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Structural Origin for the Transcriptional Activity of Human p53

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Transcriptional activation domains are known to be inherently "unstructured" with no tertiary structure. A recent NMR study, however, has shown that the transactivation domain in human p53 is populated with an amphipathic helix and two nascent turns. This suggests that the presence of such local secondary structures within the overall "unstructured" structural framework is a general feature of acidic transactivation domains. These pre-existing local structures in p53, formed *selectively* by positionally conserved hydrophobic residues that are known to be critical for transcriptional activity, thus appear to constitute the specific structural motifs that regulate recognition of the p53 transactivation domain by target proteins. Here, we report the results of a NMR structural comparison between the native human p53 transactivation domain and an inactive mutant (22L,23W→22R,23S). Results show that the mutant has an identical overall structural topology as the native protein, to the extent that the amphipathic helix formed by the residues 18T-26L within the native p53 transactivating domain is preserved in the double mutant. Therefore, the lack of transcriptional activity in the double mutant should be ascribed to the disruption of the essential hydrophobic contacts between the p53 transactivation domain and target proteins due to the (22L,23W→22R,23S) mutation.

Keywords: Mouse double minute 2, Nuclear magnetic resonance, p53, Transcriptional activation domain, Unstructured

Introduction

Transcriptional activation domains (TADs) are present in many cellular (Giniger and Ptashne, 1987; Ma and Ptashne, 1987; Fields and Jang, 1990; Lin *et al.*, 1994; Chang *et al.*, 1995; Dahlman-Wright *et al.*, 1995) and viral transcription factors (Sadowski *et al.*, 1988; Cress and Triezenberg, 1991; Donaldson and Capone, 1992; Hardwick *et al.*, 1992; O'Hare and Williams, 1992) and play an important role in the initial DNA transcription process (Triezenberg, 1995; Ptashne and Gann 1997). Determining precise three-dimensional structures of these domains, and establishing a clear relationship between structure and transcriptional activity, should be helpful in establishing a proper understanding of the transcriptional mechanism. This eventually may lead to designing effective anti-cancer therapeutics (Blommers *et al.*, 1997; Tzatsos and Papavassiliou, 1999). Unfortunately, TADs are inherently devoid of any well-defined tertiary structure. This makes it difficult to establish a solid structure-function relationship for TADs (Hahn, 1993; Cho *et al.*, 1996). Structural models of TADs, such as "negative noodles" (Sigler, 1988) or "polypeptide-lasso" (Cho *et al.*, 1996), have been proposed, mainly emphasizing the importance of predominant negative charges. However, they are inconsistent with the observation that successive mutations of the abundant acidic residues cause only a minimal decrease of activity; while replacing one of the few conserved hydrophobic residues results in a dramatic loss of activity (Hope *et al.*, 1988; Cress and Triezenberg, 1991; Lin *et al.*, 1994; Chang *et al.*, 1995). Furthermore, these models cannot account for activity loss when mutations are introduced in the potential helical regions (Schmitz *et al.*, 1994; Dahlman-Wright *et al.*, 1995).

Indeed, transcriptional activity has long been considered as governed by "some sort" of specific structural determinants (Ptashne and Gann 1997), such as an amphipathic helix (Giniger and Ptashne, 1987), rather than by the mere presence of negatively charged residues. Despite the fact that numerous attempts were made in order to find out if such specific structural elements are present in TADs, most investigators failed to present evidence for any significant structural

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element in TADs. For example, the VP16 TAD of the herpes simplex virus was shown to lack any secondary structure in water by CD spectropolarimetry and NMR spectroscopy (O'Hare and Williams, 1992). Even in 80% methanol no evidence of an α-helical structure could be found.

Another structural study on GCN4 and GAL4 TADs also concluded that these TADs are "unstructured" in an aqueous solution (Leuther *et al.*, 1993; Van Hoy *et al.*, 1993). The same study suggested that the dominant form of secondary structures of these TADs in a hydrophobic solvent should be antiparallel β-sheet rather than α-helix. Other experiments showed that the TA1 domain from NF- κ B p65 (Schmitz *et al.*, 1994), and the τ 1 core domain from the human glucocorticoid receptor (Dahlman-Wright *et al.*, 1995), are capable of forming α-helical structures albeit in helix-inducing solvents such as trifluoroethanol (TFE). However, these studies also concluded that TADs are "unstructured" in an aqueous solution. Even though only peptides, or protein fragments that inherently possess a helix-forming propensity, may form α-helical structure in TFE (Dyson *et al.*, 1988, 1992), it is difficult to establish a direct relationship between helical structures observed in TFE (McKnight *et al.*, 1997) and transcriptional activity.

Recent structural studies, employing short fragments of various TADs, elegantly demonstrated that a 15-residue fragment of p53 TAD forms an amphipathic helix when bound to a target protein mdm-2 (Kussie *et al.*, 1996). Also, a fragment of the VP16 TAD encompassing residues 472D-483L becomes α-helical upon binding to human TAF_{II} 31 (Uesugi *et al.*, 1997). Despite these results, the view has prevailed that TADs in the absence of target proteins are simply "unstructured" without any specific structural determinants that could be important for recognition of target proteins.

Recently, we applied heteronuclear multidimensional NMR techniques to the uniformly ^{15}N-enriched full-length 73-residue p53 TAD and have demonstrated that the p53 TAD is not "unstructured", but contains functionally important local structural elements in aqueous solution even in the target-free state (Lee *et al.*, 2000) (see Figure 1). p53 is an acidic type transactivator that acts as a tumor-suppressor protein and is implicated in numerous human cancers (Levine, 1997). While most tumorigenic mutations in p53 occur in the DNA-binding domain some are found in the transactivation domain (Hollestein *et al.*, 1996). Among several mutant constructs we have tested (Chang *et al.*, 1995) one double mutant (22L,23W→22R,23S) was particularly interesting since the mutation completely abolishes (less than 5% CAT activity compared to the wild type) transcriptional activity of p53 TAD. The two residues were shown to be critical for the binding of p53 to target proteins, such as mdm-2 (Kussie *et al.*, 1996). In order to understand the structural origin for the loss of transcriptional activity of the double mutant, we compared the structure of this mutant p53 TAD with that of the wild type.

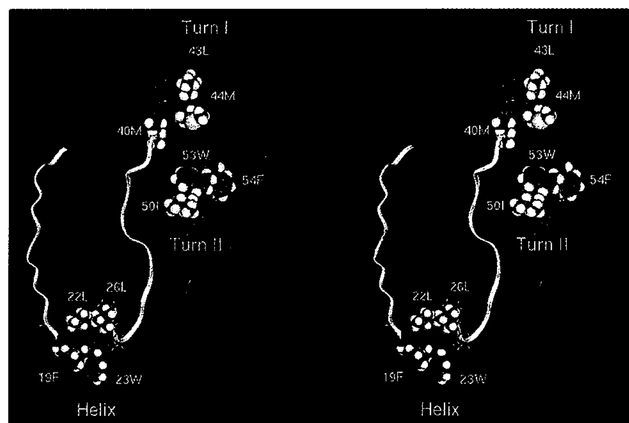

Fig. 1. A cartoon diagram for the p53 transcriptional activation domain. The model is created based upon the NMR structure of an unbound full-length p53 TAD using Insight II software (TMSI Inc.). The protein backbones of the N-terminal and C-terminal subdomain are colored in yellow and green, respectively. An amphipathic helix (red cylinder) and two turns (blue) are indicated. Sidechains of key hydrophobic residues that are known to be critical for transcriptional activity are highlighted in the space filling representation for the amphipathic helix. Sidechains of hydrophobic residues in the turns predicted to alter transcriptional activity of p53 TAD are also shown in a space-filling mode.

Experimental Procedures

Protein Purification The full-length human p53 TAD was prepared as previously described (Chang *et al.*, 1995). The DNA fragment, encoding a GS linker plus the residues 1-73 of p53, was amplified by PCR using two primers (5'-GGTCG GATCCATGGAGCCGCAGTCA-3' and 3'-GGTGAAGCTT ACACGGGGGGAGCAGCCTC-5') and subcloned into BamHI and HindIII sites of pSK(-) (Stratagene, La Jolla, USA) to construct pSK-p53-TAD. The DNA fragment of the p53 TAD in pSK(-) was subcloned into BamHI and HindIII sites of the *E. coli* expression vector pGEX-KG. The recombinant DNA containing the p53-TAD DNA fragment was introduced into the *E. coli* DH5α and subjected to expression as glutathione S-transferase (GST) fusion p53-TAD peptides. Cells were grown in 2 × YTA medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) with 0.05 mg/ml ampicillin (Sigma, St. Louis, USA) at 37°C, pH 7.0 for 4 hours. 0.3 mM IPTG was added when the $OD_{600\,nm}$ reached 0.7 and the cells were grown at 37°C for another 4 hours. The final $OD_{600\,nm}$ was approximately 1.2. Cells were centrifuged for 30 min at 8000 rpm. Pellets were re-suspended in 50 mM sodium phosphate pH 7.8, then reacted with hen egg white lysozyme (0.25 mg/mL, Sigma) for 1 hour at 4°C. The suspension was then sonicated and centrifuged at 4°C, 12,000 rpm for 20 min. The fusion protein was bound to Glutathione-Sepharose affinity resin (Pharmacia, Uppsala, Sweden) and cleaved with thrombin (Boehringer Mannheim,

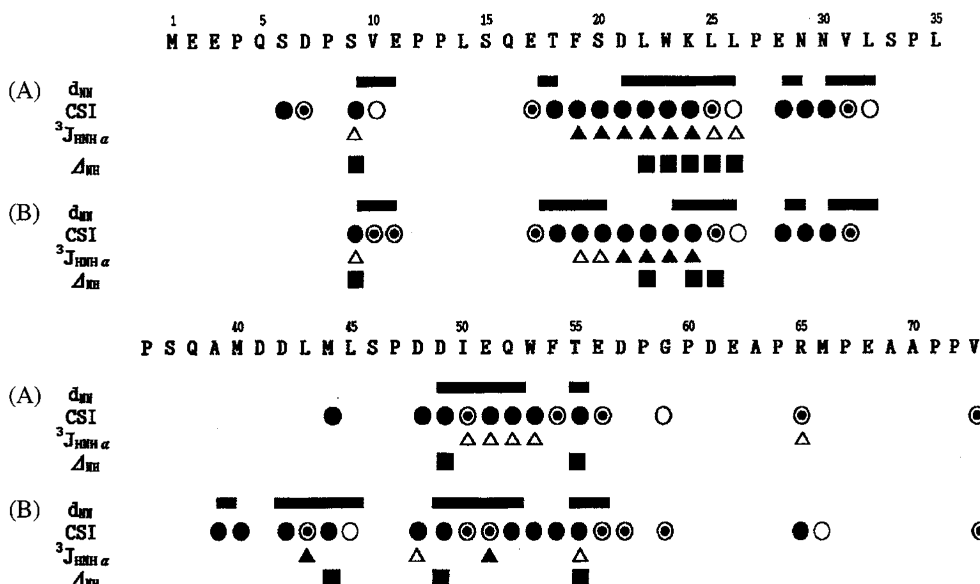

Fig. 2. A summary of NMR parameters representative of secondary structure elements are shown along with the sequence for the native human p53 TAD (A) and its inactive mutant (22L, 23W→22R, 23S) (B). Open and closed circles indicate the CSI score of +1 and -1, respectively. Double circle is used for the CSI score of 0. Filled triangles are given when $^3J_{HNH\alpha}<6.5$ Hz and open triangles when $^3J_{HNH\alpha}<7$ Hz. Filled square is used when the temperature coefficient of the amide NH (Δ_{NH}) is less than 5 ppb/K.

Indianapolis, USA) to release the p53-TAD peptide. The peptide was further chromatographically purified using SOURCE 15Q (PE 4.6 mm ID × 100 mm, Amersham-Pharmacia Biotech, Arlington Heights, USA) ion exchange and C_{18} 218TP1010 (10 mm ID × 250 mm, 10 μm particle size, Vydac) preparative reversed-phase HPLC to homogeneity. The purified peptide was subjected to amino acid composition analysis and mass spectrometry to confirm its identity.

NMR Experiments The samples for the NMR studies were prepared in 90% H_2O/10% ^2H_2O with a final concentration of approximately 1 mM at pH 5.5. Full-length p53 TAD tends to aggregate at a pH below 5.0, as has been observed in the other acidic TAD (O'Hare and Williams, 1992). The pH was measured as a direct reading from a combination microelectrode calibrated at two reference pHs. All NMR experiments were done at two temperatures (5 and 25°C) in a phase-sensitive mode using a Varian Unity 500 (Han *et al.*, 1994a), or Unity INOVA 600 spectrometer equipped with a triple-resonance probe in order to avoid spectral overlap as much as possible and to monitor temperature dependent structural changes. Hydrogen-deuterium exchange was monitored by a series of TOCSY spectra. Temperature coefficients for the backbone amide protons (Δ_{NH}) were calculated using the ^{1}H resonance assignments obtained at two temperatures (5 and 25°C). Data were processed and analyzed on a Sun SPARCstation using Varian Vnmr software. A typical 90 degree pulse width for ^{1}H was 7 μs, except for the spin-locking period of clean TOCSY experiments where it was adjusted to 16 μs (Griesinger *et al.*, 1988). Solvent suppression was carried out using selective, low-power (approximately 60 Hz field strength) irradiation of the water resonance during the relaxation delay of 1.5 s. Solvent suppression was also applied during the mixing period in the case of the NOESY (Jeener *et al.*, 1979) experiments. All peaks were referenced to a residual water signal (4.75 ppm at 25°C). Mixing times of 150 ms at 25°C and 200 ms at 5°C for NOESY and 72 ms for TOCSY experiments were used, respectively. The three-bond $^3J_{HNH\alpha}$ coupling constants were measured from phase-sensitive COSY experiments (Marion and Wuthrich, 1983; Case *et al.*, 1992). Spectral widths were 5 kHz in both dimensions. Typical 2D data consist of 2048 complex points in t_2 dimension with 512 complex t_1 increments, except for the COSY experiments where a final digital resolution of 0.6 Hz in the F2 dimension was used after a double zero-filling (Han *et al.*, 1994b).

Results

Various NMR parameters are used to delineate secondary structures in proteins. For example, the presence of sequential d_{NN} NOEs typically indicates a helix (Wuthrich, 1986). The chemical shift index (CSI) is another parameter which is highly useful for delineating secondary structures in proteins (Wishart *et al.*, 1991; Wishart *et al.*, 1992; Wishart and Sykes, 1994), especially when the resonance overlap prevents an unambiguous interpretation of NOEs. A helix is defined if four consecutive "-1s" are found, or local CSI density exceeds 70% (Wishart and Sykes, 1994). In addition, small three-bond coupling constants ($^3J_{HNH\alpha}$) are indicative of a helix or a turn. While they are not directly associated with secondary

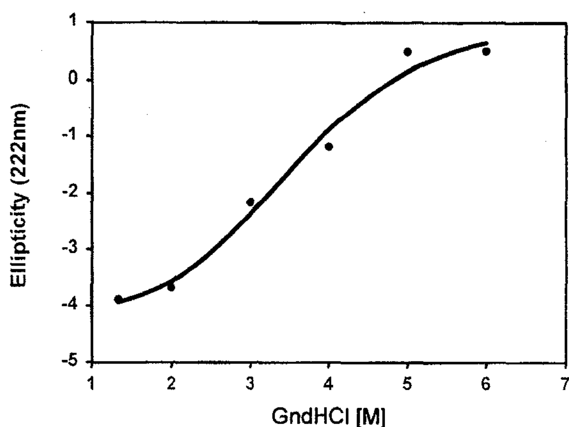

Fig. 3. A helix-melting curve for the helical region (residues 18T-26L) in human p53 TAD.

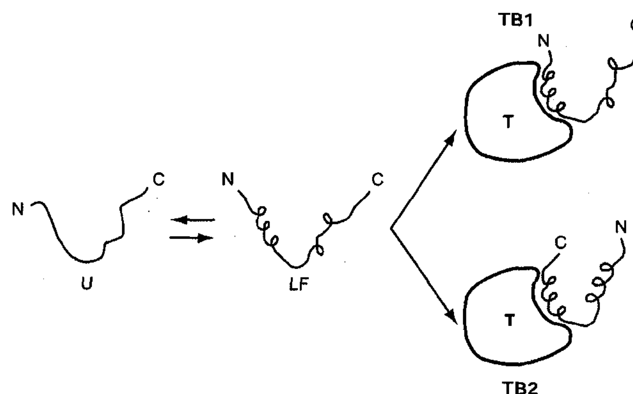

Fig. 4. A schematic drawing for the structural states of the full-length p53 TAD in the absence and in the presence of a target protein. The letters N, C and T stand for the N-, the C-terminus and a target protein, respectively. In the unbound state the p53 TAD exists in conformational equilibrium between a totally unstructured (U) state and a loosely folded (LF) state that has minimal secondary structures. The U state itself is likely to be an ensemble of several different conformers. Target-binding is proposed to occur from the LF state to result in two different target-bound forms, TB1 and TB2. In the TB1 the pre-existing amphipathic helix (residues 18-26) present in the LF state binds to the hydrophobic TAD-binding pocket of a target protein, while the two turns (turn I and turn II) remain unaffected. In the TB2 the TAD-binding pocket of a target protein is occupied by a "converted" amphipathic helix (residues 40-53), which is made from the two turns found in the LF state. In the TB2 the pre-existing helix is unaffected by target binding. Within the given description the ligand-bound fraction of the target protein is expected to be higher than when only a fragment, or a subdomain of the full-length p53 TAD interacts with the target protein.

structures, other parameters, such as small temperature coefficients of amide protons (Δ_{NH}) and slow hydrogen exchange rates, also reflect some structural rigidity in proteins. Fig. 2 shows the summary of four independent NMR parameters measured for the native and mutant p53 TADs; NOE, CSI, $^{3}J_{HNH\alpha}$ and temperature coefficients of the amide proton resonance (Δ_{NH}). The results shown in Fig. 2 for the native p53 TAD are obtained by ^{1}H two-dimensional techniques and agree with those obtained by multidimensional heteronuclear NMR methods (Lee *et al.*, 2000). For the double mutant complete resonance assignment was not possible using only ^{1}H homonuclear methods. However, resonance assignment for the critical regions of the double mutant could be achieved resorting to an analogous resonance assignment for the native p53 TAD.

Our data suggest that an amphipathic helix is formed by residues 18T-26L in the N-terminal subdomain of both native and inactive p53 TADs, as supported by all four types of parameters. The same residues were shown to form the "induced" helix when a short peptide encompassing these residues was bound to mdm2 (Kussie *et al.*, 1996). In addition to this helix, there is a region near residues 48D-55T in the C-terminal subdomain of p53 TAD that also shows structural rigidity. This region seems to contain turns rather than a helix (Fig. 1). Our observation that such local secondary structures are present in p53 TAD, even when it is examined in aqueous solution in the absence of any target proteins, makes a stark contrast to previous works reporting that unbound TADs were devoid of any significant secondary structures in the aqueous solution (Donaldson and Capone, 1992; O'Hare and Williams, 1992; Leuther *et al.*, 1993; Regier *et al.*, 1993; Van Hoy *et al.*, 1993; Schmitz *et al.*, 1994; Dahlman-Wright *et al.*, 1995; Cho *et al.*, 1996; Rahdakrisnan *et al.*, 1997; Uesugi *et al.*, 1997).

The helix-forming residues, 18T-26L, TFSDLWKLL, contains the functionally important conserved hydrophobic residues in the p53 TAD, such as 19F, 22L, 23W and 26L (Lin *et al.*, 1994; Chang *et al.*, 1995; Kussie *et al.*, 1996). Since small Δ_{NH} values are typically associated with intramolecular hydrogen bonding (Dyson *et al.*, 1988), the backbone amide protons of 22L-26L are most likely involved in hydrogen bonding within the proposed helix. The fast hydrogen-deuterium exchange rates for backbone amide protons of the helix-forming residues are consistent with the fact that TADs have no tertiary structure that exerts exchange-retarding effects (Han and LaMar, 1986). Fig. 3 shows a cooperative melting pattern of the helix by GuHCl, as observed by CD spectropolarimetry. The pre-existing helix in the N-terminal subdomain of p53 TAD is highly amphipathic as the mdm2-induced helix (Kussie *et al.*, 1996; Uesugi *et al.*, 1997), and has a hydrophobic surface formed by 19F, 22L, 23W and 26L. The turn-forming regions in the C-terminal subdomain contain hydrophobic residues 40M, 43L, 44M, 50I, 53W and 54F. Therefore, these turns display a weak amphipathic character due to clustering of these hydrophobic residues on one side of the protein backbone.

Discussion

In contrast to the early speculation, it turned out that some

unbound TADs have a certain degree of ordered local secondary structures even in aqueous solution (Hua *et al.*, 1998; Hi *et al.*, 1999; Nagadoi *et al.*, 1999; Lee *et al.*, 2000). These unbound TADs resemble a "molten globule" (Ptitsyn, 1995; Privalov, 1996) instead of being simple acid blobs. Such local structures are believed to be important for transcriptional activity as they are formed only by the functionally critical hydrophobic residues (Hua *et al.*, 1998; Hi *et al.*, 1999; Nagadoi *et al.*, 1999; Lee *et al.*, 2000). It is likely that only in such conformations do the hydrophobic residues assume an orientation optimal for interaction with target proteins. We found that the pre-existing amphipathic helix, and two nascent turns observed in the native p53 TAD, are also present in the mutant p53 TAD. Thus, what is required for manifestation of transcriptional activity in p53 are intact hydrophobic contacts with target proteins, not the amphipathic helix *per se*. Since the mutation does not induce any gross conformational disruption of the helix motif, the loss of transcriptional activity in the double mutant must be due to impaired hydrophobic interactions mediated by 22L and 23W.

The following suggests that hydrophobic interactions are important for other regions in p53 TAD to interact with target proteins. Many TADs contain multiple clusters of hydrophobic residues. In the case of p53 TAD, one cluster exists in the N-terminal subdomain and in fact forms the pre-existing helix described above. The other is located in the C-terminal subdomain and forms the two turns. Synergism in transcriptional activity is a common feature, even though its mechanism is not yet understood (Ptashne and Gann, 1997 and references therein). Between the two proposed modes of synergism, the first is an intermolecular type and involves several different or identical activation domains acting simultaneously on a target, or target proteins in the transcriptional machinery (Busby *et al.*, 1994; Joung *et al.*, 1994; Tanaka *et al.*, 1994; Scott *et al.*, 1995). On the other hand, the second type is intramolecular and arises when multiple hydrophobic clusters within one TAD, which are typically separated by 20-30 residues, interact with a target protein (Triezenberg *et al.*, 1988; Hardwick *et al.*, 1992; Regier *et al.*, 1993; Blair *et al.*, 1994; Busby *et al.*, 1994; Chang *et al.*, 1995; Dahlman-Wright *et al.*, 1995; Triezenberg, 1995). The TAD binding pocket in mdm2 is hydrophobic (Kussie *et al.*, 1996). Therefore, not only the pre-existing amphipathic helix in the N-terminal subdomain of p53 TAD, but also the nascent turns in the C-terminal subdomain, could bind to the same TAD-binding pocket in mdm2, albeit with different affinities. Since "goodness of the hydrophobic fit" between a TAD and a target protein is directly correlated with transcriptional potency (Kussie *et al.*, 1996), the N-terminal subdomain of p53 TAD should exert stronger activity than the C-terminal subdomain that contains nascent (weakly hydrophobic) turns. This is precisely what was observed (Chang *et al.*, 1995). Not only is the N-terminal subdomain of p53 TAD (containing the highly amphipathic helix) ~6 times more active than the C-terminal subdomain (containing the less amphipathic turns), but also the activity of the full-length p53 TAD is higher than the sum of the individual activities of the two subdomains. One could expect that the overall fraction of the "p53-bound" mdm-2 would be higher when mdm-2 interacts with the full-length p53 having multiple hydrophobic surfaces, than when it interacts with the N-terminal or C-terminal subdomain of p53 TAD alone. Fig. 4 summarizes such an idea, where we show a simplified model describing various structural states of the full-length p53 TAD before and after target binding.

More and more inherently unstructured or loosely folded proteins are being discovered (Schweers *et al.*, 1994; Yoo, 1995; Kriwacki *et al.*, 1996; Weinreb *et al.*, 1996; Daughdrill *et al.*, 1997; Plaxco and Gross, 1997; Davidson *et al.*, 1998; Wissmann *et al.*, 1999). The fact that the biological function of loosely folded proteins, including TADs, does not rely on tertiary structure for function, resulted in a proposal that a new view on the protein structure-function relationship has to be established (Kriwacki *et al.*, 1996; Plaxco and Gross, 1997; Lee *et al.*, 2000). It has been known for some time that NMR spectroscopy is the method of choice for the structural characterization of proteins in partially folded states (Dyson *et al.*, 1985, 1988, 1992; Wright *et al.*, 1988; Shin *et al.*, 1993; Dyson and Wright, 1995). As we have demonstrated in this report, NMR is unique in that it enables one to probe the secondary structure on a per-residue basis, even when proteins contain only a minimal amount of secondary structures, whose potential significance to function tends to be overlooked by other biophysical methods. The fact that approximately 30% of the known genes encode loosely folded proteins that are beyond structural characterization by x-ray crystallography underscores the potential role of NMR spectroscopy in structural genomics.

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