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Purification and Characterization of Human H-*ras* Proteins Expressed in *Escherichia coli*

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The full-length normal and T24 mutant human H-*ras* proteins and two truncated derivatives of the T24 mutant were expressed efficiently in *Escherichia coli*. The proteins accumulated to 1 to 5% of total cellular protein, and each was specifically recognized by anti-*ras* monoclonal antibodies. The two full-length proteins as well as a carboxyl-terminal truncated derivative (deleted for 23 amino acid residues) were soluble upon cell lysis and were purified to 90% homogeneity without the use of denaturants. In contrast, an amino-terminal truncated *ras* derivative (deleted for 22 amino acid residues) required treatment with urea for its solubilization. The guanine nucleotide binding activity of these four proteins was assessed by a combination of ligand binding on proteins blots, immunoprecipitation, and standard filter binding procedures. The full-length proteins showed similar binding kinetics and a stoichiometry approaching 1 mol of GTP bound per mol of protein. The carboxyl-terminal truncated protein also bound GTP, but to a reduced extent, whereas the amino-terminal truncated protein did not have binding activity. Apparently, the carboxyl-terminal domain of *ras*, although important for transforming function, does not play a critical role in GTP binding.

The three members of the human *ras* gene family cH-*ras*-1, cK-*ras*-2, and N-*ras* encode highly related proteins of 188 to 189 amino acid residues (p21) (3, 19, 21, 31, 37). Both qualitative and quantitative alterations in these gene products have been implicated in tumorigenesis in animals. In particular, mutant alleles of these genes which carry point changes altering amino acid residue 12 or 61 are found in a variety of tumors and tumor cell lines and, in addition, cause cellular transformation when introduced into NIH 3T3 fibroblasts (7, 10, 23, 25, 26, 29, 36, 38, 43). Likewise, elevated expression of apparently normal *ras* genes is observed in a spectrum of primary tumors (32, 33) and also may cause transformation of rodent fibroblasts (4). The *ras* proteins are synthesized in the cytoplasm and subsequently localize to the inner plasma membrane (9, 20, 30, 41). They appear to undergo posttranslational modification(s) which includes acylation (28) and results in alteration of their polyacrylamide gel electrophoretic mobility (11, 20, 30). The *ras* proteins selectively bind certain guanine nucleotides (27) and recently have been shown to hydrolyze GTP to GDP and P_i (13, 18, 35). This GTPase activity is severely impaired in products of transforming *ras* genes, and this deficiency may be responsible for the transforming activity of these *ras* genes.

Detailed biochemical and physical analyses of the *ras* proteins have been limited by the low levels of these proteins produced and obtainable from mammalian cells. One approach to circumvent these limitations has been to express the *ras* proteins in *Escherichia coli*. Several reports have described the expression in *E. coli* of *v-ras* fusion proteins, in which the *ras* polypeptide is fused to some portion of a procaryotic or synthetically derived sequence (12, 16, 34). Often the proteins made in this way are found to be insoluble, and the use of strong denaturants has been required to obtain them in a soluble form. A recent report describes the expression of nonfused authentic *ras* proteins

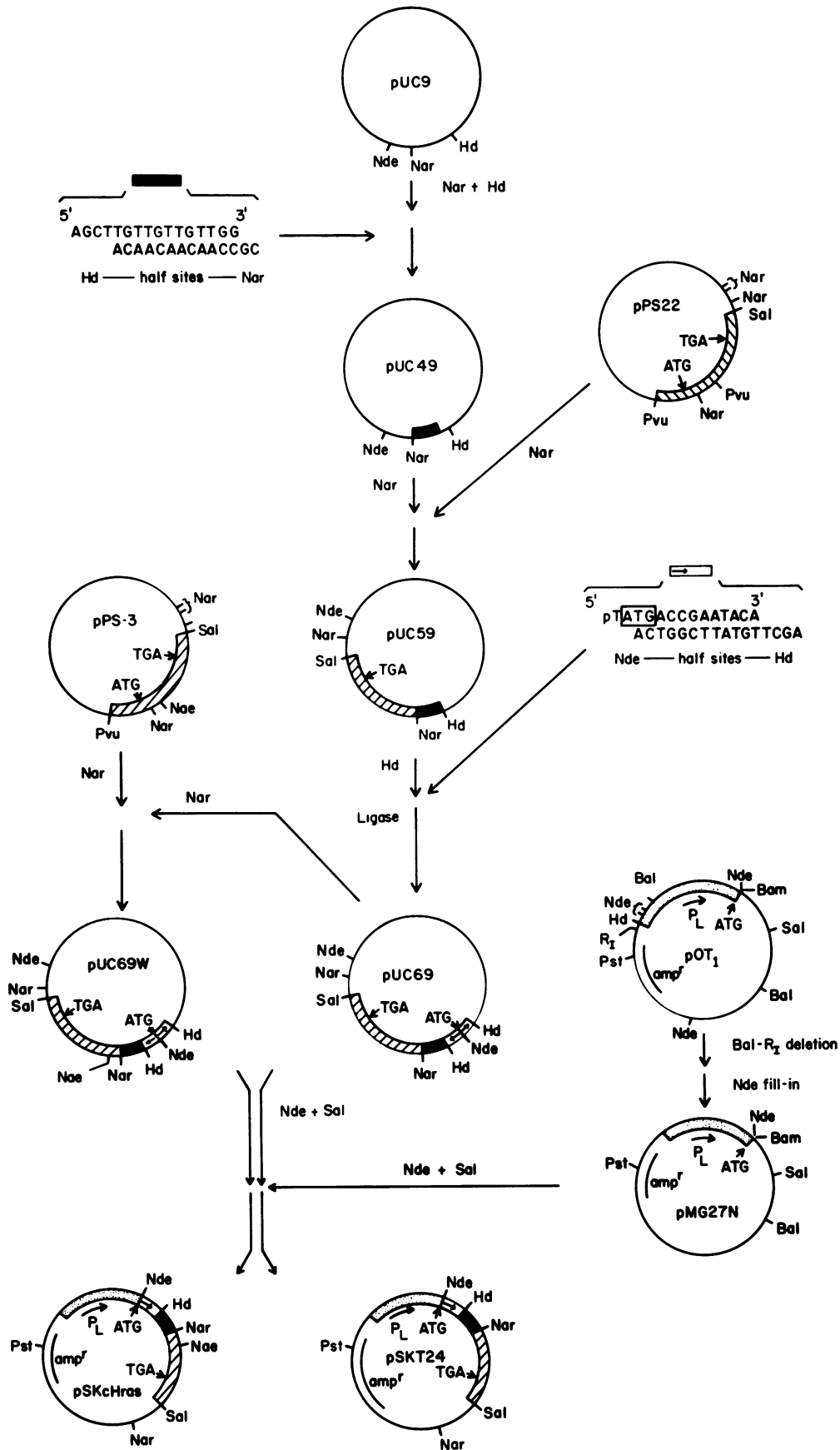
in *E. coli* (14). These proteins also were produced in an insoluble form and required treatment with urea or guanidine hydrochloride for their solubilization. Both fusion proteins and proteins which have been treated with strong denaturants may prove unacceptable for detailed analysis of their protein structure, modification, function, or a combination of these.

In this report, we describe the efficient expression and purification of an *E. coli*-synthesized, authentic, human wild-type *ras* gene product and three mutant *ras* derivatives. These include the T24 bladder carcinoma-derived *ras* variant as well as an amino-terminal truncated and a carboxyl-terminal truncated derivative of the T24 protein. All four proteins are recognized by a monoclonal antibody produced against the *v-H-ras* gene product expressed in cells transformed with Harvey murine sarcoma virus. The normal, T24, and carboxyl-terminal truncated T24 proteins are soluble upon cell lysis and were purified without the use of chaotropic agents. All three of these proteins bind guanine nucleotides. In contrast, the amino-terminal truncated T24 protein is found to be insoluble in *E. coli* and exhibits no binding activity.

MATERIALS AND METHODS

Reagents. Commercial reagents and their suppliers were: enzymes for cloning and characterization, New England BioLabs, Inc., Bethesda Research Laboratories, Inc., and Boehringer Mannheim Biochemicals; agarose, FMC Corp., Marine Colloids Div.; acrylamide reagents, Bio-Rad Laboratories and J. T. Baker Chemical Co.; nitrocellulose, Schleicher & Schuell Inc.; protein A-Sepharose, Pharmacia Fine Chemicals; rabbit anti-rat immunoglobulin G (IgG) (H+L), Cappel Laboratories; lysozyme and Freund adjuvants, Sigma Chemical Co.; [α -³²P]dGTP and riboguanosine 5'-triphosphate (rGTP) (15 TBq/mmol), ¹²⁵I-protein A (1,332 GBq/mg), [³⁵S]methionine (52 TBq/mmol), and [³H]rGDP (392 GBq/mmol), Amersham Corp. Enzymes were used according to the specifications of the manufacturers. For ol-

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igonucleotide synthesis, the silica gel solid support was obtained from Applied Biosystems, the protected nucleosides were from Cruachem Chemical Co., and all other reagents were from Aldrich Chemical Co. The hybridoma cell line producing the rat Y13-259 antibody directed against Harvey murine sarcoma virus p21 (11) was kindly provided by Mark Furth. Antibody was recovered from serum-free culture supernatants by ammonium sulfate precipitation.

Synthesis of oligonucleotide linkers. The linkers used in the plasmid constructions (Fig. 1) were synthesized on a silica gel polymer support (Fractosil) by the phosphite-triester procedure previously described (39) with some modifications. The solution containing the active phosphorylating derivative of the incoming nucleoside was passed through a column packed with derivatized silica gel to produce the coupled phosphite intermediate which was then oxidized to the phosphate form with aqueous iodine. The unreacted 5'-OH group was capped with a mixture of acetic anhydride, pyridine, and 4-dimethylaminopyridine. The dimethoxytrityl group was removed with 3% trichloroacetic acid in methylene chloride. After being washed with chloroform followed by pyridine, the resin was ready for the next cycle (cycle time was 15 min). At the end of the synthesis, the resin was removed from the column and treated with concentrated ammonium hydroxide to cleave the oligomer from the resin and to remove the protecting groups. After centrifugation, the ammonia solution was concentrated, and the residue was dissolved in water and purified on a 20% polyacrylamide gel. The oligomer was recovered from the gel by electrophoretic elution onto Whatman DEAE-81 paper, followed by elution with 1 M triethylammonium bicarbonate (pH 8.5). The triethylammonium bicarbonate was removed by coevaporation with water, and the sample was dissolved in water. The sequence of the oligomers was verified by a modification of the Maxam and Gilbert procedure (17). For cloning, complementary strands were mixed in an equal molar ratio in 0.01 M NaCl, heated to 65°C, and then allowed to cool to room temperature.

Plasmid constructions. (i) **Human H-ras cDNA plasmids.** Plasmids containing the complete protein coding and 3' noncoding sequences of the human normal (pPPS3) and T24 (pPPS22) H-ras genes were constructed (not shown) by splicing together previously described cDNA (6) and genomic (38) H-ras clones. The cDNA plasmid pRS6 contains the complete protein coding region of the transforming T24 H-ras gene (valine in place of glycine at amino acid position 12) but lacks part of the 3' noncoding sequence and has an apparent non-ras sequence upstream of the ATG initiation codon (6). The 3' region was completed by substituting a *Pst*I fragment of pRS6 with the corresponding fragment of pRS3 (6) which contains an intact 3' untranslated region and

polyadenylic acid sequence. The cDNA sequence in the resultant plasmid, pRC1, was excised at the *Pvu*II (between codons 22 and 23) and *Sal*I (end of the polyadenylic acid sequence) sites and inserted into the corresponding sites of pBR322 to yield pPS11. The 5' region was completed by cleaving pPS11 with *Pvu*II and inserting a 179-base-pair (bp) *Pvu*II fragment from the first exon of the genomic clone pT24, which carries the complete T24 variant H-ras gene on a 6.2-kilobase *Bam*HI fragment. This *Pvu*II fragment extends from codon 22 to 113 bp upstream of the ATG and encodes valine at position 12. The resultant plasmid pPPS22 is shown in Fig. 1. The corresponding plasmid encoding the normal H-ras cDNA, pPPS3 (Fig. 1), was obtained by inserting the 179-bp *Pvu*II fragment from the normal H-ras gene clone pP3 (38). This *Pvu*II fragment encodes glycine at position 12.

(ii) **pMG27N expression vector.** The 5.8-kilobase expression vector pOT₁ (5) was digested with *Eco*RI, filled in with *E. coli* DNA polymerase Klenow fragment, and then partially digested with *Bal*I (Fig. 1). The 5-kilobase *Eco*RI-*Bal*I fragment, isolated by polyacrylamide gel electrophoresis (PAGE) and electroelution, was incubated overnight with T4 DNA ligase to yield pMG27. Plasmid pMG27 was partially digested with *Nde*I, filled in, and ligated to yield pMG27N. Plasmid pMG27N has a single *Nde*I site encompassing an ATG initiation codon located 8 bp downstream of the C₁₁ ribosome binding site.

Bacterial growth, induction, and pulse-labeling. The pSKcHras, pSKT24, pSKT24₁, and pSKT24₂ expression plasmids were maintained in *ci*⁺ hosts. For expression the plasmids were transferred into *E. coli* K-12 N5151 or AR58 (J. Auerbach, unpublished data) carrying the c1857(Ts) mutation (24). The cells were grown at 32°C to an optical density at 650 nm of 0.4 (strain N5151) or 0.6 (strain AR58) and induced by rapidly increasing the temperature to 42°C. Pulse-labeling with [³⁵S]methionine was performed as previously described (24). At the indicated times after induction, 200-μl samples of the culture were pulse-labeled with 20 μCi of [³⁵S]methionine for 45 s, and the cell lysates were analyzed by sodium dodecyl sulfate (SDS)-PAGE (15). Large-scale fermentations (4 to 10 liters) were performed as previously described (24). Cell pellets were stored at -70°C.

Purification of ras proteins. Induced AR58 cells containing the normal, T24, or carboxyl-terminal truncated T24 proteins were resuspended in 7 volumes of buffer X (20 mM Tris-acetate [pH 7.5], 0.1 mM EDTA, 1 mM MgCl₂, 0.1 mM ATP, 1.0 mM 2-mercaptoethanol [2-ME]) at 0°C and lysozyme was added to a concentration of 0.2 mg/ml. After 30 min, phenylmethylsulfonyl fluoride (0.2 M in methanol) was added (final concentration, 1 mM), and the viscosity of the sample was reduced by sonication. Cell debris was

FIG. 1. Construction of vectors for the expression of the full-length normal and T24 mutant H-ras proteins. For the construction of pSKT24 a synthetic, double-stranded oligonucleotide encoding amino acid residues 6 to 10 of H-ras was ligated into pUC9 between the *Hind*III and *Nar*I sites. The resultant plasmid, pUC49, was cleaved with *Nar*I and ligated with a 900-bp *Nar*I fragment isolated from the T24 *ras* cDNA plasmid pPPS22 to give pUC59. To complete the reconstruction of the amino terminus, a second synthetic oligonucleotide was introduced into pUC59 at the *Hind*III site. The oligomer bearing the *Nde*I half site was first phosphorylated with T4 DNA kinase before annealing with its complementing strand. This singly phosphorylated, double-strand oligonucleotide was ligated with *Hind*III-cleaved pUC59. Digestion of the resultant plasmid, pUC69, with *Nde*I and *Sal*I gave an 800-bp fragment containing the complete amino acid and 3' untranslated sequences of the T24 *ras* allele. This fragment was inserted into pMG27N between the corresponding sites to yield pSKT24. The amino-terminal protein coding sequence and vector-insert boundary were verified by Maxam-Gilbert DNA sequencing. For the construction of pSKcHras the normal *ras* cDNA clone pPPS3 was digested with *Nar*I, and the 900-bp fragment encoding all but the initial 10 amino acids was isolated. Similarly, pUC69 was cleaved with *Nar*I, and the large vector fragment containing the initial 10 *ras* codons was isolated. Ligation of these two *Nar*I fragments yielded plasmid pUC69wt. The complete normal *ras* coding sequence was excised on an *Nde*I-*Sal*I fragment and ligated into the corresponding sites of pMG27N to yield the expression vector pSKcHras. The construction of the human *ras* cDNA plasmids pPPS3 and pPPS22 and the pMG27N expression vector is described in the text.

removed by centrifugation at $37,000 \times g$ for 30 min, and ammonium sulfate was added to the supernatant to 60% saturation. The ammonium sulfate pellet was collected by centrifugation, dissolved in buffer X, and applied to an Aca 34 column. Fractions containing *ras* protein, identified by protein blotting and GTP binding assays, were pooled and applied to a DEAE-Sephacel column equilibrated with buffer X containing 20 mM NaCl. The column was eluted with a linear 20 to 300 mM NaCl gradient in buffer X. The *ras* proteins eluted at 170 to 200 mM NaCl. With judicious pooling of the Aca and DEAE column fractions, the normal, T24, and C-terminal truncated *ras* proteins were obtained at about 90% homogeneity in about a 20% overall yield. The actual amount of the pure proteins recovered per gram of packed frozen cells was about 0.3 mg for the T24 and carboxyl-terminal truncated T24 proteins and about 0.15 for the normal product. Protein concentrations were measured with the Bio-Rad protein assay, using blue dye reagent and bovine serum albumin (BSA) as the standard (2). The molecular concentration of one highly purified sample (>95%) was determined by quantitative amino acid analysis, and the concentrations of the other preparations were then estimated from their relative purity as measured by SDS-PAGE. Amino acid sequencing of the normal and T24 proteins verified their predicted sequence through the initial 15 amino-terminal residues. For purification of the amino-terminal truncated H-*ras* protein, induced cells were lysed and centrifuged as above. Essentially all of this *ras* protein was found in the pellet. The pellet was successively extracted with buffer B (50 mM Tris-hydrochloride [pH 8], 2 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol) containing 0.05% deoxycholate, 1.5 M NaCl, and then 1.5% octylglucopyranoside. The T24₁ protein was then solubilized with 4 M urea in buffer B containing 0.01% Nonidet P-40. The protein remained soluble after dialysis against buffer B containing progressively lower urea and higher Tris-hydrochloride concentrations, ending with buffer B plus 0.2 M Tris-hydrochloride. This protein preparation which was about 60 to 70% pure by SDS-PAGE was used to prepare rabbit polyclonal antibody.

SDS-PAGE and immunoblot analysis. Cell extracts and partially purified *ras* proteins were incubated at 95°C for 5 min with gel loading buffer (60 mM Tris-hydrochloride [pH 8.7], 0.7 M 2-ME, 2% SDS, 10% glycerol) and analyzed by SDS-PAGE on a 15% gel. For dGTP binding assays on protein blots, samples of the purified proteins were not heated before loading on the gel. (rGTP and dGTP compete about equivalently for the GTP binding site of the *ras* protein [27; unpublished data]. The use of [α -³²P]dGTP in these initial assays was determined by availability.) For immunoblot analysis, the proteins were electrophoretically transferred to nitrocellulose in 25 mM Tris-192 mM glycine-20% methanol for 2 to 6 h at 200 mA with a Bio-Rad Transblot apparatus. The protein blot was rinsed twice in phosphate-buffered saline for 5 min each and then treated successively as follows: 3% BSA in phosphate-buffered saline for 1 h at 25°C; RIPA (20 mM Tris-hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% SDS) twice for 2 min each at 25°C; monoclonal antibody Y13-259 in RIPA for 45 min at 37°C; rabbit anti-rat IgG in RIPA for 30 min at 37°C; ¹²⁵I-protein A in RIPA for 20 min at 37°C; RIPA rinse five times for 5 min each at 37°C.

GDP-dGTP binding assays. (i) **Immunoprecipitation.** Assays were carried out at 4°C in 100 μ l of buffer A (20 mM Tris-hydrochloride [pH 7.4], 100 mM NaCl, 5 mM MgCl₂) containing 1% Triton X-100 and 10 μ M [³H]GDP (1 Ci/mmol)

or [α -³²P]dGTP (1 Ci/mmol) essentially as described by Furth et al. (11). The protein sample was added to the assay solution and incubated for 20 min. The primary antibody (rabbit anti-T24₁, prebled antiserum or rat monoclonal 259) was added for 30 min, followed by rabbit anti-rat IgG (in assays with the monoclonal antibody) for 30 min and then by protein A-Sepharose beads for 1 h with mixing. The beads were then washed extensively with buffer A, eluted by heating at 95°C for 5 min with gel loading buffer, and centrifuged. A portion of the eluted sample was counted in a scintillation counter.

(ii) **GTP binding on a protein blot.** After SDS-PAGE and electrotransfer as described above, the protein blots were incubated successively in the following solutions with gentle agitation: 3% BSA in phosphate-buffered saline containing 5 mM sodium PP_i for 1 h at 25°C, twice in buffer A for 15 min at 4°C, and finally in buffer A containing 5 mM [α -³²P]dGTP (2.7 Ci/mmol) and 1 mM sodium PP_i for 30 min at 4°C. Monoclonal antibody 259 was added for 30 min at 4°C, followed by the addition of rabbit anti-rat IgG for 20 min at 4°C. Then the blot was washed five times with buffer A containing 1 mM sodium PP_i at 4°C.

(iii) **Filter binding.** The normal and T24 protein samples were incubated with [α -³²P]GTP in buffer A containing 0.12 M ammonium sulfate at 25°C. Samples of these reactions were added to 2 ml of buffer A at 4°C containing 10 μ g of BSA per ml. The samples were filtered through nitrocellulose (pore size, 0.45 μ m) and washed with 20 ml of buffer A. The filters were counted in a scintillation counter at settings for Cerenkov radiation.

Antibody preparation. Polyclonal antibody directed against the purified amino-terminal truncated protein (T24₁) was prepared in New Zealand White rabbits. Rabbits were injected subcutaneously at 10-day intervals, and the antibody was titrated by an enzyme-linked immunosorbent assay procedure with goat anti-rabbit IgG conjugated to horseradish peroxidase.

RESULTS

Vector constructions for the expression of normal and mutant *ras* proteins. The constructions of the plasmid vectors for the expression of the authentic normal (pSKcHras) and T24 variant (pSKT24) human H-*ras* gene products are diagrammed in Fig. 1. The basic expression vector, pOT₁ (5), is a derivative of pAS1 (24) and uses transcriptional and translational control elements derived from phage lambda to regulate expression of inserted foreign genes. We further modified the pOT₁ vector by deleting three of its four *Nde*I restriction sites (Fig. 1). The resulting vector, pMG27N, retains a single *Nde*I site which overlaps the translation initiation codon (CATATG) provided on the vector. Plasmids containing the complete protein coding sequences of the human normal (pPPS3) and T24 (pPPS22) H-*ras* genes in a cDNA configuration were constructed by splicing together cDNA (6) and genomic H-*ras* (38) clones as described above (Fig. 1).

To introduce the complete T24 *ras* coding sequence into pMG27N at the unique *Nde*I site, we first reconstructed the N-terminus of the T24 *ras* cDNA with synthetic oligonucleotide linkers. Two overlapping linkers were used to replace the first 28 bp of the *ras* gene sequence and to position an *Nde*I site at the initiation codon (Fig. 1). The linker sequences also introduced nucleotide changes into the *ras* coding sequence; however, none of these changes altered the amino acid sequence encoded by the gene. The nucleotide changes created codons which were more fre-

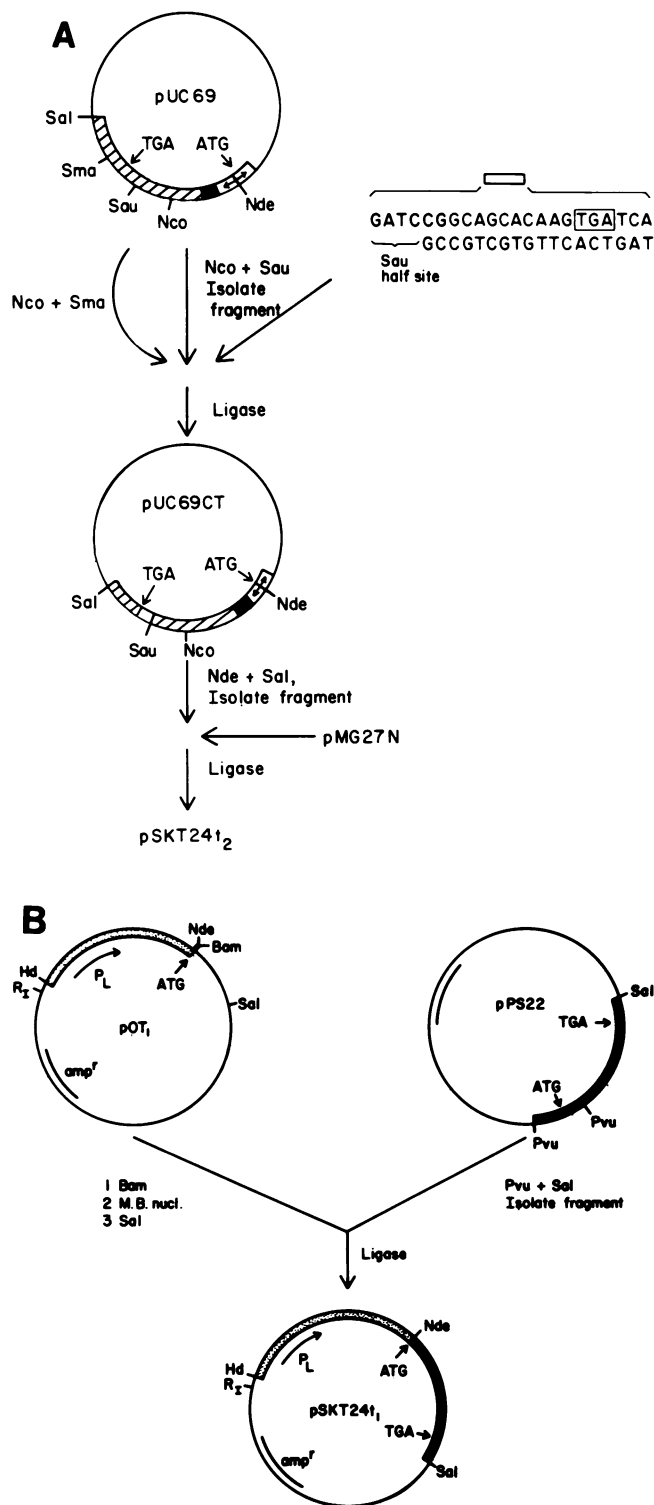


FIG. 2. Construction of vectors for the expression of truncated H-ras proteins. A. For the construction of pSKT24_{t1} plasmid pPS22 was cleaved with *PvuII* and *SalI*, and the 800-bp fragment lacking the initial 22 codons was isolated. The expression vector pOT₁ was digested with *BamHI* and subsequently treated with mung bean nuclease to create a flush end terminating with ATG. The vector was cleaved with *SalI* and ligated with the 800-bp *ras* fragment to yield the pSKT24_{t1} expression plasmid. The *ras* coding sequence is deleted in frame between codons 1 and 23. B. For the construction of pSKT24_{t2} the activated *ras* plasmid pUC69 was

quently used by *E. coli* and also introduced a unique *HindIII* restriction site at position 13 in the T24 *ras* gene sequence. The remainder of the *ras* coding sequence, extending from a unique *NarI* site, was obtained from the T24 cDNA plasmid pPPS22. The reconstructed *ras* coding sequence was excised on an *NdeI-SalI* DNA fragment and inserted into the corresponding *NdeI* and *SalI* sites on pMG27N. Direct DNA sequencing verified that the synthetic 5' terminus of the gene and the junctions of the insert on the final expression vector, pSKT24, were correct. The corresponding plasmid containing the wild-type H-ras allele, pSKcHras, was made by exchanging a DNA segment, which included the T24 mutation, with the same DNA segment obtained from plasmid pPPS3 containing the normal *ras* cDNA sequence (Fig. 1). The final *ras* expression vectors are thus identical, except for a sequence difference at position +34 (guanine versus thymine, glycine in wild type versus valine in T24 at codon 12) of the *ras* coding sequence. We also constructed vectors for the expression of an amino-terminal truncated and a carboxyl-terminal truncated derivative of the T24 *ras* gene. Plasmid pSKT24_{t1} encoding the amino-terminal deletion was constructed by inserting a *PvuII-SalI* fragment from pPPS22 directly into the pOT₁ expression vector between the *BamHI* and *SalI* sites (Fig. 2A). The *BamHI* site of the vector was first blunt ended with mung bean nuclease (24), thereby allowing direct fusion of the *PvuII* end of the fragment insert. This fusion places codon 23 of *ras* in frame with the ATG initiation codon provided on the vector and thus leads to expression of a variant *ras* protein deleted between amino acid residues 1 and 23 (T24_{g1}). Vector pSKT24_{t2} expressing the carboxyl-terminal deletion was constructed by first introducing a linker encoding a TGA termination signal after amino acid residue 166 in pUC69. This *ras* sequence, deleted of 23 C-terminal residues, was then transferred to the *NdeI-SalI* window of the pMG27N expression vector (Fig. 2B).

Expression of wild-type and variant *ras* proteins. The *ras* expression plasmids pSKcHras, pSKT24, pSKT24_{t1}, and pSKT24_{t2}, as well as the control vector pMG27N, were transfected into *E. coli* lysogens, which carry a defective prophage encoding a temperature-sensitive repressor (*cI857*). Cultures were grown at 32°C and induced at 42°C, and protein expression was initially examined by pulse-labeling with [³⁵S]methionine. Each of the *ras* vectors selectively induced the synthesis of a prominent labeled 20 to 22-kilodalton (kDa) protein, which was absent in cultures containing the control vector (Fig. 3, results for pSKT24_{t2} are not shown). The induced products were identified directly as *ras* proteins by an immunoblot (i.e., Western) analysis with a monoclonal antibody (11) developed against the viral H-ras protein. Total induced bacterial lysates were fractionated by SDS-PAGE and transferred electrophoretically to nitrocellulose paper. The Western analysis (Fig. 4) identified the same 20- to 22-kDa protein bands observed in the pulse-labeling experiments.

restricted with *NcoI* and *Sau3A*, and a 156-bp fragment was gel purified. A linker was synthesized to reconstitute exactly the DNA sequence from the *Sau3A* site (amino acid 161 to 162) to DNA position 501 (amino acid 166). Codon 167 was replaced with a termination signal (TGA). The vector was prepared by restricting pUC69 with *NcoI* and *SmaI*. The final construction of the carboxyl-terminal truncated T24 gene was achieved by ligation of the 156-bp *NcoI-Sau3A* fragment, the *Sau3A-TGA* linker, and the *NcoI-SmaI* vector fragment.

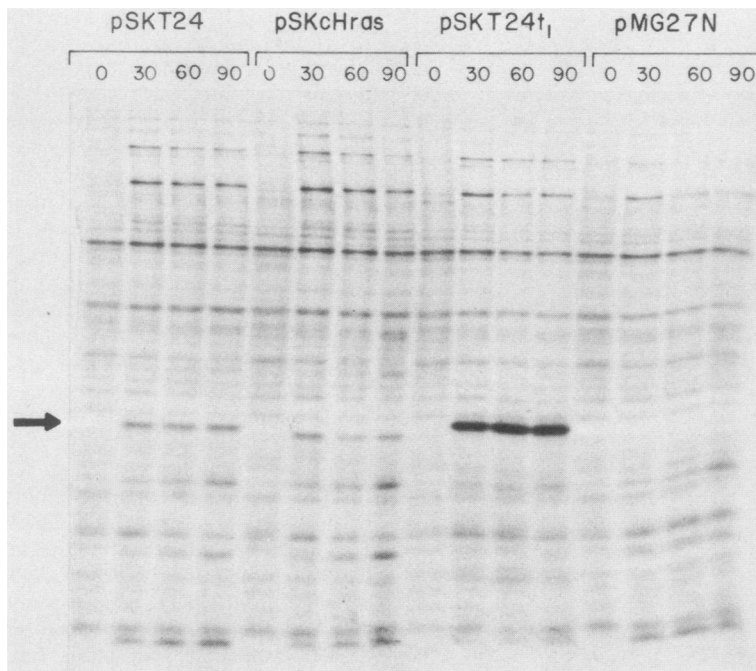


FIG. 3. [^{35}S]methionine pulse-labeling. Cultures of *E. coli* AR58 containing the designated plasmids were assayed at the indicated times after heat induction for the synthesis of *ras* proteins by pulse-labeling with [^{35}S]methionine (see the text). The zero-time sample was taken just before induction. Equivalent amounts of acid-precipitable radioactivity were loaded in each lane. The gel was dried and exposed in direct contact with Kodak XAR film at -20°C . The arrow marks the unique induced bands at about 22,000 kDa.

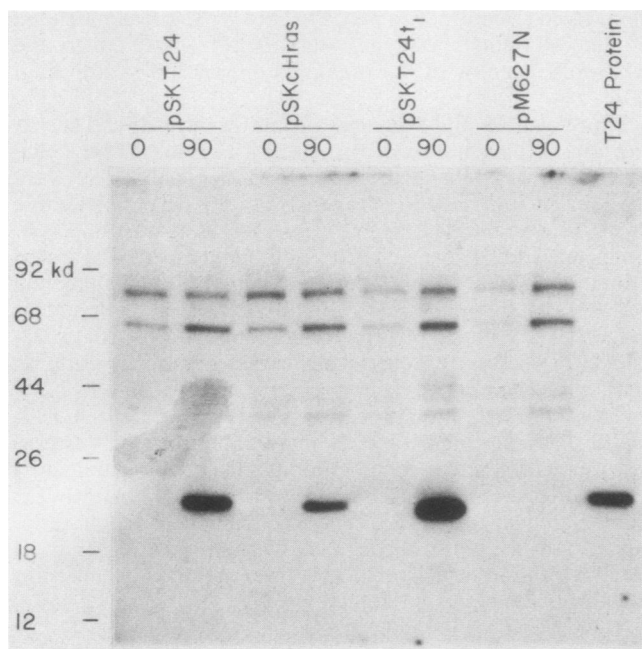


FIG. 4. Identification of *ras* proteins with anti-vH-*ras* monoclonal antibody. At the indicated times, the optical density at 650 nm of cultures of N5151 containing the designated plasmids was measured, and 1-ml samples were removed and centrifuged at $10,000 \times g$ for 1 min. The cell pellets were solubilized, and an equivalent of 0.006 unit (optical density at 650 nm) of cells was fractionated by SDS-PAGE and on a 15% gel and assayed for *ras* protein by an immunoblot procedure (see the text). The purified T24 protein (40 ng) was included as a reference. The processed blot was exposed to X-ray film for 8 days at -70°C with an intensifying screen. kd, kDa.

To examine the stability of the various *ras* gene products in *E. coli*, we carried out pulse-chase-labeling experiments. Induced cultures were pulse-labeled with [^{35}S]methionine for 1 min and then chased with excess unlabeled methionine in the presence of chloramphenicol. Samples were examined at various times after the chase. The results (data not shown) indicate that the four different *ras* proteins have similar stabilities in *E. coli* with a half-life of approximately 10 to 15 min. We examined several different *E. coli* K-12 strains for their ability to synthesize and stabilize the *ras* proteins, and all were found to exhibit similar levels of expression.

The efficient expression of all four *ras* gene constructs resulted in the accumulation of sufficient protein to enable the development of a simple and rapid protocol for *ras* purification (detailed above). Of note, upon lysis the wild-type, T24, and T24₂ *ras* proteins were found exclusively in the soluble portion of the cell extract. Curiously, the full-length *ras* proteins produced in other *E. coli* expression systems are insoluble and require the use of strong denaturants to obtain them in soluble form (14). Only our N-terminal truncated *ras* derivative, T24₁, was found to be insoluble upon initial cell lysis and required the use of denaturants for its solubilization. However, upon removal of the urea by dialysis, this truncated derivative remains soluble (see above). A gel profile of the purified normal, T24 and T24₁, proteins is shown in Fig. 5. The difference in the mobilities of the purified normal and T24 proteins in SDS-denaturing gels is consistent with previous comparisons of similar proteins produced in mammalian cells (11, 20, 30). Thus, this altered mobility is a consequence of primary sequence rather than differential modification in mammalian cells. Corresponding results were reported recently by Lacal et al. (14). We also note that the mobility of T24₁ is similar to that of T24, although the former is presumably deleted of 22 amino-terminal residues (Fig. 3). We have not directly deter-

mined the amino or carboxy terminus of the T24_{t1} protein, but based on our experience with other proteins expressed from pAS-1 or derivative vectors, we think it unlikely that the T24_{t1} differs from its predicted primary sequence. We thus presume that the aberrant migration of T24_{t1} results from conformational properties in SDS.

Guanine nucleotide binding of the wild-type and T24 ras proteins. One well-characterized biochemical property of the *ras* proteins is their ability to selectively bind certain guanine nucleotides (e.g., GTP, GDP, and dGTP). We have utilized three different procedures to assess the guanine binding properties of our bacterially produced *ras* proteins. First, we developed a simple qualitative ligand binding assay which can be applied either to complex protein mixtures such as total *E. coli* cell lysates or to purified preparations of the *ras* proteins. In addition, this assay allows analysis of both soluble and insoluble protein preparations. The protein(s) was resolved by SDS-PAGE, transferred to nitrocellulose, and then incubated with [α -³²P]dGTP and the *ras* antibody (see above for details). Both the wild-type and T24 proteins, in whole cell extracts or as purified proteins, bound dGTP (Fig. 6A and B).

The second assay uses an immunoprecipitation procedure (27) to examine *ras*-related guanine nucleotide binding. Soluble protein extracts were prepared from induced cultures producing the wild-type and T24 proteins. Portions of these extracts were incubated with [³H]GDP or [α -³²P]dGTP and subsequently immunoprecipitated with antibody and protein A-Sepharose (see above). The results (Table 1) indicate that both the normal and T24 proteins exhibit GDP-dGTP binding. This binding was competed selectively by GTP and not by ATP. In this assay only about 10 to 20% of our *ras* protein exhibited binding; however, this does not reflect saturation values and thus is only a minimum estimate of binding efficiency.

Our ability to purify milligram quantities of both the wild-type and T24 proteins allowed us to accurately measure GTP binding by a nitrocellulose-filter retention assay (see above). We compare the binding kinetics of these two proteins by this assay (Fig. 7). The results show that the T24 protein exhibits a two- to threefold-slower initial rate of binding than does the normal p21, although both proteins

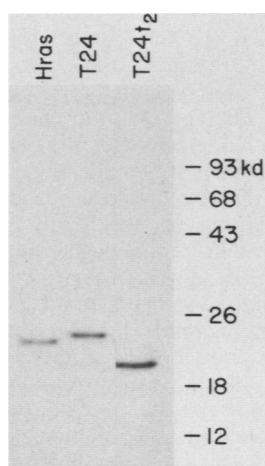


FIG. 5. SDS-PAGE profile of purified normal, T24, and carboxyl-terminal truncated (T24_{t2}) proteins. Each lane contained about 1.5 μ g of the indicated protein purified through the DEAE-Sepharose step (see the text). kd, kDa.

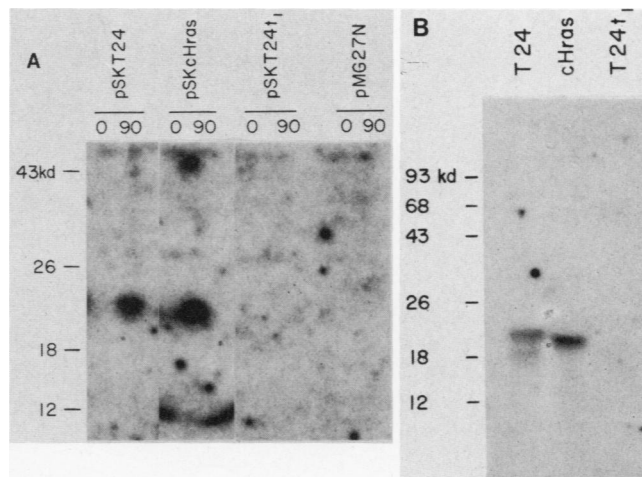


FIG. 6. dGTP binding to the normal and T24 polypeptides on protein blots. A. Samples of the solubilized cells described in the legend to Fig. 4 (0.06 unit [optical density at 650 nm] of cell culture) were electrophoresed on a 15% acrylamide gel, transferred to nitrocellulose, and assayed for [α -³²P]dGTP binding as detailed in the text. The film was exposed at -70°C with an intensifying screen for 7 days. B. About 0.4 μ g of the purified normal, T24, and T24_{t1} proteins were processed as described for panel A. The film was exposed for 3 days. kd, kDa.

attain equivalent equilibrium binding. This difference between the two proteins indicates that the substitution of valine at position 12 causes a subtle alteration of the GTP binding site. The amount of GTP bound was about 0.7 mol/mol of p21 (the protein concentration was determined by quantitative amino acid analysis). This value represents saturation binding since comparable results were obtained even at a 20-fold-lower GTP concentration (not shown). In

TABLE 1. Immunoprecipitable guanine nucleotide binding activity

Sample ^a	pmol of nucleotide ^b bound
Expt 1	
T24	
- 259	0.4
+ 259	7.9
+ 18-7 prebleed	0.2
+ 18-7 Ab	8.2
H-ras	
- 259	0.1
+ 259	17.8
BSA (20 μ g)	0.2
Expt 2	
T24	
- 259	0.5
+ 259	9.6
+ 259, 80 mM rGTP	3.6
+ 259, 100 mM rATP	9.5
BSA (20 μ g)	0.2

^a Lysates of AR58 cells containing the H-ras or T24 proteins were purified through the ammonium sulfate step, and then 2- μ l samples were assayed for immunoprecipitable guanine nucleotide binding as described in the text. The fractionated lysates (10 ml) were prepared from 10 g of the H-ras or from 4 g of the T24 cell pellets. 259, Monoclonal antibody Y13-259; 18-17 prebleed, preimmunization rabbit sera; 18-17, Ab, anti-T24_{t1}, rabbit antisera.

^b [³H]rGDP in experiment 1; [α -³²P]dGTP in experiment 2.

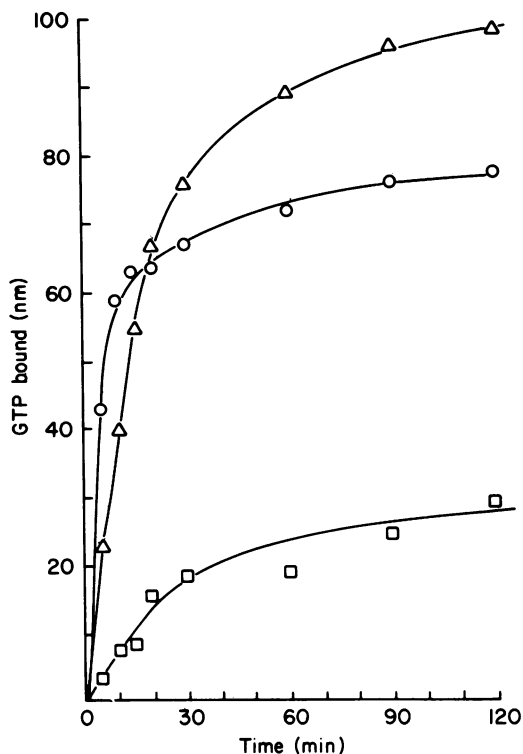


FIG. 7. GTP binding kinetics of purified *ras* proteins. The normal (100 nM, ○), T24 (120 nM, △), and C-terminal truncated T24 (250 nM, □) proteins were incubated with 10 μ M [α - 32 P]GTP (2 Ci/mmol) in buffer A-0.12 M ammonium sulfate at 25°C. Duplicate 20- μ l portions were removed at the indicated times and assayed for GTP bound to protein by filtration through nitrocellulose (see the text). The molar concentration of the *ras* proteins was estimated as described in the protein purification section of the text.

other assays we have determined that the efficiency of retention on nitrocellulose is only 70 to 80% (35). Thus, most (if not all) of our purified wild-type and T24 mutant *ras* proteins are capable of guanine nucleotide binding.

Guanine nucleotide binding of the *ras* truncates. Since the N-terminal truncated protein is expressed in an insoluble form, we assessed its GTP binding activity only by the protein blot-ligand binding assay. This assay allows us to compare directly the qualitative GTP binding of this N-terminal truncate with the full-length forms of *ras* because whole cell extracts are examined after identical treatment. The T24t₁ protein showed no binding activity under conditions in which both the full-length normal and T24 products did bind GTP (Fig. 6).

In contrast, the C-terminal truncated *ras* derivative, T24t₂, does retain its GTP binding activity. As measured by the filter binding assay (Fig. 7), the kinetics of binding are similar to those of the full-length T24 product; however, the overall extent of binding is significantly reduced. This non-stoichiometric binding was not altered by increasing the GTP concentration fivefold (not shown), suggesting that only a fraction of our purified protein preparation is active. Nonetheless, these results suggest that the carboxyl-terminal 23 amino acids of *ras* are not essential for its GTP binding ability.

DISCUSSION

We described the construction of *E. coli* expression vectors which encode the authentic wild-type human H-*ras*

protein, its T24 variant, and two different truncated forms of the T24 protein. One of the T24 truncated proteins, T24t₁, was deleted between residues 1 and 23 at the amino terminus, whereas the other truncated protein, T24t₂, was deleted for 23 C-terminal residues. To precisely introduce the wild-type and T24 cDNA sequences into the expression vector, the 5' end of the *ras* gene was reconstructed with synthetic linkers. In doing so, we inserted a unique *Hind*III restriction site at codon positions 4 and 5 in the human gene without altering the amino acid coding sequence. Creation of this restriction site provides a new entry site into the N-terminal sequence of the human gene which has been useful for making additional gene alterations. In addition, we made several codon changes within this region of the gene to introduce codons more frequently used in *E. coli*. The four different *ras* gene constructs were shown to express efficiently in *E. coli*, and the *ras* products accumulated to levels ranging from 1% (cH-*ras*) to 5% (T24t₁) of total cellular protein. Pulse-chase analyses indicated approximately equivalent half-lives for these proteins, although these assays would not readily detect a 1.5- to 2-fold difference. Thus, the efficiency of transcription or translation, or both, of these plasmids probably differs slightly. The induced protein products were shown to be *ras* related by immunoblot analysis with a monoclonal antibody raised against the authentic mammal-derived p21 protein (Fig. 4).

The wild-type, T24, and T24t₂ (C-terminal truncated) proteins were produced in soluble form in *E. coli* and were purified to homogeneity without the use of chaotropic agents. Only the T24t₁ (N-terminal truncated) protein was found to be insoluble in crude cell lysates and required treatment with urea for its initial solubilization. This report constitutes the first description of an extensive purification of nonfused *ras* proteins from bacteria without denaturation of the protein. We point this out to emphasize our concern that the biochemical, physical, and biological characteristics of proteins which are fused to other proteins, denatured during their isolation, or both may not accurately reflect the properties of the native protein. As evidenced above, this does not appear to be the case with the proteins described here.

We used our purified *ras* protein preparations to characterize certain biochemical parameters, as well as biological activities, of these *E. coli*-synthesized molecules. In this report, we described the guanine nucleotide binding capabilities of these proteins. We demonstrated (by several different assay systems) that the full-length *ras* wild-type and T24 variant proteins appropriately bind certain guanine nucleotides and that the stoichiometry of nucleotide binding was essentially equimolar. This quantitative assessment of nucleotide binding was made possible only because of our ability to obtain these proteins in a sufficient quantity and in pure enough form to assay by standard nitrocellulose filter binding techniques. Moreover, we found that the C-terminal truncated *ras* protein retained its ability to bind GTP, although the extent of binding was reduced. Apparently, the C-terminal 23 amino acid residues are not a crucial part of the nucleotide binding site. This C-terminal truncate, however, has lost its transforming ability (unpublished data). Willumsen et al. (42) have shown that mutations in the C-terminal portion of *ras* dramatically reduce its transforming ability and result in the inability of *ras* to localize properly to the inner cell membrane. Our results are consistent with these conclusions and further suggest that C-terminal altered molecules will retain their nucleotide binding capabilities. In contrast to the results obtained with the C-terminal truncate, we found that the N-terminal truncate

was completely deficient in nucleotide binding. Since this protein was insoluble upon initial lysis of the cells, our conclusion is based only on comparing its guanine binding ability to that of the other three *ras* proteins by the qualitative Transblot ligand binding assay (Fig. 6). The inability of this derivative to bind nucleotide is consistent with protein modeling studies (22, 40) which implicate the region around amino acid 12 as part of the nucleotide binding site.

It recently was reported (35) that the normal *ras* protein, synthesized and purified as described in this report, has an intrinsic GTPase activity which yields GDP and P_i. In contrast, the T24 transforming derivative was markedly deficient in this activity. Presumably, this defect is responsible for its transforming properties. Preliminary data suggest that the C-terminal truncate, described here, exhibits GTPase properties similar to those found for the full-length T24 derivative. This result further suggests that the carboxyl end of *ras* does not play a critical role in either GTP binding or GTP catalysis.

It also has been demonstrated that *E. coli*-produced and -purified *ras* proteins exhibit authentic biological activities when they are introduced into mammalian cells (8). The T24 oncogenic form of *ras* caused rapid morphological changes and induced cell proliferation when microinjected into quiescent, confluent NRK, REF52, and NIH 3T3 rodent cells. In contrast, the wild-type protein exhibited little, if any, effect on these cells. We conclude that the *ras* proteins synthesized in *E. coli* are appropriate substrates for studying both the biochemical and biological activities of these molecules. We have thus undertaken the purification of sufficient pure protein for crystallographic analysis of both the wild-type and T24 variant proteins.

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