pentamers, [lanes 21–25] the RGC site, [lanes 26–30] the mutant RGC site. (B) The results of the competition experiments were quantitated, and the amount of binding observed was plotted against the logarithm [base 10] of the amount of competitor used in each reaction. To facilitate comparisons, we list the amount of each competitor required to give a 50% reduction in binding. In calculating the molar excess of the pentamer competitors, we included the fourth, imperfect pentamer of the RGC site.

The carboxy-terminal structural domain contains the tetramerization activity of p53

Previous studies [Shaulian et al. 1992, Stürzbecher et al. 1992] had mapped the oligomerization activity of p53 to the carboxy-terminal portion, and Stürzbecher et al. [1992] have reported that a stretch of basic residues in this region is required for the conversion of dimers to tetramers. The proteolytic digestion pattern that we observe indicates the presence of a 53-residue structural domain [residues 311–363] in this carboxy-terminal region of the protein. The short basic region implicated in oligomerization [residues 368–387] occurs immediately after this domain and is connected to it by a linker highly sensitive to subtilisin.

To determine whether the 53-residue structural domain has any oligomerization activity and to investigate the role of the basic region in oligomerization, we expressed the structural domain [residues 311–367] and a peptide consisting of this domain plus the basic region [residues 311–393] in E. coli, and assayed the oligomerization activity of the purified peptides. Each peptide elutes from a Superdex 75 gel-filtration column as a single, high-molecular mass complex, and the two peptides seem to have similar oligomerization states [Fig. 5A]: The shorter peptide [residues 311–367] elutes at an estimated molecular mass of 37 kD (~6.4 molecules/oligomer) and the longer peptide [residues 311–393] at 53 kD (~5.9 molecules/oligomer). Since the elution profile on a gel-filtration column may be influenced by the molecular shape, we also performed glutaraldehyde cross-linking experiments to determine whether these oligomeric
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tent of the basic region, it also is conceivable that the basic region is involved in the association of tetramers to form higher-order oligomers observed with intact p53 [Stenger et al. 1992]. However, such high-molecular-mass forms were not observed in our gel-filtration experiments (Fig. 5A), and the significance of these bands is not clear.

Since it has been proposed that the DNA-binding domain of p53 may reside in the carboxyl terminus [Foord et al. 1991], we also tested our carboxy-terminal peptides for DNA binding. We found that the longer peptide [residues 311–393] has considerable affinity for DNA, but the shorter peptide [residues 311–367] which is missing the basic region, has no detectable affinity for DNA (data not shown). However, the interaction of the longer peptide with DNA is not specific for the p53-binding site, because we find that the mutant RGC site or nonspecific calf thymus DNA can effectively compete with the wild-type RGC site for binding. Although the affinity of this carboxy-terminal peptide for DNA may be an in vitro artifact, it is also possible that the basic region makes auxiliary contacts as p53 binds to DNA, or binds nonspecifically and helps regulate the activity of p53 [Hupp et al. 1992].

Discussion

Our proteolytic digestion experiments and our studies of fragments produced in E. coli provide new insights into the structural and functional organization of the p53 protein. The key finding of our study is that the central portion of p53 [residues 102–292] constitutes the sequence-specific DNA-binding domain of the protein. This finding reveals that the DNA-binding domain coincides with the major mutation hot spots (Fig. 6), and this helps us to understand why the tumor-derived mutant p53 proteins are defective in DNA-binding.

Several lines of evidence demonstrate that the core domain is the sequence-specific DNA-binding domain of p53. First, the core domain binds tightly to the RGC site, with an affinity comparable to that of intact p53. Second, competition experiments show that the core domain, like intact p53, strongly prefers to bind to the wild-type RGC site over the mutant RGC site. Third, methylation interference experiments show that the core domain makes many of the key DNA contacts made by intact p53. The methylated guanines that interfere most strongly with the binding of intact p53 are precisely the ones that interfere with the binding of the core domain. Fourth, the pentamer sequence motif that is required for the binding of intact p53 also is necessary for the binding of the core domain.

Although the core domain appears to be a monomer in solution, several observations suggest that it may bind as a dimer. A monomer might be expected to bind weakly, but we find that the core domain binds to the RGC site with an affinity only five- to sixfold lower than that of intact p53. We also find that the core domain requires at least two adjacent pentamers for specific binding. Again these results suggest that the core domain may form complexes.
Our studies of peptides from this region show that the shorter carboxy-terminal domain is the tetramer. Third, Shaulian et al. (1992) have shown that a peptide corresponding to our carboxy-terminal domain [residues 302–360] has transforming properties very similar to those of a peptide containing the basic region [residues 302–390], suggesting that the two peptides have very similar oligomerization activities. [The transforming ability of these peptides seems to involve the formation of mixed oligomers with wild type p53.]

Our results, in conjunction with previous work from other groups, allow a clear delineation of the major domains of p53. The amino-terminal region contains the trans-activation domain, the central region contains the sequence-specific DNA-binding domain, and the carboxy-terminal region contains sites responsible for oligomerization and nonspecific DNA-binding. However, certain puzzles remain, and these may reflect [1] possible regulatory mechanisms, [where the presence of one domain may help to modulate the activity of another domain], or [2] additional minor roles of the domains, [thus the core domain could have some weak dimer contacts, the carboxy-terminal domain might be able to make some auxiliary contacts with the DNA]. Further experiments will be needed to fully understand these effects. Nevertheless, the results presented here help us to understand the role of the conserved central region and the effects of mutations in this region. The identification of the structural domains of p53 also should aid in its structural characterization by crystallographic or nuclear magnetic resonance (NMR) spectroscopic methods.

Materials and methods
Proteolytic digestion and identification of the fragments
Proteolysis reactions contained 60–200 µg/ml of human p53 protein immunopurified from SF21 cells expressing the recombinant baculovirus pEV55hwt (Bargonetti et al. 1992), in a buffer of 10 mM HEPES-Na at pH 7.5, 5 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, and the protease subtilisin [the p53 preparations that we used in this study typically contain a number of minor contaminant proteins that have not been characterized]. The reactions were typically carried out for 25 min and were stopped by the addition of 1 mM PMSF. For analysis, the fragments were either purified by reverse-phase HPLC on a C4 column, or separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Amino acid analyses were per-
formed on an ABI 420H amino acid analyzer with PITC derivatization, and amino-terminal sequencing was performed on an ABI 477A pulsed liquid microsequencer with on-line phenylthiodydantoin analysis. The mass of the purified fragments was determined either on an electrospray ionization triple-stage quadrupole mass spectrometer (Finnigan) or on a matrix-assisted laser desorption time of flight mass spectrometer (Vestek and Finnigan). The analyses were performed at the Harvard Microchemistry Facility (Cambridge, MA) and at the Sloan-Kettering Microchemistry Facility (New York, NY). It should be noted that although we have identified most of the major fragments, there are several minor proteolytic fragments that we have not characterized.

Expression and purification of the core domain

The portion of the human p53 gene encoding residues 102–292 was amplified in a PCR, and the amplified product was cloned into the pET3d expression vector (Novagen). The core domain peptide was produced in E. coli BL21(D3) cells at room temperature. Cells were harvested by centrifugation and lysed by sonication in 40 mM MES-Na at pH 6.0, 200 mM NaCl, and 5 mM DTT. The lysate was clarified by centrifugation, diluted fivefold, loaded onto a Mono S cation-exchange column (Pharmacia) in 40 mM MES-Na at pH 6.0, 5 mM DTT, and was eluted by a NaCl gradient to yield the core domain at >85% purity. For most of the experiments, the Mono S fraction was precipitated by 80% saturated ammonium sulfate and was purified further by gel-filtration chromatography on a Superdex 75 gel-filtration column (Pharmacia) in 50 mM bis-trispropane-HCl at pH 6.8, 100 mM NaCl, and 5 mM DTT to yield the core domain at >98% purity.

Gel mobility shift and methylation interference assays

Binding reactions (15 µl) contained 25 mM Tris-HCl [pH 7.2], 175 mM NaCl, 5 mM MgCl₂, 5 mM DTT, 5% glycerol, 50 µg/ml of bovine serum albumin, 28 µg/ml of nonspecific DNA, and 1–2 nM radiolabeled RGC site DNA. The reactions were equilibrated at room temperature for 20 min, and were electrophoresed at 10 V/cm in a 4.5% polyacrylamide gel running 0.5× Tris-borate buffer. Typically, 50% binding was obtained with reactions that contained 8.5 µg/ml of intact p53 or 23 µg/ml of core domain. For the competition experiments, the radiolabeled RGC site DNA was first mixed with the appropriate unlabeled DNA and was then equilibrated with the protein. The amount of bound and free DNA was quantitated using a PhosphorImager (Molecular Dynamics). The DNA fragments used in this study were constructed by annealing purified synthetic oligonucleotides. The sequences of these fragments, reading along one strand, are as follows (the pentamer sequences are indicated in bold letters): RGC (42 bp), GATCCGATTGCTTACTGCGCATGCTTGCGGCTTGCCCTTG; mutant RGC (42 bp), GATCCGATCTCCTTGCCCTTGCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTTGCCCTT
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