physiologic temperatures. For steady-state anisotropy measurements, the spectrofluorometer was configured in the T-format, labeled suspensions were excited at 360 nm, and emission light was measured through Schott long-pass filters. Observation of a narrow emission band was unnecessary since the fluorescence anisotropy of DPH is constant across the emission spectrum. Steady-state fluorescence anisotropy (r_s) was calculated from observed fluorescence intensities (I) by the following equation:

$$r_s = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \text{ where } G = \frac{I_{hv}}{I_{hh}}$$

First and second subscripts refer to the vertical (v) or horizontal (h) positions of the excitation and emission polarizers, respectively. This formula corrects for any asymmetry in the detection of vertically and horizontally polarized light. The usefulness of the steady-state anisotropy of DPH in labeled membranes as an inferential index of membrane "fluidity" or "order" has been documented widely in the literature.

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28. We thank J. Moossy, A. J. Martinez, and G. Rao for providing the autopsy-proved cases of Alzheimer's disease; the clinicians and staff of the Alzheimer's Disease Research Center at the University of Pittsburgh, especially F. J. Huff; and S. Belle, R. Gibbons, and J. Beyer for statistical consultations. Supported by Alzheimer's Disease Research Center grant AG05133 (G.S.Z. and F.B.) and Mental Health Clinical Research Center grant MH30915 (G.S.Z.); program grants AG03705 (G.S.Z. and F.B.) and MH36224 and MH31154 (B.M.C.); and project grant MH38313 (B.M.C). G.S.Z. was the recipient of National Institute of Mental Health Research Scientist Development Award MH00540.

8 April 1987; accepted 24 July 1987

A Cytoplasmic Protein Stimulates Normal N-*ras* p21 GTPase, but Does Not Affect Oncogenic Mutants

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The role of guanine nucleotides in *ras* p21 function was determined by using the ability of p21 protein to induce maturation of *Xenopus* oocytes as a quantitative assay for biological activity. Two oncogenic mutant human N-*ras* p21 proteins, Asp12 and Val12, actively induced maturation, whereas normal Gly12 p21 was relatively inactive in this assay. Both mutant proteins were found to be associated with guanosine triphosphate (GTP) in vivo. In contrast, Gly12 p21 was predominantly guanosine diphosphate (GDP)–bound because of a dramatic stimulation of Gly12 p21-associated guanosine triphosphatase (GTPase) activity. A cytoplasmic protein was shown to be responsible for this increase in activity. This protein stimulated GTP hydrolysis by purified Gly12 p21 more than 200-fold in vitro, but had no effect on Asp12 or Val12 mutants. A similar factor could be detected in extracts from mammalian cells. It thus appears that, in *Xenopus* oocytes, this protein maintains normal p21 in a biologically inactive, GDP-bound state through its effect on GTPase activity. Furthermore, it appears that the major effect of position 12 mutations is to prevent this protein from stimulating p21 GTPase activity, thereby allowing these mutants to remain in the active GTP-bound state.

Ras P21 PROTEINS BIND GUANINE nucleotides and convert bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP) by an intrinsic guanosine triphosphatase (GTPase) activity (*1, 2*). In these respects, *ras* proteins resemble signal-transducing G proteins, as well as initiation and elongation factors. All of these proteins cycle between active, GTP-bound forms, and inactive, GDP-bound forms (*3*). The observation that many oncogenic mutants of p21 are reduced in GTPase activity led to the proposal that transformation by p21 is the result of abnormal levels of p21 in an active, GTP-bound state (*2*). However, analysis of GTPase activities associated with a large number of p21 mutants has failed to reveal a quantitative relationship between GTPase activity and transforming potential (*4, 5*). It therefore appears that biochemical properties of p21 proteins measured in vitro do not adequately account for their different biological properties. This could be because in vitro measurements do not accurately reflect conditions in vivo, so that estimates of GTPase activities are misleading, or because oncogenic mutations have additional effects that have not yet been identified. To resolve these issues, and to develop a quantitative model for the effects of oncogenic mutations, we examined the roles of guanine nucleotide binding and hydrolysis on p21 function in living cells. *Xenopus* oocytes were used because of the relative ease with which these large cells can be manipulated and because it has been shown previously that human p21 proteins are able to induce oocyte maturation, thus providing a convenient quantitative assay for biological activity (*6*).

As shown in Fig. 1a, Asp12 and Val12 N-*ras* p21 proteins efficiently induced maturation of stage VI *Xenopus* oocytes, as determined by the fraction of oocytes that undergo germinal vesicle breakdown (GVBD) after injection of p21 protein. Normal Gly12 p21 was much less potent, requiring at least 150 ng of pure protein per oocyte to induce maturation. These effects are similar to those of Gly12 and Val12 H-*ras* p21 (*6*).

The effects of guanine nucleotides on the ability of p21 proteins to induce oocyte maturation were examined by binding either GDP or Gpp(NH)p (a nonhydrolyzable GTP analog) to p21 proteins prior to injection into oocytes. GDP had no effect on the ability of normal Gly12 p21 to induce maturation. However, binding of Gpp(NH)p greatly increased its biological activity (Fig. 1b). Under these conditions, Gly12 p21 was as active as either the Asp12 or the Val12 mutant (*7*). These results indicate that the function of normal Gly12 p21 is controlled by association with GDP or GTP. They also suggest that the different biological potencies of normal and mutant p21 proteins may reflect their differing association with GDP and GTP in vivo.

To determine whether GTP or GDP became associated with injected p21 proteins in vivo, [^{32}P]phosphate was co-injected so that cellular nucleotide pools would rapidly become radiolabeled (*8*), and p21 proteins were recovered from oocyte extracts by immunoprecipitation with monoclonal antibody to p21 (anti-p21) Y13-259 (*9*). Immune complexes were treated with EDTA and SDS and analyzed for radiolabeled nucleotide by thin-layer chromatography. Nor-

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mal, Gly12 p21 was predominantly associated with GDP 3 hours after injection, whereas both Asp12 and Val12 p21 proteins were associated with GTP (Fig. 2a). Identical results were obtained if extracts were made 5 hours after injection, indicating that steady-state conditions had been achieved. When p21 protein was added to extracts from [^{32}P]phosphate-labeled oocytes, no GTP binding was detected (Fig. 2, control). This was because free GTP was rapidly degraded in these extracts and indicates that guanine nucleotides bound to injected proteins in vivo rather than during extraction and immunoprecipitation. From these results, we conclude that different biological activities of normal and mutant proteins are indeed a result of their association with GDP and GTP, respectively.

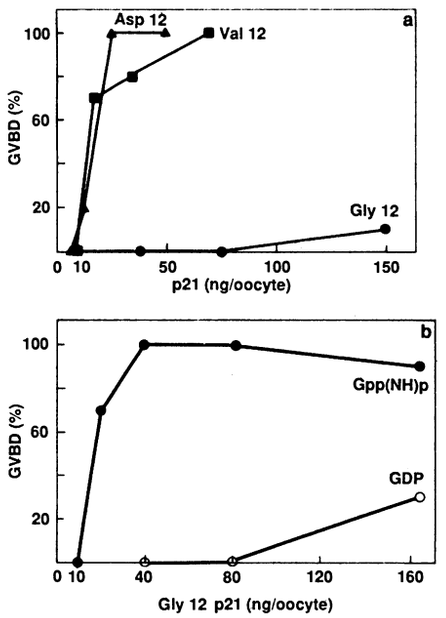

Fig. 1. Biological activity of N-*ras* p21 proteins in *Xenopus* oocytes. (**a**) Maturation-inducing activity of Gly12, Asp12, and Val12 p21 proteins. Human N-*ras* p21 expressed in *Escherichia coli* (*4*) was purified on an antibody affinity column to greater than 90% homogeneity. The mouse monoclonal antibody, directed against a peptide spanning amino acids 29 to 44 of p21, has been described (*15*). The p21 protein eluted from this column in 100 m*M* sodium carbonate, *p*H 10.6, was free of bound nucleotide [as determined by its ultraviolet spectrum (*12*)] and was able to bind and hydrolyze GTP. The p21 proteins purified in this manner were dialyzed into buffer A (80 m*M* β-glycerophosphate, 5 m*M* $MgCl_2$, 1 m*M* DTT, *p*H 7.5) and microinjected into stage VI oocytes (*8*). Oocytes were obtained from sexually mature female *Xenopus laevis* stimulated with 25 units of pregnant mare serum gonadotrophin (PMSG) 24 hours before surgical removal of the ovary (*16*). Injected oocytes were incubated at room temperature for 23 hours in modified Ringer's saline (100 m*M* NaCl, 1.8 m*M* KCl, 2 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 4 m*M* $NaHCO_3$, *p*H 7.8). Maturation was judged by germinal vesicle breakdown (GVBD), which results in the appearance of a well-defined white spot at the pigmented animal pole (*17*). In ambiguous cases oocytes were fixed in 5% trichloroacetic acid and split open to verify the presence or absence of the germinal vesicle. (**b**) Effects of bound Gpp(NH)p and GDP on the biological activity of Gly12 p21. Gly12 p21 protein in buffer A was incubated with 2 m*M* Gpp(NH)p (closed symbols) or 3 m*M* GDP (open symbols) at 4°C for 72 hours. Various concentrations of nucleotide-bound p21 were microinjected, and GVBD was scored as described above (*7*).

It was surprising to observe such a dramatic difference between Gly12 and Asp12 p21 in their association with guanine nucleotides in vivo. Analysis of these proteins in vitro revealed no significant differences in GTP or GDP binding affinities and less than a threefold difference in GTPase activities (*4*). For direct comparison with results obtained in vivo (Fig. 2a), p21 proteins were incubated at physiological GTP concentrations and at room temperature in vitro. Normal and mutant proteins were predominantly GTP-bound after a 3-hour incubation period (Fig. 2b), as expected from previous estimates of their GTPase activities (*4*). Therefore, Asp12 and Val12 mutants appeared to behave similarly in vivo and in vitro, whereas Gly12 p21 displayed strikingly different properties in these two situations.

Estimates of guanine nucleotide pool sizes in *Xenopus* oocytes indicated that the concentration of GTP is greatly in excess of that of GDP (*10*). Under these conditions, it is unlikely that GDP binds directly to p21. This suggests that GDP associated with Gly12 p21 (Fig. 2a) was derived from hydrolysis of bound GTP and that the GTPase activity of Gly12 p21 in vivo may be much higher than that expected from in vitro measurements. To test this possibility, the rate of hydrolysis of GTP bound to Gly12 p21 in vivo was measured directly. The p21 protein was incubated with [α-^{32}P]GTP and was injected into oocytes. Conversion of bound GTP to GDP could be detected within 5 minutes of injection (Fig. 3). Quantitative analysis of radiolabeled GTP and GDP bound to Gly12 p21 revealed that 70% bound GTP was lost during this 5-minute period, and all of this radiolabel was recovered in bound GDP. We therefore estimate that the hydrolytic half-time of GTP bound to Gly12 p21 in vivo is approximately 2 to 3 minutes. This corresponds to a rate constant of 0.23 to 0.35 moles GTP hydrolyzed per mole of p21 per minute at room temperature $t_{1/2}$ = ln 2/rate constant). The extent of conversion of GTP to GDP during 5 minutes of incubation in vivo was considerably greater than that during a 3-hour incubation in vitro (Fig. 2b). In contrast to Gly12 p21, hydrolysis of GTP bound Asp12 p21 was not clearly detectable, even during 3 hours of incubation in vivo (Fig. 3). During this period, radiolabeled GTP bound to Asp12 p21 decreased by about 40% (472 count/min to 192 count/min), whereas radiolabeled GDP increased from 122 count/min to 157 count/

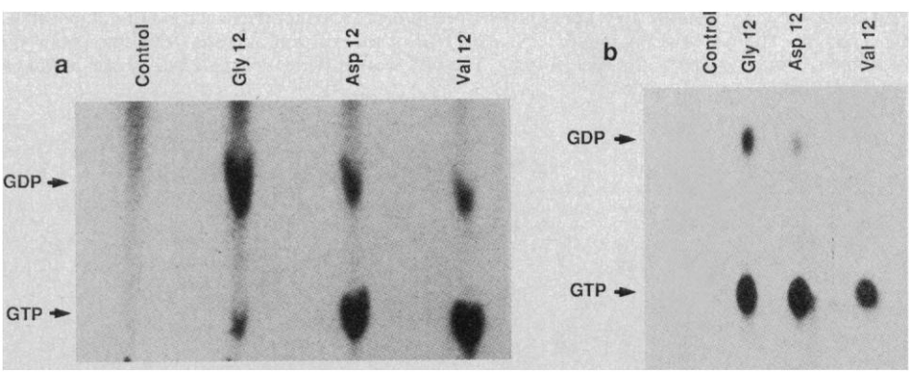

Fig. 2. Association of guanine nucleotides with p21 protein in vivo and in vitro. (**a**) Nucleotide state of N-*ras* p21 injected into *Xenopus* oocytes. The p21 proteins (2.0 to 3.0 mg/ml in buffer A) purified as described in Fig. 1 were mixed with [^{32}P]phosphate (200 mCi/ml, carrier-free, 285 Ci/mg P, ICN) and microinjected (50 nl per oocyte) into 50 stage VI oocytes from PMSG-treated *Xenopus laevis*. After 3 hours at room temperature, oocytes were crushed in buffer B (20 m*M* tris-HCl, *p*H 8.0, 100 m*M* NaCl, 5 m*M* $MgCl_2$) with 1% NP40. Lysates were centrifuged at 15,000*g* for 10 minutes at 4°C. Supernatants were diluted to 0.5% NP40 and reacted with monoclonal antibody Y13-259. All immunoprecipitation steps were carried out at 0°C. The immune complexes were collected with goat anti-Rat Ig–Protein A Sepharose and washed ten times in buffer B with 0.5% NP40. To the control lysate ([^{32}P]phosphate injected alone), 5 μg of purified p21 was added before the Y13-259 antibody to determine the extent of binding and hydrolysis during the immune precipitation reaction. Nucleotides were eluted from p21 with 1% SDS, 20 m*M* EDTA at 65°C for 5 minutes and chromatographed on PEI Cellulose in 1*M* LiCl. GTP and GDP spots were visualized with autoradiography and identified by means of standard solutions. (**b**) Nucleotide state of p21 protein in vitro. Nucleotide-free p21 (*18*) (2 μ*M* in buffer A) was incubated with 200 μ*M* [α-^{32}P]GTP (final specific activity 8 Ci/mmol), 3 m*M* adenosine triphosphate (ATP), and bovine serum albumin (BSA) (2 mg/ml). After 3 hours at room temperature, p21 was immunoprecipitated and associated nucleotides were analyzed as described above. Control, no p21 added.

min. It therefore appears that GTP was lost by dissociation as well as by conversion to GDP, and that the relative contributions of these two parameters cannot be easily estimated from these data. However, estimates of Asp12 and Val12 GTPase activities in vivo by indirect methods (*11*) suggest hydrolytic half-times of over 1000 minutes (rate constant of 0.00069 mole of GTP per mole of p21 per minute) for both mutants. This is consistent with in vitro estimates; for example, Fig. 2b shows a very small fraction of bound GTP was converted to GDP by either mutant protein during a 3-hour incubation, suggesting a hydrolytic half-time of several hours. From these results we conclude that the GTPase activity of normal p21 in vivo is at least 300 times higher than that of either mutant. We also propose that this high GTPase activity is responsible for the association of Gly12 with GDP and, hence, for the low biological activity of this protein in oocytes relative to oncogenic mutants.

Stimulation of normal p21 GTPase did not appear to require localization of injected p21 into cellular membranes. It was shown previously that injected p21 takes about 2 hours to become membrane-associated (*6*), whereas GTPase stimulation occurs within minutes of injection. To test the possibility that a cytoplasmic factor stimulates normal p21 GTPase activity, an extract was made by subjecting oocytes to high-speed centrifugation. Under these conditions, cells ruptured and released their cytoplasmic content. This supernatant was tested for its ability to stimulate p21 GTPase activity. The p21 proteins were first incubated with [α-^{32}P] GTP at 37°C for 30 minutes. During this period, guanine nucleotides that remained bound to p21 during conventional chromatographic purification (*4, 12*) were exchanged for radiolabeled GTP, some of which underwent hydrolysis to GDP. The p21 proteins bound to radiolabeled GTP or GDP were then incubated with the cytoplasmic extract. This prior incubation step was necessary because the extract rapidly degraded free GTP, so that no GTP binding to p21 could be detected when p21, GTP, and extract were incubated simultaneously. Conversion of GTP to GDP bound to Gly12 p21 was dramatically stimulated by the extract (Fig. 4a). We estimated that this stimulation was more than 200-fold. In contrast, no effect on either mutant was seen. Stimulation of Gly12 p21 GTPase was blocked by pre-incubating p21 with the monoclonal antibody, Y13-259 (Fig. 4b). Centrifugation of extracts at 100,000*g* for 1 hour did not significantly diminish their activity. Furthermore, the activity was recovered in the excluded volume of a G-75 Sephadex gel filtration column, indicating that stimulation of p21 GTPase did not require the presence of low molecular weight cellular components. Incubation of partially purified factor with trypsin-agarose beads (10 units of trypsin, 2 hours at 20°C) inactivated the factor, as did heating to 50°C for 5 minutes. These results indicate that the factor is a protein; we will refer to this protein as GAP (GTPase activating protein).

To determine whether mammalian cells contain a GAP-like activity, detergent extracts were prepared from mouse NIH 3T3 cells and from human peripheral blood lymphocytes. Both extracts contained GAP activity. Stimulation of GTPase activity was again specific for Gly12 p21; Asp12 p21 was not significantly affected by these extracts (Fig. 5).

In summary, we have shown that the biological activity of normal Gly12 p21 is controlled by guanine nucleotides, and that in vivo this protein is maintained in the inactive, GDP-bound state through stimulation of GTP hydrolysis. The factor responsible for this stimulation appears to be a soluble cytoplasmic protein, the identity of which is currently under investigation. The physiological role of this protein, referred to as GAP, is not yet known. It is possible that under certain conditions the ability of this protein to stimulate p21 GTPase is diminished, thus allowing p21 to remain in the active, GTP-bound state. If so, GAP could

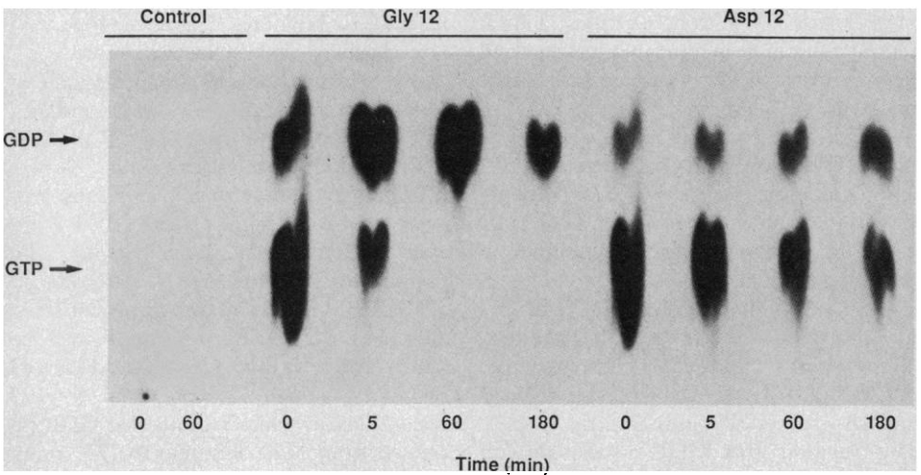

Fig. 3. GTPase activity of normal p21 is stimulated in vivo. Purified p21 (*18*) was incubated with [α-^{32}P]GTP at room temperature for 3 hours as described in Fig. 2b, except the final concentration of BSA was 1 mg/ml. The p21 was immunoprecipitated (1 μl of the reaction mixture; zero time point) or microinjected into oocytes (50 nl per oocyte). The p21 was recovered from 20 oocytes at the times indicated. Control, no p21 added.

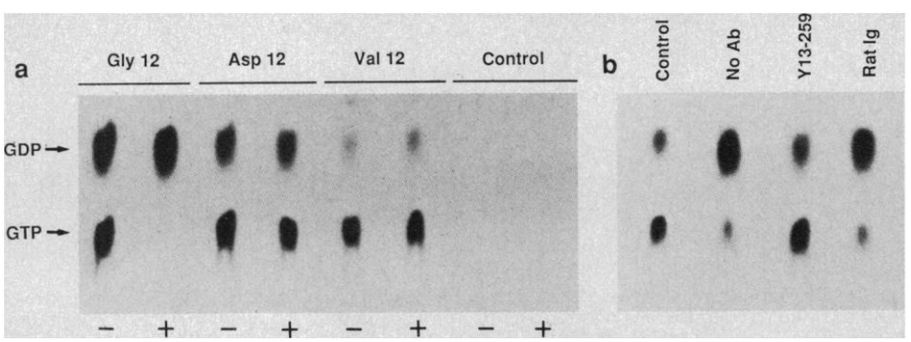

Fig. 4. Stimulation of p21 GTPase activity in vitro. (**a**) Stimulation of Gly12, but not Asp12 or Val12 p21 GTPase activity. Conventionally purified p21 (4 μM in buffer A) was incubated with 255 μM [α-^{32}P]GTP (16 Ci/mmol), 4 mM ATP, and BSA (2.5 mg/ml) at 37° for 30 minutes. Two microliters of this reaction mixture was then incubated with 20 μl of buffer A or oocyte extract. Extracts were prepared from stage VI oocytes treated with collagenase (2.5 mg/ml) for 12 hours, washed with Ringer's saline, packed by low-speed centrifugation, and lysed by centrifugation at 16,000*g* for 30 minutes. After 1 hour at room temperature, p21 was immunoprecipitated and associated nucleotides were analyzed as in Fig. 2a. Control, no p21 added; +, oocyte extract added; −, buffer A added. (**b**) In vitro stimulation of p21 GTPase is blocked by Y13-259 monoclonal antibody. Gly12 p21 was incubated with [α-^{32}P]GTP as described in (a). Two microliters of the binding reaction was incubated with 1 μg of rat monoclonal antibody Y13-259, nonimmune rat IgG, or buffer (no antibody) for 15 minutes at room temperature. A 10-μl portion of partially purified extract (prepared by gel filtration of extracts on G-75 Sephadex) or buffer A (control) was added, and the reaction proceeded for 1 hour. Y13-259 was added to the control samples, and p21 was immunoprecipitated and analyzed as in Fig. 2a.

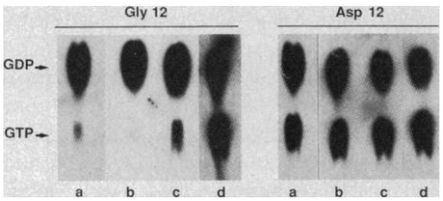

Fig. 5. Stimulation of Gly12 p21 GTPase by mammalian cell extracts. Packed cell pellets were washed with phosphate-buffered saline and lysed with buffer A + 0.5% NP40 (1 vol cell pellet : 0.5 vol lysis buffer). Extracts were mixed with Gly12 or Asp12 p21·GTP and GTPase activities measured as in Fig. 4. Panel a, human peripheral blood lymphocytes; panel b, mouse NIH 3T3 cells; panel c, 1:10 dilution of NIH 3T3 extract in buffer A + 0.5% NP40; and panel d, buffer control.

be considered to be directly upstream from p21 in a signal transduction pathway depending on p21 activation. It is interesting to note that bacterial elongation factor EF-Tu, which may be structurally related to p21 within the GTPase domain (*13*), has virtually no detectable GTPase activity in vitro. As with p21, EF-Tu GTPase activity is greatly stimulated in vivo; in this case by association with ribosome components (*14*).

A striking property of GAP is its failure to stimulate GTPase activity of Asp12 or Val12 p21 proteins. As a result, they are able to remain GTP-bound in vivo. Estimation of GTPase activities of these mutants suggests that they are several hundredfold lower than the GTPase activity of Gly12 p21 in vivo. These differences reflect the different biological potencies of these mutant and normal p21 proteins. We therefore conclude that the major role of Asp12 and Val12 mutations is to prevent interaction with GAP, and that the effects of these mutations on intrinsic GTPase activities measured in vitro are not biologically significant.

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10. The cellular GTP pool size in stage VI oocytes has been determined previously by others [M. J. LaMarca, L. D. Smith, M. C. Strobel, *Dev. Biol.* **34**, 106 (1973)] to be on average 257 pmol per oocyte. We attempted to determine the GDP pool size by incubating oocytes in [^{3}H]pool guanosine for 18 hours and resolving labeled nucleotides on PEI Cellulose in 1*M* LiCl. No [^{3}H]GDP could be detected in cellular extracts whereas GTP was labeled efficiently. From these data an upper limit for the GDP pool size can be set at 1/30 of the GTP pool. The GDP and GTP pools rapidly equilibrate as was shown by monitoring the fate of injected [^{3}H]GDP and [^{3}H]GTP. [^{3}H]GDP equilibrated with the GTP pool within 5 minutes. [^{3}H]GTP equilibration with the GDP pool was slower, occurring between 5 and 30 minutes after injection.
11. The ratio of p21·GTP/p21·GDP is a function of GTPase activity (k_{cat}) and GDP dissociation (k_{off}):

$$\text{GTP} + \text{p21} \leftrightarrow \text{p21·GTP} \xrightarrow{k_{cat}}$$

$$\text{p21·GDP} \xrightarrow{k_{off}} \text{p21} + \text{GDP}$$

assuming that when GTP>>GDP, direct binding of GDP to p21 is negligible. At steady state, p21·GTP × k_{cat} = p21·GDP × k_{off}, so, p21·GTP/p21·GDP = k_{off}/k_{cat}. The k_{off} for Gly12, Asp12 and Val12 p21 proteins was measured as follows: 50 μ*M* p21 protein was incubated with 500 μ*M* [8-^{3}H]-GDP (9.3 Ci/mmol, NEN) at 20° for 4 hours. Unbound GDP was removed by dialysis, and p21 GDP was injected into oocytes, as in Fig. 1. We estimated that after dialysis, free [8-^{3}H]GDP amounted to less than 3 pmol per oocyte; this was considered insignificant compared to endogenous guanine nucleotide pools [200 pmol per oocyte (*10*)]. Radiolabeled GDP bound to p21 was recovered as in Fig. 2b. Dissociation $t_{1/2}$'s of about 200 minutes were obtained in each case (k_{off} = ln 2/200). The ratio of p21·GTP/p21·GDP in the experiment shown in Fig. 2a was greater than 5.0. From this, we estimated the hydrolytic half-time to be 1000 minutes.
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19. We thank R. J. Milley for the gift of the mouse monoclonal affinity column, R. Clark for the affinity purification protocol, M. Wu and Associates for oocyte injections, A. Sampson-Johannes for human peripheral blood lymphocytes, D. Lowy for useful discussions, and W. Bloch for critical review of the manuscript.

18 June 1987; accepted 10 August 1987

Germline Organization of the Murine T Cell Receptor β-Chain Genes

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The complete germline organization of the β-chain genes of the murine T cell receptor was elucidated in order to obtain the structural basis for understanding the mechanisms of somatic DNA rearrangements. Twenty of the 22 known variable (V_β) genes are clustered within 250 kilobases of DNA 5′ to the constant region (C_β) genes. These V_β genes share the same transcriptional orientation as the diversity (D_β), joining (J_β), and C_β genes, which implies that chromosomal deletion is the mechanism for most V_β to D_β-J_β rearrangements. Within this V_β cluster, the distance between the most proximal V_β gene and the D_β-J_β-C_β cluster is 320 kilobases, as determined by field-inversion gel electrophoresis. The large distance between V_β and D_β, relative to that between D_β and J_β, may have significant implications for the ordered rearrangement of the T cell receptor β-chain genes.

THE VERTEBRATE IMMUNE SYSTEM uses multiple rearranging gene families to generate clonal diversity for its antigen-specific receptors. These gene families, including the heavy- and light-chain (κ and λ) genes of the B cell receptor (immunoglobulin) and the α-, β-, and γ-chain genes of the T cell receptor, undergo developmentally regulated somatic DNA rearrangements to generate functional transcription units during B cell or T cell differentiation (*1*). T cell receptors involved in the recognition of antigen and major histocompatibility complex molecules are disulfide-linked heterodimers composed of α and β chains, each divided into variable (V) and constant (C) regions (*2*). The V region of the β chain, like that of the immunoglobulin heavy chain, is encoded by three separate DNA segments, V_β, diversity (D_β), and joining (J_β), that recombine somatically to form a complete V_β gene (*3*, *4*). The β-gene family contains two closely linked C_β genes,

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