culture medium was 88 m*M* NaCl, 1.0 m*M* KCl, 2.4 m*M* $NaHCO_3$, 10 m*M* Hepes, 0.82 m*M* $MgSO_4$, 0.33 m*M* $Ca(NO_3)_2$, 0.91 m*M* $CaCl_2$, penicillin (0.01 mg/ml), and streptomycin (0.01 mg/ml); the *p*H was 7.3 to 7.4.

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18. The measured value of the reversal potential would be in error by the amount of the liquid junction potential arising at the tip of the voltage-sensing electrode as it penetrated an oocyte. The value of the liquid junction potential is likely to be at least several millivolts, because a potential of up to about −10 mV (typically −2 to −3 mV) could be observed on withdrawing the pipette, and this potential often decayed toward zero over several minutes as the electrode sat in the bathing fluid. It is unclear if the rectification in the positive limb of the current-voltage curve in Mg^{2+} (Fig. 1B) represents an actual difference from the properties of neuronal NMDA receptors, because a similar phenomenon has been observed in neurons (M. L. Mayer, personal communication).
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Autoreactive Epitope Defined as the Anticodon Region of Alanine Transfer RNA

CHRISTOPHER C. BUNN* AND MICHAEL B. MATHEWS

Autoantibodies to aminoacyl–transfer RNA (tRNA) synthetases are common in the human autoimmune diseases polymyositis and dermatomyositis. Sera of the PL-12 specificity contain separate antibodies reacting with alanyl-tRNA synthetase and alanine tRNA ($tRNA^{Ala}$). The antibodies to tRNA recognize at least six distinguishable human $tRNA^{Ala}$ species grouped into two sequence families. The antibody-reactive determinants on the tRNA were identified through ribonuclease protection and oligonucleotide binding experiments. The antibody binding site is a seven- to nine-nucleotide sequence containing the anticodon loop and requires an intact anticodon. No requirement for anticodon stem structure or sequence is observed, although the 5′ portion of the stem is protected from nuclease attack. Antibodies from several patients appear to share the same specificitym, indicating that the antibodies are induced by a unique sequence feature in the immunogen.

NATIVE NUCLEIC ACIDS DO NOT stimulate significant antibody production when injected into experimental animals, but such antibodies can be generated against nucleic acid in association with protein (*1*) and are common in human systemic lupus erythematosus (SLE) and its murine equivalents. In these diseases, where the nature of the immunogen is unknown, characterization of the reactive epitopes may provide clues to the identity of potential etiologic agents. Antibodies to DNA are particularly prevalent in SLE and have been extensively investigated. They are heterogeneous and can recognize numerous features, such as heterocyclic bases, nucleosides, nucleotides, simple repeating sequences, and the configuration of the sugar-phosphate backbone (*2, 3*). Similarly, RNA molecules, including ribosomal, transfer, and U1 species, are the target of the autoimmune response in up to 30% of patients with autoimmune disorders (*4–9*). Here both sequential and conformational antigenic determinants are thought to occur, but they are less well defined than those in DNA. Three antibody systems that involve transfer RNA (tRNA) occur in patients with myositis, an inflammatory muscle disease. In two such systems, known as Jo-1 and PL-7, the antigenic components are associated protein molecules, the aminoacyl-tRNA synthetases specific for histidine (Jo-1) and threonine (PL-7) (*7*). In the third system, PL-12, antibody recognizing alanyl-tRNA synthetase coexists with another antibody that directly recognizes alanine tRNA ($tRNA^{Ala}$) (*8*). This unusual interaction of an autoantibody with a specific set of tRNA species has allowed us to identify the antibody binding site (epitope) of an autoantigen.

The RNA antigen precipitated by the PL-12 antibody is predominantly, if not exclusively, $tRNA^{Ala}$, and other sera containing antibodies to DNA, RNA, or ribonucleo proteins (RNPs) do not precipitate these molecules (*6, 8, 9*). We fractionated the tRNA in the precipitate and determined the primary nucleotide sequences of two related species and the anticodon stem and loop sequence of a third species (*9*). The antibody recognizes two families of human $tRNA^{Ala}$, named "slow" and "fast" for their relative electrophoretic mobility in denaturing polyacrylamide gels. They contain the same anticodon loop sequence but differ in the anticodon stem region and in other parts of the molecule. To determine whether the binding of antibody could confer resistance to ribonuclease digestion, we formed immune complexes between uniformly radiolabeled tRNA and the PL-12 antibody from several patients. The complexes were isolated on protein A–Sepharose and digested with ribonuclease T_1, ribonuclease A, or a mixture of both. After the matrix had been washed to remove released digestion products, the residual bound RNA was eluted. In all cases, gel electrophoresis revealed the presence of protected fragments ~20 nucleotides in length.

Using antibody from one patient and $tRNA_1^{Ala}$, a purified slow RNA, we isolated the fragments protected from digestion by either ribonuclease T_1 or A. Further digestion to completion with these enzymes followed by a two-dimensional electrophoretic separation of the resultant oligonucleotides (*10*) yielded simple "fingerprint" patterns on autoradiography. The oligonucleotides were isolated and characterized by redigestion, and their locations in the molecule are shown in Fig. 1A, lines a and b. The protected region includes the anticodon stem and loop. The same result was obtained with $tRNA_2^{Ala}$, another member of the slow family, and with $tRNA_3^{Ala}$, which belongs to the fast family and contains two pairs of base changes in the anticodon stem. This implies that the stem sequence is not critical for antigenicity but leaves open the possibility that a duplexed stem structure is required.

In the naked state, the anticodon loop is exposed and susceptible to nuclease attack, so its presence in the protected fragment is particularly striking. To confirm that the antibody blocks digestion in this region and to obtain more detailed definition of the binding site, we performed "footprint" experiments to reveal nuclease-sensitive sites that are shielded by antibody. Immunoprecipitated $tRNA^{Ala}$ was labeled at its 3′ end, and individual species were isolated by gel electrophoresis (*9*). The $tRNA_2^{Ala}$ species was subjected to mild ribonuclease digestion in the presence or absence of PL-12 antibody and analyzed by gel electrophoresis (Fig. 2). The antibody blocked digestion of bonds in the anticodon loop by the single-strand–specific ribonucleases T_1, *Bacillus cereus*, and T_2 (which are specific for G residues, for pyrimidines, and are not base-specific, respectively). Similarly, digestion by the double-strand–specific ribonuclease V_1 of bonds in the stem on the 5′ side of the

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724.

*Present address: Royal Postgraduate Medical School, Hammersmith Hospital, DuCane Road, London W12 OHS, United Kingdom.

anticodon was also blocked (Fig. 1A, line c). Digestion on the opposite side of the stem was weaker and not blocked by antibody. It seems likely, therefore, that protection of the stem against attack by single-strand–specific ribonucleases is partly due to its duplex nature rather than to antibody binding. Sensitive sites in other regions of the molecule were generally unaffected, and control antibody did not protect the anticodon region.

These data demonstrate that the anticodon arm, including the loop and possibly part of the stem, interacts with PL-12 antibody in such a way as to hinder access to nucleases. The data do not say how much sequence is needed for antibody binding, nor whether higher order structure is required. To address these issues we first isolated the uniformly labeled ~20-nucleotide protected fragments from ribonuclease T_1–digested immune complexes by deproteinization and gel electrophoresis, mixed them with intact tRNA (as internal control), and tested their immunoprecipitation with PL-12 antibody. The isolated protected fragments efficiently (>75%) bound to antibody (Fig. 3A), showing that the determinants are entirely located in the anticodon stem-loop structure. To see if antigenicity requires the integrity of this region, we took advantage of its high susceptibility to nuclease attack under mild conditions. The $tRNA_1^{Ala}$ species was partially digested with ribonuclease T_1, and the mixture of oligonucleotides, supplemented with intact RNA, was reacted with PL-12 antibody. The intact molecules were immunoprecipitated, while the submolecular fragments, including the 5′ and 3′ terminal halves, remained in the supernatant (Fig. 3B). The same result was obtained regardless of whether the RNA had been deliberately denatured to separate the base-paired half molecules. We conclude that cleavage in the anticodon loop, and specifically at the I or G residue of the anticodon IGC, destroys antibody recognition of the RNA. This result implies that the anticodon itself is an important feature of the antigenic determinant and also that the determinant is not merely one of the minor bases in the molecule.

To determine the minimal sequence needed for antibody binding, we digested $tRNA^{Ala}$ to completion with either ribonuclease T_1 or A and tested the ability of the resulting mixture to interact with antibody. No detectable binding was observed, suggesting that the anticodon itself is not sufficient (IGC is released by ribonuclease A). To obtain longer fragments that might bind antibody, we formed immune complexes with uniformly labeled RNA and subjected them to ribonuclease T_1 digestion under more severe conditions. The ~20-nucleotide fragment was broken down into a series of shorter antibody-bound oligonucleotides. The shortest of these, 11 nucleotides, was isolated by gel electrophoresis. It bound to antibody and was analyzed by fingerprinting and further digestion with ribonucleases T_1 and A. It proved to have lost all of the 5′ side of the anticodon stem except for one base (Fig. 1A, line d). We conclude that the antigenic determinant is the anticodon loop, that the anticodon stem structure is not essential, and that if 3′ stem nucleotides are also involved, this region is short and not rigidly constrained in sequence.

For a more precise definition of the sequences required for binding, end-labeled tRNA was nicked with alkali to generate a family of submolecular fragments, which were then reacted with PL-12 antibody. With molecules labeled at the 5′ end, immunoprecipitability declined sharply near the junction between the anticodon loop and the stem on its 3′ side (Fig. 4A). The shortest antibody-bound fragment extended from the 3′ side of the anticodon loop to the 5′ end of the tRNA, containing the entire anticodon loop and only two bases from the 3′ side of the anticodon stem (Fig. 1A, line e). Similarly, with 3′ end–labeled tRNA, precipitability declined abruptly at the junction between the anticodon loop and the stem on its 5′ side, the shortest antibody-

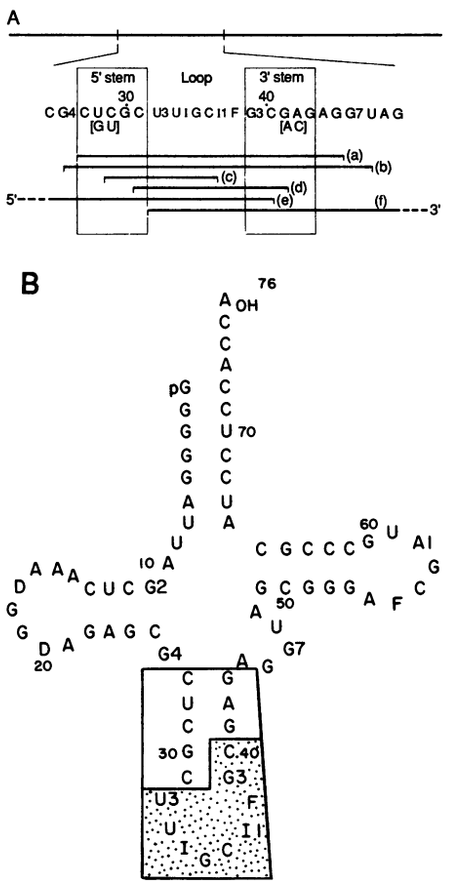
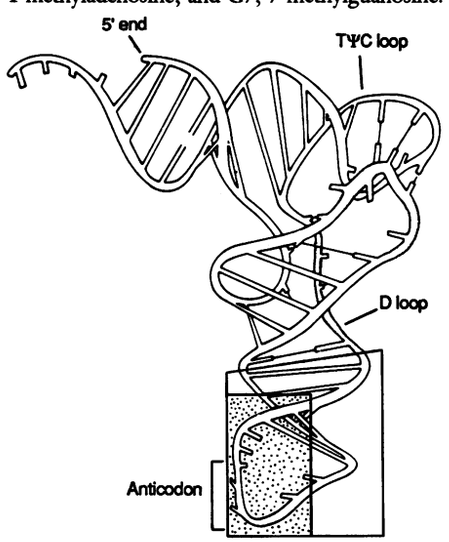


Fig. 1. Location of antibody binding site on $tRNA^{Ala}$ molecule. (**A**) Linear representation of $tRNA_1^{Ala}$ sequence, with $tRNA_3^{Ala}$ sequence in square brackets (*9*). The bars indicate (a and b) fragments protected from ribonuclease T_1 and A; (c) region protected from V_1, T_1, T_2 and *B. cereus* nucleases (Fig. 2); (d) shortest ribonuclease T_1–generated fragment that bound to antibody; (e and f) shortest 5′ end–labeled and 3′ end–labeled fragments that bound to antibody (Fig. 4). (**B**) Binding site (stippled box) and protected region (entire box) superimposed on the $tRNA_1^{Ala}$ cloverleaf structure and the $tRNA^{Phe}$ tertiary structure model (*17*). Modified nucleosides: U3, 2′-*O*-methyluridine; I, inosine; II, 1-methylinosine; F, pseudouridine; G3, 2′-*O*-methylguanosine; A1, 1-methyladenosine; and G7, 7-methylguanosine.



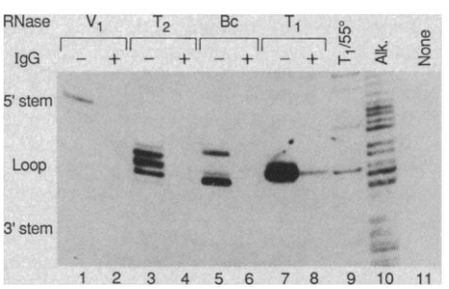


Fig. 2. Protection of nucleotides in the anticodon arm from nuclease digestion. The $tRNA_2^{Ala}$ labeled at its 3′ end with [^{32}P]pCp and T4 RNA ligase was purified and digested either in the presence or absence of 2 μg of PL-12 immunoglobulin G with (lanes 1 and 2) nuclease V_1, 0.001 U; (lanes 3 and 4) T_2, 0.5 U; (lanes 5 and 6) *B. cereus* (Bc), 0.1 U; or (lanes 7 and 8) T_1, 0.001 U, in the presence of 3 μg of high molecular weight RNA carrier at 37°C for 15 minutes. (Lane 9) A partial T_1 digest was produced by incubation at 55°C for 12 minutes and (lane 10) partial hydrolysis products were generated by incubating [^{32}P]RNA with 1 μg of carrier RNA at 90°C for 6 minutes in 0.5*M* carbonate buffer, *p*H 9.0. Lane 11 contains intact tRNA. Oligonucleotides were resolved in a 10% polyacrylamide-urea gel, of which only the portion corresponding to the region of the anticodon arm is shown.

bound fragment extending from the first base of the anticodon loop to the 3′ end of the molecule (Fig. 4B and Fig. 1A, line f). In each case, fragments one nucleotide shorter might also react with antibody, but the resistance of the U3-U and G3-C bonds to alkali precludes further resolution in this experiment.

Taken together, our data define the binding site as the anticodon loop, with one or two additional bases on its 3′ side **(U3)-U-I-G-C-I1-F-G3-(C)**, where the anticodon loop is shown in bold and the anticodon is underlined (Fig. 1). Nucleotides that have uncertain significance for antibody binding are in parentheses. This epitope differs from those described previously for nucleic acids in several ways. First, it consists of a short nucleotide sequence. A stable conformation is probably not required, in contrast to the tRNA structures recognized by other human (*5*) and murine (*11*) antibodies. Furthermore, unlike the minor-base–specific antibodies generated by immunization with a complex between $tRNA^{Phe}$ and bovine γ-globulin (*12*), the entire seven- to nine-nucleotide sequence seems to be required because antigenicity is destroyed by cleavage at either end or internally in the anticodon. The minor bases may contribute to antibody recognition but they are not unique to $tRNA^{Ala}$ and are not immunodominant. Finally, the size of the epitope is rather larger than the four to six nucleotides deduced as the minimum size for antibodies to DNA on the basis of competition and protection experiments (*3*).

On the other hand, the existence of a wide range of sequence-specific antibodies directed against proteins makes the discovery of a sequence-specific antibody directed against a nucleic acid less surprising. Indeed, comparison of the epitope recognized by the antibody to tRNA with the characteristics of epitopes recognized on protein immunogens reveals a marked correspondence. The most frequently recognized peptide sites generally exhibit high mobility, convex surface shape, and negative electrostatic potential (*13*). All of these are also features of the anticodon loop, which forms a protrusion in the presumptive three-dimensional structure of the tRNA molecule (Fig. 1B), is not rigidly fixed in space (*14*), and is obviously negatively charged.

Failure to maintain tolerance in autoimmune disease may be due to intrinsic regulatory defects in the immune system or to the action of extrinsic factors such as infectious agents. The similarity between the $tRNA^{Ala}$ epitope and protein immunogenic sites lends support to the idea that these autoantibodies are generated as an antigen-driven response to an extrinsic immunogen, although we do not yet know what the immunogen is. There are also implications for the anti-idiotype hypothesis, a special case of the antigen-driven response that explains autoantibodies as secondary antibodies generated against primary antigen-directed antibodies (*15*). As noted previously (*8*), the coexistence in PL-12 sera of antibodies to $tRNA^{Ala}$ and to alanyl-tRNA synthetase could be accounted for by such a model. On this basis, we could expect the synthetase, like the antibodies to tRNA, to react with the anticodon loop. This is a testable prediction and one for which precedents are known, although synthetases more commonly contact their cognate tRNAs elsewhere (*16*).

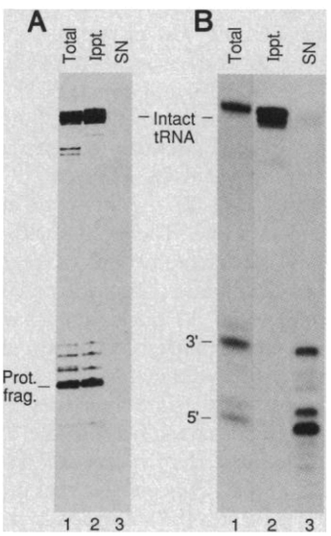


Fig. 3. Binding of $tRNA^{Ala}$ fragments to antibody. (**A**) Binding of protected fragment. A mixture of intact, uniformly labeled $tRNA^{Ala}$ with the protected RNA fragments remaining after ribonuclease T_1 digestion of antibody-bound [^{32}P]$tRNA^{Ala}$ was precipitated with PL-12 antibody. Equivalent portions of (lane 1) the starting mixture, (lane 2) immunoprecipitated RNA, and (lane 3) supernatant RNA were resolved in a 15% polyacrylamide-urea gel. The protected fragment (Prot. frag.) and intact tRNA are indicated. (**B**) Lack of binding of partial tRNA digest. A mixture of intact, uniformly labeled $tRNA^{Ala}$ with the products of its partial ribonuclease T_1 digestion (1:150 enzyme to RNA incubated at 15°C for 30 minutes) was immunoprecipitated with PL-12 antibody. (Lane 1) The starting mixture, (lane 2) immunoprecipitated RNA (Ippt.), and (lane 3) supernatant RNA (SN) were resolved in a 10% polyacrylamide-urea gel. The 5′ and 3′ half molecules resulting from cleavage in the anticodon are indicated. Lanes 2 and 3 were loaded with equivalent amounts of RNA and lane 1 contained one-tenth of this amount but was exposed longer to film.

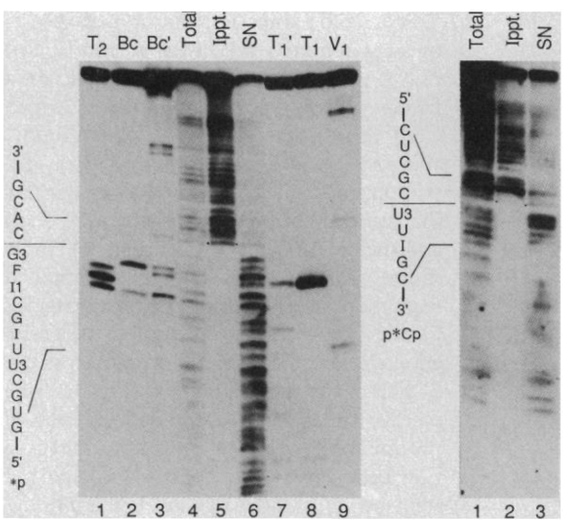


Fig. 4. Binding of fragmented $tRNA^{Ala}$ to antibody. (**A**) $tRNA_3^{Ala}$ was labeled at its 5′ end with ^{32}P by using [^{32}P-γ]ATP (adenosine triphosphate) and polynucleotide kinase, digested partially with alkali to generate a "ladder," and reacted with PL-12 antibody. The immunoprecipitated RNA (Ippt.), nonprecipitated RNA remaining in the supernatant (SN), and a sample of the alkali digested RNA (Total) were resolved in a 10% polyacrylamide-urea gel (lanes 5, 6, and 4 respectively). Markers comprised $tRNA_3^{Ala}$ digested with (lane 1) ribonuclease T_2 at 37°; (lanes 2 and 3) *B. cereus* at 37°C and 55°, respectively; (lanes 7 and 8) ribonuclease T_1 at 55° and 37°C, respectively; and (lane 9) nuclease V_1 at 37°C. (**B**) Similar analysis with $tRNA_2^{Ala}$ labeled at its 3′ end by using [^{32}P]pCp and RNA ligase.

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Microtubule Gelation-Contraction: Essential Components and Relation to Slow Axonal Transport

RICHARD C. WEISENBERG, JAMES FLYNN, BAOCHONG GAO, SHUAIBU AWODI, FRANK SKEE, STEVEN R. GOODMAN, BEAT M. RIEDERER

Preparations of microtubule proteins isolated by assembly and disassembly undergo gelation-contraction after addition of adenosine triphosphate (ATP). A particulate fraction from these preparations that is required, along with purified tubulin, to produce ATP-dependent microtubule gelation-contraction in vitro has been isolated. The particulates exhibited microtubule-stimulated adenosine triphosphatase activity and moved slowly (about 1 micrometer per minute) along microtubule walls in the presence of ATP. The particulates contained tubulin, neurofilament, and spectrin polypeptides. The composition, solubility, and motility of the particulates are consistent with those of slow component a of axonal transport.

CRUDE MICROTUBULE PROTEINS isolated from calf brains by cycles of assembly and disassembly will undergo gelation-contraction in the presence of adenosine triphosphate (ATP) (*1*). Microtubule gelation-contraction is slow, becoming visible about 30 minutes after ATP addition, and requires an hour or more to reach completion. Microtubule gelation-contraction involves the movement, at about 1 μm per minute, of particulate material in association with microtubules (*2*).

A particulate fraction has now been isolated that in combination with purified tubulin will undergo gelation-contraction in the presence of ATP. The composition, solubility, and rate of movement of the isolated particulates are similar to the composition, solubility, and rate of movement of the slowest component of axonal transport, termed slow component a or SCa (*3*). These similarities suggest a relation between the two processes. In the following discussion we will adopt as a provisional name the term slow component a particulates, or SCAPs, for the particulates required for microtubule gelation-contraction.

For these experiments crude microtubule proteins were purified by three cycles of assembly and disassembly as previously described (*1, 2*). Unless otherwise indicated, all experiments were done in a standard buffer of 0.1*M* MES (*4*) and 1 m*M* DTT at *p*H 6.6 (MES-DTT buffer) or in a microtubule assembly buffer of MES-DTT plus 1 m*M* EGTA, 0.5 m*M* $MgCl_2$, and 1 m*M* GTP. Crude microtubule proteins, isolated by cycles of assembly and disassembly, have been shown to contain a particulate component (*2, 4*). We isolated the particulate fraction by centrifugation in the presence of high (1 to 2*M* NaCl) salt concentrations (Fig. 1). In the presence of high salt concentrations tubulin ring aggregates (which could contaminate these preparations) are broken down. Particulates stable in high salt concentrations could also be isolated directly from whole brain homogenates by differential centrifugation, without the need to perform cycles of assembly and disassembly (Fig. 1). The particulates isolated directly were able to induce microtubule gelation-contraction, but these particulates had a more complex protein composition than particulates that copurified with microtubules and were not used further in the experiments reported here.

Neither the isolated particulates (SCAPs) nor purified tubulin will by themselves undergo gelation-contraction in the presence of ATP. However, when SCAPs and tubulin are mixed together in the proper ratio, gelation-contraction occurs and is indistinguishable from the process that occurs with crude third-cycle microtubule proteins (Fig. 1). Concentrations of about 1 mg of particulates per milliliter and 3 mg of tubulin per milliliter are optimal for the occurrence of gelation-contraction. Gelation-contraction will occur if SCAPs are either added to tubulin prior to inducing microtubule assembly or if microtubules are first assembled and the SCAPs added at the same time as ATP.

A reproducible pattern of polypeptides was observed after one- or two-dimensional gel electrophoresis of active SCAP preparations isolated from cycled microtubule proteins in high salt concentrations (Fig. 2). The presence of 1% Triton X-100 (a detergent commonly used to extract cellular membranes) in the isolation medium had no significant effect on the composition of the isolated particulates or on their ability to induce gelation-contraction (Fig. 3).

The major component of the particulates migrates as a doublet of molecular sizes 55 and 53 kD and is identifiable as tubulin.

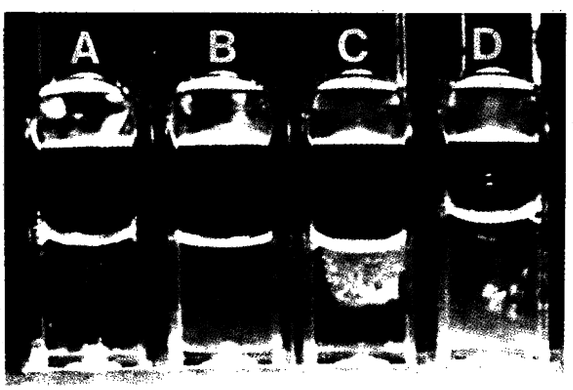

Fig. 1. Adenosine triphosphate–dependent gelation-contraction of mixtures of microtubules and SCAPs. Microtubules were assembled from phosphocellulose-purified tubulin, and SCAPs were prepared from high-salt centrifugation of crude microtubule proteins. Sample A contained microtubules only, sample B contained SCAPs only, and samples C and D are one-to-one mixtures of microtubules (A) and SCAPs (B). Samples A, B, and C contained 2 m*M* ATP and sample D contained standard assembly buffer only. The photograph was taken about 1 hour after ATP addition. Contraction of the microtubule gel occurred only in the complete mixture of tubulin, SCAPs, and ATP (*26*).

R. C. Weisenberg, J. Flynn, B. Gao, S. Awodi, F. Skee, Department of Biology, Temple University, Philadelphia, PA 19122.
S. R. Goodman and B. M. Riederer, Cell and Molecular Biology Center, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, PA 17033.