Nucleic Acids Res. **19**, 4817 (1991).
30. J. Felsenstein, *Evolution* **39**, 783 (1985).
31. D. L. Swofford and G. J. Olsen, in *Molecular Systematics*, D. M. Hillis and C. Moritz, Eds. (Sinauer, Sunderland, MA, 1990), pp. 411–501.
32. D. L. Swofford, PAUP version 3.0s (Illinois Natural History Survey, Champaign, IL, 1989).
33. Sequences have been submitted to GenBank and carry accession numbers LO6455, MV-1; LO6456, MC-1; LO658, MMP 1990; and LO6457, MMP 1991.
34. E. F. DeLong, G. S. Wickham, N. R. Pace, *Science* **243**, 1360 (1989).
35. We thank P. A. Eden, T. M. Schmidt, R. P. Blakemore, J. McN. Sieburth, and N. R. Pace for sharing unpublished information and for helpful discussions; D. G. Franks for technical assistance; and J. F. Stolz for critically reading this manuscript. This work was supported by the U.S. Office of Naval Research (ONR) grant N00014-91-J-1290; E.F.D. was the recipient of an ONR Young Investigator Award N00014-90-J-1917. D.A.B. is also supported by National Science Foundation grant MCB-9117694.

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Probing the Structure and Mechanism of Ras Protein with an Expanded Genetic Code

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Mutations in Ras protein at positions Gly^{12} and Gly^{13} (phosphate-binding loop L1) and at positions Ala^{59}, Gly^{60}, and Gln^{61} (loop L4) are commonly associated with oncogenic activation. The structural and catalytic roles of these residues were probed with a series of unnatural amino acids that have unusual main chain conformations, hydrogen bonding abilities, and steric features. The properties of wild-type and transforming Ras proteins previously thought to be uniquely associated with the structure of a single amino acid at these positions were retained by mutants that contained a variety of unnatural amino acids. This expanded set of functional mutants provides new insight into the role of loop L4 residues in switch function and suggests that loop L1 may participate in the activation of Ras protein by effector molecules.

The proteins encoded by the mammalian *ras* proto-oncogene act as a molecular switch, passing extracellular signals for cell growth and differentiation to one or more intracellular effector molecules (*1–4*). The chemical basis for switch function involves the cycling of the protein between the inactive guanosine diphosphate (GDP)–bound state and the active guanosine triphosphate (GTP)–bound state. Point mutations that result in a decrease in the intrinsic guanosine triphosphatase (GTPase) activity of Ras or the GTPase activity stimulated by GTPase-activating protein (GAP) (*5–8*) are associated with approximately 30% of human tumors. To gain greater insight into the molecular mechanisms of switch inactivation (and oncogenic activation), we systematically substituted residues in loop L1 (the phosphate-binding loop) and loop L4 (the switch II region) with a series of unnatural amino acids (Figs. 1 and 2). The ability to precisely vary the steric or electronic properties of a given residue (*9–14*) has allowed us to test mechanistic and structural issues not addressable by conventional mutagenesis studies.

Incorporation of unnatural amino acids into Ras protein was accomplished by in vitro suppression of TAG nonsense mutations with a chemically aminoacylated suppressor tRNA (*15–17*). As a control, wild-type protein and a $Gly^{12} \rightarrow Pro$ mutant were generated in vivo and in vitro by suppression of the $Gly^{12} \rightarrow TAG$ mutant with Gly and Pro, respectively (Fig. 3). The resulting proteins were purified to homogeneity from in vitro transcription-translation reactions (1 ml) in approximately 20% yield by sequential chromatography on DEAE-sephacel and an immunoaffinity column. The purified proteins had the same chromatographic properties, intrinsic GTPase activity, and GAP-stimulated GTPase activity as the corresponding Ras protein synthesized in vivo (Table 1). In vitro protein synthesis reactions with wild-type plasmid (pRG) yielded active protein (40 to 50 μg/ml); suppression efficiencies are listed in Table 1. In vitro synthesis reactions that contained the nonsense mutants in the absence of suppressor tRNA or in the presence of full-length unacylated suppressor $tRNA_{CUA}$ yielded less than 0.5% of Ras protein. The amount of Ras synthesized was determined by polyacrylamide gel electrophoresis, immunoprecipitation of [^{35}S]methionine-labeled protein, and GTPase activity assays (Fig. 3).

Mutations at Gln^{61} are among the most common that lead to impaired intrinsic GTPase activity and, in many cases, oncogenic activation (*18*). On the basis of biochemical studies (*19*) and the three-dimensional x-ray crystal structures of the GTP-, Gpp(CH_2)p-, and Gpp(NH)p-bound forms of Ras (*20–27*), it has been proposed that Gln^{61} is critical for γ-phosphate binding and catalysis. Specifically, it has been proposed that the γ-carboxamide of Gln^{61} either polarizes water 175 for attack on the γ-phosphate (*24*) or stabilizes the incipient pentacoordinate transition state (*25*). In order to test these mechanistic hypotheses, we substituted Gln^{61} with the Gln homolog homoglutamine (HGln) or with the isoelectronic, isosteric nitro analog (NGln) (Table 1 and Fig. 2). Whereas 17 natural mutants at position 61 have reduced GTPase activity and are not activated by GAP, the $NGln^{61}$ mutant had GTPase activity similar to that of the wild-type protein and was activated by GAP. The fact that this mutant retained activity even though the nitro group is a much poorer base than a carboxamide group [the protonated form of the nitro group has a pK_a (negative loga-

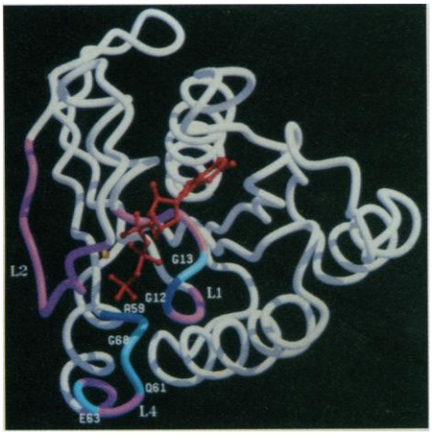

Fig. 1. Model of Ras protein with bound GTP (*25*).

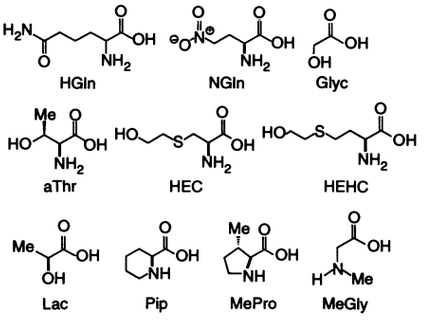

Fig. 2. Structures of amino acids (*47*). The α-amino group of all the amino acids was protected as the NVOC derivative, whereas the α-hydroxyl group of the hydroxy acids was protected as the *o*-nitrobenzyl derivative. The free acids were activated for acylation to pdCpA as cyanomethyl esters (*15*). Me, methyl group.

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rithm of the equilibrium constant for association) some 10 log units lower than the carboxamide group] (*27*) suggests that Gln^{61} is not acting as a general base to activate H_2O. Moreover, the activity of the mutant did not increase with increasing pH, which indicates that the deprotonated form of NGln is not catalytically active.

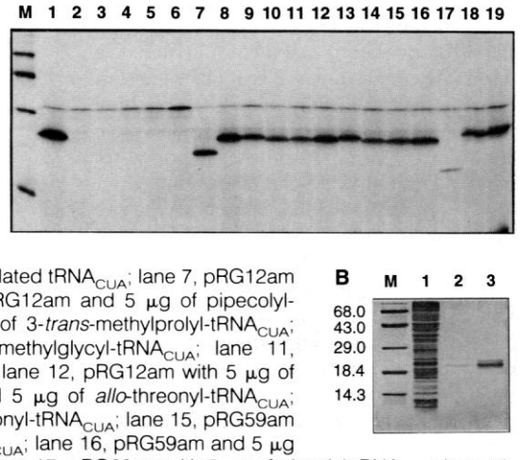

Fig. 3. (**A**) Autoradiograms of in vitro suppression reactions (30 μl) labeled with L-[^{35}S]methionine and containing the following plasmids and tRNAs: lane 1, pRG (wild type, in vivo); lanes 2 through 6, pRG12am (G12am refers to an amber mutation at position 12 in the wild-type gene) and pRG13am, pRG59am, pRG60am, and pRG61am, respectively, with 5 μg of full-length, unacylated $tRNA_{CUA}$; lane 7, pRG12am and 5 μg of lactyl-$tRNA_{CUA}$; lane 8, pRG12am and 5 μg of pipecolyl-$tRNA_{CUA}$; lane 9, pRG12am and 5 μg of 3-*trans*-methylprolyl-$tRNA_{CUA}$; lane 10, pRG12am with 5 μg of *N*-methylglycyl-$tRNA_{CUA}$; lane 11, pRG12am with 5 μg of glycyl-$tRNA_{CUA}$; lane 12, pRG12am with 5 μg of prolyl-$tRNA_{CUA}$; lane 13, pRG13am and 5 μg of *allo*-threonyl-$tRNA_{CUA}$; lane 14, pRG59am with 5 μg of *allo*-threonyl-$tRNA_{CUA}$; lane 15, pRG59am with 5 μg of hydroxyethylcysteinyl-$tRNA_{CUA}$; lane 16, pRG59am and 5 μg of hydroxyethylhomocysteine-$tRNA_{CUA}$; lane 17, pRG60am with 5 μg of glycolyl-$tRNA_{CUA}$; lane 18, pRG61am with 5 μg of nitroglutaminyl 3-$tRNA_{CUA}$; and lane 19, pRG61am with homoglutaminyl-$tRNA_{CUA}$. Cleared supernatants from terminated in vitro reactions were incubated with ribonuclease A and analyzed by SDS-PAGE (15% gel). Mutants were constructed in a M13mp19 phage (containing the 578-bp Cla I–Sal I insert) and reintroduced into the expression vector pRG in which p21 ras is under the control of the trp promoter (*48*). In vitro suppression experiments were performed as described (*16*) with the exception that the in vitro–transcribed $tRNA_{CUA}$ was purified by phenol extraction and ethanol precipitation. The band at 30 kD is β-lactamase. (**B**) Silver-stained SDS-PAGE (15% gel) showing purification of wild-type Ras synthesized in vitro with plasmid pRG. Lane 1, crude in vitro reaction; lane 2, Ras purified by sequential chromatography on DEAE-sephacel and immunoaffinity chromatography (with immobilized antibody 1F6); lane 3, wild-type Ras purified from *Escherichia coli* with pRG. Lane M, molecular size markers in kilodaltons.

Table 1. Suppression efficiencies, intrinsic and GAP-activated GTPase activities, and autophosphorylation activities of mutant Ras proteins (*46*, *48–50*). The GAP activation is a comparison of the percentage conversion of Ras (GTP) to Ras (GDP) in the presence of 40 nM recombinant GAP after 2 min; – indicates no increase in activity over the wild-type protein. The autophosphorylation rate constant for Thr^{59} (*51*) is 6.5×10^{-6} s^{-1}. ND, not determined; NA, not applicable.

Mutant	Suppression efficiency (%)	Intrinsic GTPase activity* (k_{rel})	GAP activation (%)	Autophosphorylation
Wild-type†	–	1.0	100	NA
$Gly^{12} \rightarrow Gly$	35	1.0	100	NA
$Gly^{12} \rightarrow Pro$†	–	1.10	–	NA
$Gly^{12} \rightarrow Pro$	30	1.10	–	NA
$Gly^{12} \rightarrow Val$†	–	0.15	–	NA
$Gly^{12} \rightarrow Lac$	50	0.95	–	NA
$Gly^{12} \rightarrow Pip$	40	0.84	–	NA
$Gly^{12} \rightarrow MePro$	35	0.19	–	NA
$Gly^{12} \rightarrow MeGly$	40	1.0	–	NA
$Gly^{13} \rightarrow Thr$	–	2.6	–	NA
$Gly^{13} \rightarrow aThr$	25	2.8	–	NA
$Gly^{13} \rightarrow Ser$†	–	2.8	–	NA
$Ala^{59} \rightarrow Thr$†	–	0.10	ND	1.0
$Ala^{59} \rightarrow Ser$†	–	0.10	ND	1.0
$Ala^{59} \rightarrow Val$†	–	0.10	ND	–
$Ala^{59} \rightarrow aThr$	30	0.07	ND	0.18
$Ala^{59} \rightarrow HEC$	35	0.50	ND	10.6
$Ala^{59} \rightarrow HEHC$	45	0.60	ND	3.0
$Gln^{61} \rightarrow HGln$	25	1.37	95	NA
$Gln^{61} \rightarrow NGln$	30	1.0	86	NA
$Gly^{60} \rightarrow Glyc$	15	0.10	–	NA
$Gln^{61} \rightarrow Glu$, $Glu^{63} \rightarrow Gln$†	–	0.2	–	NA

*The catalytic rate constant (k_{cat}) of wild-type Ras is 2.3×10^{-4} s^{-1}. †Protein synthesized in vivo; all other proteins were synthesized by the suppression of the corresponding nonsense mutations.

The nitro group is also less efficient at stabilizing the trigonal bipyramidal transition state than glutamine (*25*) because the nitro group cannot act as a hydrogen bond donor and is a much weaker hydrogen bond acceptor than the carboxamide group. Although the carboxylate group of Glu is structurally similar to the nitro group, its pK_a is roughly 6 log units lower and it exists predominantly in the anionic form (*28*). Consequently, the diminished GTPase activity of the $Gln^{61} \rightarrow Glu$, $Glu^{63} \rightarrow Gln$ double mutant (Table 1) may result from repulsive electrostatic interactions between the γ-phosphate and the anionic carboxylate group that displace loop L4 toward the GDP-bound, inactive conformation (*19*).

Substitution of Gln^{61} with HGln, which extends the γ-carboxamide group by one methylene unit, resulted in a 30% increase in intrinsic GTPase activity as well as a high degree of GAP-activated activity (Table 1 and Fig. 3). The modeling of HGln into the structure of the GTP·Ras complex suggests that the δ-carboxamide of HGln cannot adopt the same geometry as that of Gln^{61} relative to water 175, the γ-phosphate, and Glu^{63}. X-ray diffraction studies may further clarify the catalytic role of this residue. However, the observation that mutants in which Gln^{61} is replaced with HGln and NGln retain intrinsic and GAP-stimulated GTPase activity (in contrast to natural amino acid mutants) indicates that the γ-carboxamide of Gln^{61} is not unique in its function. Possible roles for these polar side chains may involve distortion of GTP toward the trigonal bipyramidal transition state or stabilization of a catalytically competent conformation of loop L4 not seen in the crystal structures (*29*).

The Asp^{57}-X-X-Gly^{60} sequence motif (where X is any amino acid) in the GTP binding pocket of Ras is conserved in all GTPase proteins (*1–4*). It has been proposed that the backbone NH group of Gly^{60} forms a hydrogen bond to the trigonal bipyramidal transition state (*25*). To test this notion, we converted Gly^{60} to glycolic acid (Glyc) (Table 1 and Fig. 2). Substitution of the N–H bond by a lone pair [ester bonds, like amide bonds, are strongly biased toward the planar trans configuration (*30*)] resulted in a loss of intrinsic GTPase activity. This result supports the notion that the hydrogen bond between the backbone NH group and the phosphoryl oxygens is important for GTPase activity. The $Glyc^{60}$ mutant was hydrolyzed completely at the ester bond under the conditions of polyacrylamide gel electrophoresis (PAGE), which confirms that this mutant is not contaminated with any natural amino acids at position 60.

Oncogenic Ras proteins encoded by retroviral Ras genes have $Ala^{59} \rightarrow Thr$ and $Gly^{12} \rightarrow Arg$ mutations and can be auto-

phosphorylated at Thr^{59} (*31, 32*). The structure of the $Gpp(CH_2)p$- Ras complex and modeling studies show that the Thr hydroxyl group is close to the γ-phosphate and probably directly attacks the phosphoryl group (*26*). The $Ala^{59} \rightarrow$ Ser mutant autophosphorylates to a similar extent as the Thr^{59} mutant, but simple inversion of the geometry of the secondary alcohol from the *R* (Thr) to the *S* configuration [*allo*-threonine (aThr)] resulted in diminished autophosphorylation activity (Table 1 and Fig. 2). This activity therefore appears to be a function of the spatial disposition of the secondary hydroxyl group rather than steric bulk at the β-carbon, behavior unlike that of the corresponding nonenzymatic reactions. Mutation of Ala^{59} to hydroxyethylcysteine (HEC) or hydroxyethylhomocysteine (HEHC), in which the hydroxyl group is extended an additional three or four bond lengths (4 to 6 Å), resulted in autophosphorylation activities ten and three times, respectively, more than that of the Thr^{59} mutant (Table 1 and Fig. 2). The x-ray crystal structure of wild-type Ras protein (*20, 21*) suggests that the transition state for phosphoryl transfer to HEC or HEHC cannot be accommodated without a conformational change in loop L4. This result is consistent with crystallographic and fluorescence studies (*22, 25, 28*) and suggests that loop L4 is conformationally flexible in solution and that changes in loop L4 conformation can affect GTPase activity.

Mutation of Gly^{12} to any natural amino acid other than Pro results in diminished intrinsic GTPase activity and is commonly associated with oncogenic activation (*33*). Gly^{12} occurs in a highly conserved type II β-turn [Gly^{11}-X-X-X-Gly-Lys-(Ser or Thr)], a phosphate-binding loop found in many nucleotide-binding proteins (*34, 35*). Krengel *et al.* (*26*) have shown that loop L1 conformation in the GTP·Ras($Gly^{12} \rightarrow$ Val) complex is pushed slightly away from the γ-phosphate but propose that the main effect of the Val^{12} mutation is to push Gly^{60} and Gln^{61} (L4) away from the γ-phosphate. Privé *et al.* have argued that side chains at position 12 interfere with the formation of the trigonal bipyramidal transition state (*25*). Neither proposal adequately accounts for the wild-type intrinsic GTPase activity of Pro^{12} Ras versus the diminished activity of the Ala^{12} mutant (*33*).

To more fully understand the role of Gly^{12} mutants in switch function, we inserted a number of unnatural amino acids at this site, including lactic acid (Lac), pipecolic acid (Pip), *trans*-3-methylproline (MePro), and *N*-methylglycine (MeGly). The pipecolic acid mutant, which was expected to have a much more negative φ value than Gly^{12} (within peptides, the carboxyl group of pipecolic acid occupies the axial position in the chair conformation) (*36*), retained GTPase activity similar to that of wild-type Ras (Table 1 and Fig. 2). The MeGly mutant also had wild-type GTPase activity (N_α methyl substitution also decreases φ values) (*37*). Whereas the Ala^{12} mutant had reduced GTPase activity, the isosteric Lac^{12} mutant, in which the backbone amide linkage is replaced by an ester linkage, had normal GTPase activity (and is hydrolyzed completely under basic conditions) (Fig. 3). Although the ester bond is biased toward the planar trans conformation, the rotational barrier around the C–O bond is reduced some 5 to 7 kcal/mol relative to amides (*30*), which allows some main chain flexibility. The fact that only the Pro^{12}, Pip^{12}, Lac^{12}, and $MeGly^{12}$ mutants, all of which can adopt unusual backbone conformations, were active suggests that a conformational change in loop L1 may relieve unfavorable side chain–transition state interactions at position 12. One might expect that this conformational change could be neutralized by the introduction of sufficient steric bulk at the β position of Pro or pipecolate. In fact, the $MePro^{12}$ mutant had GTPase activity similar to that of the Val^{12} mutant (Table 1).

Even though the Pro^{12}, Lac^{12}, Pip^{12}, and $MeGly^{12}$ mutants had intrinsic GTPase activity similar to that of wild-type Ras, they were not activated by GAP. Moreover, the $Gly^{13} \rightarrow$ Thr, $Gly^{13} \rightarrow$ aThr, and $Gly^{13} \rightarrow$ Ser mutants, which have intrinsic GTPase activity two to three times that of wild-type Ras (attributable to the β hydroxyl group), were not activated by GAP and were transforming in a germinal vesicle breakdown assay with *Xenopus laevis* oocytes (*38*). These Gly^{13} mutants, like the Gly^{12} mutants, might also be expected to have altered loop L1 conformations relative to wild-type Ras because of β branching effects. One interpretation of these results is that loop L1 can adopt two or more conformations in solution and that its positioning by one or more effector proteins modulates GTPase activity and oncogenic activation. The unusual backbone structures of the Gly^{12} and Gly^{13} mutants may prevent switch function. Nuclear magnetic resonance studies should provide additional information concerning the conformational lability of loop L1 (*39*).

REFERENCES AND NOTES

1. M. Barbacid, *Annu. Rev. Biochem.* **56**, 779 (1987).
2. J. L. Bos, *Cancer Res.* **49**, 4682 (1989)
3. J. A Glomset, M. H. Gelb, C. C. Farnsworth, *Trends Biochem. Sci* **15**, 139 (1990).
4. D Spandidos, *Ras Oncogenes* (Plenum, New York, 1989).
5. M Trahey *et al*, *Science* **242**, 1697 (1988)
6. M. Trahey and F. McCormick, *ibid* **238**, 542 (1987).
7. G A. Martin *et al*, *Cell* **63**, 843 (1990).
8. G Xu *et al*, *ibid*, p 835
9. C J. Noren, S. J. Anthony-Cahill, M C Griffith, P G Schultz, *Science* **244**, 182 (1989)
10. J. A Ellman, D. Mendel, P G Schultz, *ibid.* **255**, 197 (1992).
11. D Mendel *et al.*, *ibid*, **256**, 1798 (1992).
12. D Mendel, J. A. Ellman, P G Schultz, *J. Am. Chem Soc* **113**, 2758 (1991).
13. J A. Ellman, B Volkman, D Mendel, P G Schultz, D A Wemmer *ibid.* **114**, 7959 (1992).
14. J D. Bain *et al.*, *Biochemistry* **30**, 5411 (1991).
15. S. A. Robertson, J A. Ellman, P G Schultz, *J. Am Chem. Soc.* **113**, 2722 (1991).
16. J. A. Ellman, D. Mendel, C J. Noren, S Anthony-Cahill, P. G. Schultz, *Methods Enzymol* **202**, 301 (1991)
17. C. J. Noren, *Nucleic Acids Res.* **18**, 83 (1990).
18. Diminished intrinsic GTPase activity and GAP-stimulated GTPase activity are not always associated with in vivo transforming activity.
19. C. J Der, T Finkel, G M. Cooper, *Cell* **44**, 167 (1986).
20. A M. de Vos *et al.*, *Science* **239**, 888 (1988)
21. E. F. Pai *et al.*, *Nature* **341**, 209 (1989)
22. L Tong, M V Milburn, A M de Vos, S -H Kim, *Science* **245**, 244 (1989).
23. M. V Milburn *et al*, *ibid* **247**, 939 (1990).
24. E F Pai *et al.*, *EMBO J.* **9**, 2351 (1990)
25. G G. Privé *et al.*, *Proc. Natl Acad Sci. U S A* **89**, 3649 (1992)
26. U Krengel *et al*, *Cell* **62**, 539 (1990).
27. I Schlichting *et al*, *Nature* **345**, 309 (1990)
28. T H Lowry and K S Richardson, *Mechanism and Theory in Organic Chemistry* (Harper and Row, New York 1976)
29. S E Neal, J F. Eccleston, M. R. Webb, *Proc Natl Acad. Sci. U.S.A.* **87**, 3562 (1990)
30. K. B. Wiberg and K E Laidig, *J. Am Chem Soc* **109**, 5935 (1987)
31. T Y Shih, P. E. Stokey, G. W. Smythers, R Dhar, S Vroszalan, *J Biol Chem.* **257**, 11767 (1982)
32. J John, M. Frech, A Wittinghofer, *ibid.* **263**, 11792 (1988).
33. P H Seeburg, W W. Colby, D J Capon, D V Goeddel, A. D. Levison, *Nature* **312**, 71 (1984)
34. N J Gay and J E Walker, *ibid.* **301**, 262 (1983)
35. R K. Wierenga, P Terpstora, W G Hol, *J Mol Biol* **187**, 101 (1986)
36. I D Rae and H A Scheraja, *Int J Pept. Protein Res.* **13**, 304 (1979)
37. M Parthasarathy and F. A Momany, *Biopolymers* **19**, 1943 (1980)
38. C Birchmeier, D Broek, M. Wigler, *Cell* **43**, 615 (1985).
39. S Yokayama *et al.*, unpublished results
40. Y Kasiro, *Biochim. Biophys. Acta.* **95**, 505 (1978)
41. Y. N. Belokoń *et al*, *J Chem Soc Perkin Trans I*, 2075 (1988).
42. J Hooz and S. S H Gilani, *Can J Chem.* **46**, 86 (1968).
43. R Matsueda, T. Kimura, E T Kaiser, G. R. Matsueda, *Chem Lett.* 737 (1981).
44. D. J. Suich, thesis, University of California, Berkeley (1992).
45. J.-W Han, F McCormick, I. G. Macara, *Science* **252**, 576 (1991)
46. GTPase activity was determined by incubation of immunopurified Ras (1 μM) with 100 μM [α-^{32}P]GTP (Amersham, 5000 cpm/pmol) in 50 mM tris-HCl (pH 7 5), 1 mM $MgCl_2$, 5 mM EDTA, 10 mM NH_4Cl, and 5 mM dithiothreitol (DTT) buffer containing bovine serum albumin (20 μg/ml) (reaction volume was 100 μl). At various times, 5-μl samples were mixed with 5 μl of stop mix [2 mM EDTA (pH 8.0), 0.5% SDS, and 4 mM GTP, GDP, and guanosine monophosphate (GMP)]. The conversion of GTP to GDP was monitored by chromatography on polyethyleneimine (PEI) plates (Sigma) that were developed with 1 M LiCl. After autoradiography of the PEI plates, the labeled GDP nucleotide was excised and quantitated by liquid scintillation counting The rate constants were calculated as described (*40*).
47. L-Pipecolic acid, L-homoglutamine, L-*allo*-threonine, *N*-methylglycine (sarcosine), and L-lactic acid are all commercially available. The *trans*-3-methylproline was prepared by the method of

Belokoń *et al.* (*41*). Nitrobenzyl-L-lactic acid (*10*) was prepared by reaction between L-lactic acid and *o*-nitrobenzyl bromide in the presence of two equivalents of vinyl magnesium bromide in tetrahydrofuran (THF) at 60°C. For synthesis of *o*-nitrobenzyl–protected glycolic acid, ethylene glycol was first monoalkylated with *o*-nitrobenzyl bromide, and then the hydroxyl group was oxidized with pyridinium dichromate. Bromination of the mono-*o*-nitrobenzyl–protected ethylene glycol (*42*) followed by reaction with *N*-NVOC-L-cysteine (*43*) and *N*-NVOC-L-homocysteine gave nitrobenzyl-protected *N*-NVOC-L-hydroxyethylcysteine and *N*-NVOC-L-hydroxyethylhomocysteine, respectively (NVOC, nitroveratryloxycarbamyl). The hydroxyl group of *N*-NVOC-L-*allo*-threonine was protected as the *tert*-butyl-dimethylsilyl (TBDMS) derivative. Removal of the TBDMS group after acylation of the protected amino acid to pdCpA was effected with a mixture of acetic acid–H_2O-THF (3.1·1). The nitro analog of glutamic acid was synthesized by D Suich (*44*).

48 K. Miura *et al.*, *Cancer Res.* **77**, 45 (1986)

49. Suppression efficiencies were determined by scintillation counting of Ras immunoprecipitated with monoclonal antibody 1F6 (which was prepared by standard methods and shown not to affect the intrinsic GTPase activity of cellular H-Ras)

50 GAP-mediated GTPase activity was measured by a modification of the procedure described by Han *et al.* (*45*). Immunopurified Ras (100 nM) was incubated for 15 min at 25°C with 50 μCi of [α-^{32}P]GTP (410 Ci/mmol; Amersham) in buffer I [50 mM Hepes (pH 7.4), 200 mM sucrose, 1 mM $MgCl_2$, 5 mM EDTA, 0.2% NaN_3, and 1 mM DTT]. The [α-^{32}P]GTP-bound Ras was purified on a PD10 column (Pharmacia LKB). The GTPase reaction was initiated by the addition of 10 mM $MgCl_2$ in the presence or absence of recombinant GAP (total assay volume was 1 ml) Incubation time was 10 min at 30°C Ras was collected by filter binding (0.2-μm pore size; Millipore) and released from the filter by incubation with 20 mM EDTA, 1% SDS, 4 mM GTP, 4 mM GDP, and 4 mM GMP at 65°C for 5 min The bound guanine nucleotides were resolved by chromatography on PEI plates as described (*46*) The labeled nucleotides were quantitated by scintillation counting The percent GTP remaining was calculated as [(counts per minute of GTP)/(counts per minute of GTP + counts per minute of GDP)] × 100. Results are representative of duplicated experiments.

51 Autophosphorylation activity of Ras was assayed at 37°C in the presence of 10 μM [γ-^{32}P]GTP in 100 μl of reaction buffer containing 50 mM tris-HCl (pH 7 5), 10 mM MgCl, 1 mM DTT, and 1 mM NaN_3. At various times, portions (10 μl) were removed and quenched with 100 μl of cold quenching solution [50 mM tris-HCl (pH 7.5), 2 mM GTP, 2 mM GDP, 5 mM potassium inorganic phosphate, 5 mM EDTA, and 1 mM DTT]. The sample was filtered through a nitrocellulose membrane filter (Schliecher and Schuell, Keene, NH) and washed six times with 1 ml of rinsing buffer [50 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NH_4Cl, and 1 mM 2-mercaptoethanol]. The radioactivity retained on the filter was measured by liquid scintillation counting.

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Reduction in Size of the Myotonic Dystrophy Trinucleotide Repeat Mutation During Transmission

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Myotonic dystrophy (DM) is an autosomal-dominant disorder that affects 1 in 8000 individuals. Amplification of an unstable trinucleotide CTG repeat, located within the 3′ untranslated region of a gene, correlates with a more severe DM phenotype. In three cases, the number of CTG repeats was reduced during the transmission of the DM allele; in one of these cases, the number was reduced to within the normal range and correlated at least with a delay in the onset of clinical signs of DM. Haplotype data of six polymorphic markers in the DM gene region indicate that, in this latter case, two stretches of the affected chromosome had been exchanged with that region of the wild-type chromosome.

The most prevalent inherited neuromuscular disease in adults, myotonic dystrophy (DM), is a multisystem disorder that is characterized by progressive muscle weakness and myotonia (*1*). This disease is variable both in severity and age of onset; those most severely affected express the disease congenitally. The genetic defect in DM is thought to be an amplified trinucleotide CTG repeat located in the 3′ untranslated region of a gene, which putatively encodes a serine-threonine protein kinase (*2–4*). The number of CTG repeats in normal individuals, although quite polymorphic, is both mitotically and meiotically stable within a lineage. From 5 to 30 repeat copies are observed in normal alleles (*2, 4*). In contrast, the number of CTG repeats on DM chromosomes can be unstable and become extremely large. Mildly affected patients have 50 to 80 CTG repeats, whereas severely affected individuals have 2000 or more copies (*4*). Amplification of the CTG repeat has been proposed to be the molecular basis in this disease for genetic anticipation, a phenomenon in which the severity of an inherited disease increases in successive generations of an affected family (*5*).

In our Canadian DM kindreds, positive correlation was observed between increased numbers of CTG repeats and earlier age of disease onset (*6, 7*). One of these kindreds (Fig. 1) was haplotyped for a total of 18 restriction fragment length polymorphisms (RFLPs) at 12 loci, located between the gene encoding apolipoprotein C2 (APOC2) and the marker p134C, although only four markers are shown (*8*). This kindred possesses the most common DM haplotype of our affected population, one that occurs in approximately 30% of DM kindreds tested, and is quite rare among normal chromosomes: the calculated frequency is less than 0.000015 (*9*). We have not observed this haplotype on more than 2000 normal chromosomes analyzed to date.

Genetic data indicated that the affected chromosome of individual II-8 (Fig. 1) represented the most common DM haplotype. The DNA of individual II-8 was analyzed by genomic Southern (DNA) blot and polymerase chain reaction (PCR) with primers flanking the CTG repeat region (*2*). The extent of his expanded DM chromosome was in the range of 1.5 to 3.0 kb (Fig. 2A), and the heterogeneity is typical of DM individuals assayed by PCR (Fig. 2B). This was consistent with both the genetic and clinical data. Clinical symptoms of hand weakness and myotonia were originally noted in II-8 from the mid-to-late teens, and a myopathic facies, bitemporal narrowing, and early pattern baldness were apparent from photographs from that age.

The daughter of II-8 (III-10, Fig. 1) inherited her father's DM chromosome. However, no CTG trinucleotide repeat amplification was detected on Southern blot analysis (Fig. 2, A and B). Repeat blood samples were obtained and the initial findings were confirmed. Unlike her father, III-10 showed no evidence of CTG heterogeneity (Fig. 2B). Genomic DNA was amplified with the use of primers immediately flanking the CTG repeat (Fig. 2C). Two alleles, one with 13 and one with 22 CTG repeats, were detected in III-10. The 22-repeat allele was maternally derived. A reduction in the number of CTG repeats down to the normal number and an increase in their stability had occurred on her DM chromosome. The number of CTG copies in the unaffected (non-DM) allele of II-8 is the same as that observed for the paternally derived DM allele in III-10.

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