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12 May 1989; accepted 8 August 1989

Bacterial Blight of Soybean: Regulation of a Pathogen Gene Determining Host Cultivar Specificity

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Soybean cultivars resistant to *Pseudomonas syringae* pathovar *glycinea* (*Psg*), the causal agent of bacterial blight, exhibit a hypersensitive (necrosis) reaction (HR) to infection. *Psg* strains carrying the *avrB* gene elicit the HR in soybean cultivars carrying the resistance gene *Rpg1*. *Psg* expressing *avrB* at a high level and capable of eliciting the HR in the absence of de novo bacterial RNA synthesis have been obtained in in vitro culture. Nutritional signals and regions within the *Psg hrp* gene cluster, an approximately 20-kilobase genomic region also necessary for pathogenicity, control *avrB* transcription.

GENETIC STUDIES OF PLANT HOSTS and their parasites suggest that host resistance to disease requires specific pathogen recognition. Many interactions between particular host cultivars and particular natural variants ("races") of a pathogen culminate in disease resistance only when a specific avirulence gene in the pathogen and a resistance gene in the host are present (*1*). Interactions of this type are designated gene-for-gene host-parasite relationships.

Infection of soybean, *Glycine max* (L.) Merr., by *Pseudomonas syringae* pv. *glycinea* (*Psg*), the causal agent of bacterial blight, provides a model for investigating the molecular basis of a gene-for-gene interaction (*2*). *Psg* multiplies in the leaf intercellular fluid of susceptible soybean cultivars, where it induces the appearance of spreading, water-soaked, chlorotic lesions. In resistant soybean cultivars, the phenotype of disease resistance is typified by the hypersensitive reaction (HR): leaf mesophyll cells near the invading bacteria rapidly collapse to produce a dessicated necrotic lesion. In this reaction, bacterial multiplication is inhibited and the pathogen is localized at the site of infection (*3*). Soybean cultivars carrying the dominant resistance gene *Rpg1* produce hypersensitive lesions when infected by *Psg* races carrying the avirulence gene *avrB* (*2, 4*). Thus, the interaction between *avrB* and *Rpg1* represents a gene-for-gene relation. Here we address the relation between *avrB* and other *Psg* genes required for the bacterium to elicit the HR.

Psg race 0 (*Psg*0), which contains *avrB*, is avirulent (that is, HR-inducing) on the *Rpg1*-containing soybean cultivar (cv.) Harosoy and virulent on cv. Centennial (*2, 5*). The dark brown lesions characteristic of the *avrB-Rpg1* interaction begin to appear approximately 9 hours after inoculation of *Psg*0 into a Harosoy leaf. To detect *avrB* expression, we used antibodies to a portion of the 36-kD polypeptide encoded by the *avrB* open reading frame (ORF) (legend to Fig. 1) in immunoblot analysis (*6*) of total lysates of *Psg* isolated from infected soybean leaves. An ~36-kD protein in *Psg*0 isolated from leaves of either the susceptible soybean cv. Centennial (Fig. 1, lane 2) or the resistant cv. Harosoy (lane 5) was detected. The signal was not produced by *Psg*0 grown in standard rich broth, King's B (KB) medium (*7*) (Fig. 1, lane 1), or by a *Psg*0 strain in which the *avrB* gene had been inactivated by a Tn5 insertion (*2*) (lanes 3 and 6). When the plasmid pPg0-01-17 bearing the cloned *avrB* gene (Fig. 2) was introduced into the *avrB* mutant strain, the race-specific HR-inducing activity and the ability of the bacteria to produce the 36-kD protein were restored (Fig. 1, lanes 4 and 7).

The 36-kD *avrB* gene product can be detected in bacteria grown in planta but not in KB broth. Soybean leaf extracts added to bacteria growing in KB broth [which contains peptone, glycerol, potassium phosphate, and $MgSO_4$ (7)] failed to induce *avrB* (*8*). In contrast, manipulating the carbon source in a defined culture medium did affect transcription from the *avrB* promoter. Expression of a plasmid-borne translational fusion between the 5′ end of the *avrB* gene and the *Escherichia coli lacZ* gene encoding β-galactosidase (Fig. 2) was measured in *Psg*0 grown in minimal medium (MM) containing one of 14 different carbon sources utilizable by *Psg*0. Depending on the carbon source, the level of β-galactosidase activity varied 89-fold (Fig. 3). Bacteria grown in the presence of fructose, sucrose, or mannitol showed the greatest β-galactosidase ac-

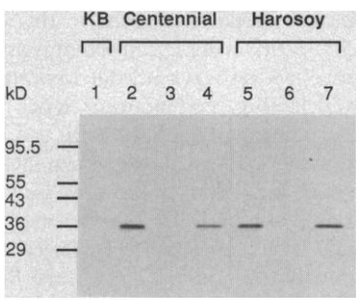

Fig. 1. Detection of *avrB* protein in *Psg*0 recovered from infected soybean leaves. *Psg*0 (lanes 1, 2, and 5), *Psg*0Rif^r *avrB*::Tn5 (lanes 3 and 6), and *Psg*0Rif^r*avrB*::Tn5 carrying pPg-0-01-17 (Fig. 2), a plasmid containing a functional cloned copy of *avrB* (lanes 4 and 7), were grown in KB broth or soybean cvs. Centennial or Harosoy. For growth in plants, *Psg* grown in KB medium were suspended in 10 m*M* $MgSO_4$ [~3 × 10^8 colony-forming units (CFU) per milliliter], inoculated into 2- to 3-week-old soybean seedlings by vacuum infiltration, and the infected plants were incubated (*16*). Leaf intercellular fluid containing bacteria was harvested 15 to 48 hours after inoculation (*17*). Total bacterial proteins were separated on a 10% SDS-polyacrylamide gel (*18*), transferred to nitrocellulose (*6*), and challenged with antibodies to the *avrB* protein prepared as follows. Rabbit antibodies to a fusion protein consisting of the COOH-terminal 281 amino acids encoded by the 963-nucleotide *avrB* ORF (*19*) fused to the COOH-terminus of *E. coli* β-galactosidase (*20*) were affinity-purified to the fusion protein immobilized on nitrocellulose (*21*). Antibodies to the β-galactosidase portion of the fusion protein were removed by adsorption to an *E. coli* extract containing β-galactosidase. The remaining antibodies were used in immunoblot analysis at a dilution of 1:8000 relative to the original serum. Bound antibody was visualized by reaction with alkaline phosphatase–conjugated goat antibodies to rabbit immunoglobulin G (Promega Biotec).

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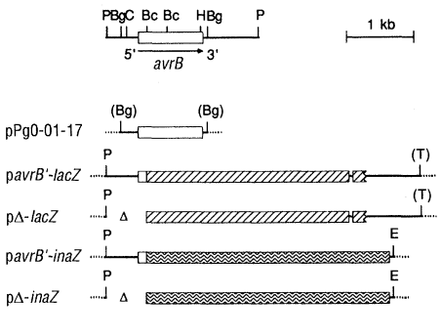

Fig. 2. Maps of the *Psg*0 genomic region containing *avrB* (top line) and plasmids and fusions. The plasmids, polylinker-containing derivatives of pRK290, were transferred from *E. coli* to wild-type or mutant *Psg*0 strains with the helper plasmid pRK2013 (*22*). Open box, *avrB* ORF (*19*); hatched box, *lacZ* structural gene from the eighth codon followed by truncated *lacY* gene and pBR322 segment (*23*); wavy box, *P. syringae inaZ* gene from nucleotide number 775 to 4458 (*11*) preceded by seven nucleotides of synthetic DNA; Δ, deleted segment. ORFs are shown reading left to right. The *Psg*0 DNA fragment used in the construction of the *avrB'-lacZ* and *avrB'-inaZ* translational fusions extends 476 nucleotides upstream of the putative translational start site and includes the NH_2-terminal 41 amino acids encoded by the *avrB* ORF. P, Pst I; Bg, Bgl I; C, Cla I; Bc, Bcl I; H, Hind III; T, Tth 111I; and E, Eco RI. In parentheses, sites destroyed during plasmid construction.

tivity, whereas those grown in the presence of citrate, succinate, L-glutamate, or peptone showed the least. The *avrB* promoter activity reflected the amount of *avrB* protein present in the bacteria, as determined by immunoblot analysis (*8*). In general, *avrB* promoter activity was inversely related to the rate of bacterial growth.

In *Pseudomonas*, bacteria in which energy-yielding metabolism is respiratory rather than fermentative, synthesis of carbohydrate utilization enzymes and transport systems are subject to catabolite repression by tricarboxylic acid (TCA) cycle intermediates, which are the preferred carbon sources (*9*). To determine whether particular carbon sources repress *avrB* induction, we measured *avrB* promoter activity in *Psg*0 growing in MM containing each of the 14 carbon sources individually in addition to mannitol. Citrate, succinate, L-glutamate, and peptone effectively repressed *avrB* induction, whereas L-aspartate, L-glutamine, pyruvate, sugars, and sugar alcohols had less or no effect (Fig. 3). Degradation of carbohydrates by pseudomonads occurs via the Entner-Doudoroff pathway to produce pyruvate, which enters the TCA cycle (*9*). Growth substrates entering this pathway at the pyruvate step or earlier appear not to repress *avrB*. TCA cycle intermediates and substrates readily converted to TCA cycle intermediates (for example, L-glutamate), support rapid growth rates, and repress *avrB*.

Next we addressed the question of whether the conditions in defined media that permit high-level *avrB* transcription are related to those that induce *avrB* in *Psg*0 in planta. We used regulatory mutants affecting *avrB* promoter activity to test whether the mutations have the same affect on *avrB* promoter activity in *Psg*0 grown in defined medium and in planta. Candidates for mutations affecting *avrB* promoter activity were previously identified *hrp* mutations. The *hrp* mutations abolish the ability of *Psg* to cause the leafspot disease in normally susceptible soybean cultivars as well as the ability to induce the HR in resistant soybean cultivars and in nonhost plant species (*10*).

The effects of 22 Tn5 insertion mutations defining the ~20-kb *Psg*0 *hrp* gene cluster (Fig. 4A) on *avrB* promoter activity were tested by expression of reporter genes. To examine bacteria in the defined medium, we used the *avrB'-lacZ* fusion (Fig. 2). Soybean leaves, however, contain β-galactosidase activity; for in planta studies we used a fusion between the 5′ end of *avrB* and the *P. syringae* gene *inaZ* (Fig. 2), which encodes a protein conferring bacterial ice nucleation activity (*11*). The *hrp* mutant strains containing the plasmid-borne *avrB*–reporter gene fusions were grown in KB medium and then inoculated either into MM containing mannitol (MM-mannitol) (legend to Fig. 3) or into soybean cv. Centennial seedlings. One mutation at the left end of the *hrp* gene cluster (designated E10-18) and three mutations at the right end (E43, E62, and E2) reduce *avrB* promoter activity in MM-mannitol to the basal level obtained in KB medium (Fig. 4B). Of the 16 *hrp* mutants tested in planta, the four mutations that abolished *avrB* induction in defined medium also abolished *avrB* induction in planta, assayed by ice nucleation activity (Fig. 4C). Therefore, it is likely that *Psg*0 uses the same mechanism to modulate *avrB* expression in the two environments. The presence of an intact *avrB* gene was not required for high-level transcription from the plasmid-borne *avrB* promoter (Fig. 4, B and C). It is interesting that *hrp* mutations E10-18, E43, E62, and E2 also abolish elevated transcription of some regions within the *hrp* gene cluster in defined medium and in planta (*12*). The striking influence of carbon source on transcription of the *hrp* and *avrB* genes suggests that regulatory functions encoded at the termini of the *hrp* gene cluster have roles in integrating information about carbon source availability.

We next asked whether bacteria grown in media that induce *avrB* expression transcribe all other genes necessary to elicit the HR. Simultaneous inoculation of the bacterial RNA polymerase inhibitor rifampicin (200 μg/ml) and KB-grown *Psg*0 into soybean cv. Harosoy inhibited HR induction (Fig. 5, site 3). [The sensitivity of HR development to rifampicin is a property of the bacterium and not the host, because a rifampicin-resistant isolate of *Psg*0 induced the HR in the presence of the drug (*8*)]. Bacteria grown in MM-fructose, however, were able

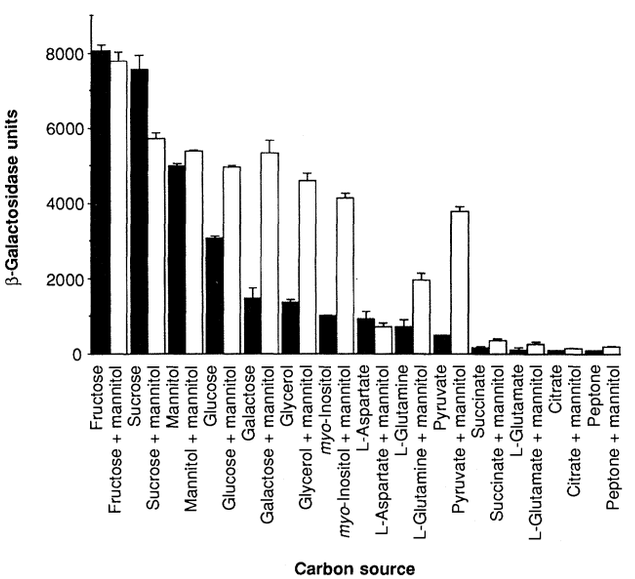

Fig. 3. Composite of *avrB* promoter activity in *Psg*0 grown in MM containing various carbon sources. *Psg*0(p*avrB'-lacZ*) or *Psg*0-(pΔ-*lacZ*) (Fig. 2) were grown to mid-log phase in KB at 24°C, washed twice in 10 m*M* $MgSO_4$, and resuspended at an optical density of 600 [OD_{600}] = 0.1 (~6 × 10^7 CFU/ml) in MM [50 m*M* potassium phosphate buffer, 7.6 m*M* $(NH_4)_2SO_4$, 1.7 m*M* $MgCl_2$, and 1.7 m*M* NaCl, *p*H 5.7 (*p*H optimum for induction)] containing tetracycline (10 μg/ml) and carbon sources as indicated. Carbon sources were used at 10 m*M* except for the following: glycerol, 20 m*M*; pyruvate, 20 m*M*; and Difco proteose peptone no. 3, 2% (w/v). Cultures (1 ml) were shaken (250 rpm, 24°C) for 12 hours and then assayed for β-galactosidase activity (standardized to CFU). Assays were performed on toluene-treated bacteria (*24*) in Z buffer supplemented with bovine serum albumin (100 μg/ml). β-galactosidase activity of *Psg*0 containing the *lacZ* construction lacking the *avrB* promoter (pΔ-*lacZ*) was zero units for each medium. Values are means +1 standard deviation for three independent experiments. Induction of the *avrB* promoter did not occur if the medium lacked a utilizable carbon source. Solid bars, carbon source; blank bars, mannitol + carbon source.

to induce the HR in Harosoy in the presence of rifampicin (Fig. 5, site 4). HR development in the presence of rifampicin required both *avrB* and the resistance gene *Rpg1*, because the HR failed to occur in response to the *avrB* mutant (Fig. 5, site 6) or in the susceptible soybean cv. Centennial (*8*). Therefore, growth in a defined medium that induces *avrB* permits the transcription of all bacterial genes required to induce the HR. For *Psg*, the presence of the host is apparently not required for the expression of genes involved in interactions with the host.

The HR obtained in the presence of rifampicin is usually weak (Fig. 5, compare sites 2 and 4). Most likely, development of an intense HR requires continued bacterial RNA synthesis. When bacteria grown in MM-fructose are exposed to rifampicin (200 μg/ml), their ability to induce the HR decays over a period of 10 min (*8*). The rapid turnover time of the HR-inducing activity suggests that certain classes of bacterial molecules, for example, cell surface polysaccharides (*13*), are unlikely to determine by themselves the ability to induce the specific HR.

Determining conditions that permit *avrB* expression has clarified several aspects of how the soybean host and this bacterial parasite interact:

1) Induction of *avrB*. During infection, the *avrB* gene is expressed at high levels in *Psg*0 in both resistant and susceptible soybean cultivars (Fig. 1). Therefore, the host resistance gene *Rpg1* does not control the expression of the pathogen gene *avrB*. The specific interaction between *avrB* and *Rpg1* must occur at a later stage of infection. Transcription of *avrB* is also induced when *Psg*0 is inoculated into either of two nonhost plants, tobacco and bean (*12*), further demonstrating that a soybean-specific molecule is not required for *avrB* induction. It is difficult to determine directly what plant molecule, or molecules, induce *avrB* during infection. In defined media we find that particular carbon sources (fructose, sucrose, and mannitol) stimulate *avrB* transcription. Of these saccharides, sucrose is the most abundant in leaf tissue and probably contributes to the environment that induces *avrB* during infection. It is interesting that two *hrp* gene products (*14, 12*) that appear to regulate *avrB* (defined by mutations E43, E62, and E2) share homology with the transcriptional regulatory proteins in two-component systems controlling bacterial gene expression in response to environmental stimuli (*15*).

2) Repression of *avrB*. Transcription of *avrB* is subject to catabolite repression by

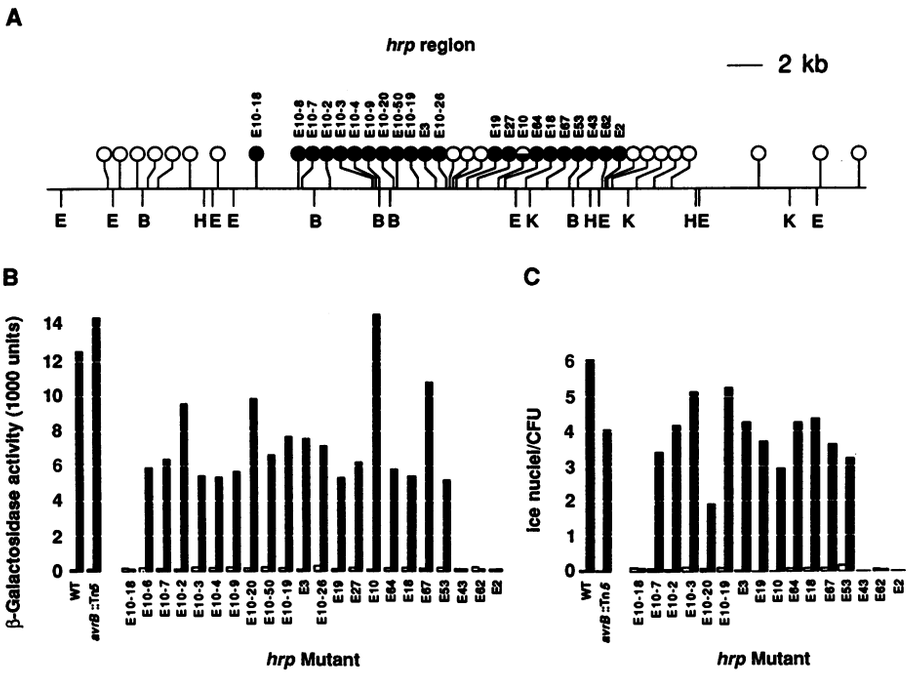

Fig. 4. Composite of *avrB* promoter activity in wild-type (WT), *avrB* mutant (*avrB*::Tn5), and *hrp* mutant backgrounds. (**A**) The *Psg*0 *hrp* region. DNA probes from the cloned *hrp* region of *P. syringae* pv. *phaseolicola* (*25*) were used to isolate homologous clones in a pLAFR3 (*2*) cosmid library of *Psg*0 DNA. Positive clones were mutagenized in *E. coli* by transposon Tn5. Individual Tn5 insertion mutations were introduced into the *Psg*0Rif^r (*2*) genome by marker exchange (*25*) and restriction mapped. Sites of individual Tn5 insertions are indicated above the map. Symbols for phenotypes caused by individual Tn5 insertions: open circle, no effect on pathogenicity or HR-inducing ability; closed circle, nonpathogenic (unable to multiply to normal levels or cause disease symptoms) and non–HR-inducing; half-filled circle, nonpathogenic and weakly HR-inducing. E, Eco RI; B, Bgl II; H, Hind III; and K, Kpn I. (**B**) *avrB* promoter activity in *Psg* strains grown in KB medium (open bars) or in MM-mannitol (solid bars). *Psg*0 wild-type and mutant strains carrying p*avrB'-lacZ* or pΔ-*lacZ* were grown in KB broth then transferred to MM-mannitol (initial OD_{600} = 0.5) for 12 hours (time of maximum induction) and assayed for β-galactosidase as in Fig. 3. The β-galactosidase activity of each strain containing the *lacZ* construction lacking the *avrB* promoter (pΔ-*lacZ*) was zero units. Values are means of four simultaneous replicates. Three independent experiments gave similar results. (**C**) The *avrB* promoter activity in *Psg* strains grown in KB medium (open bars) or in planta (solid bars). *Psg*0 wild-type and mutant strains carrying p*avrB'-inaZ* or pΔ-*inaZ* (Fig. 2) were grown on KB agar containing tetracycline (10 μg/ml) at 24°C, and then inoculated into soybean cv. Centennial seedlings by vacuum infiltration. Immediately or 5 hours after inoculation (time of maximum induction), two leaf disks were removed from separate plants with a number 3 cork borer and homogenized in 10 m*M* potassium phosphate buffer (*p*H 7.0). For each data point, ice nucleation frequency at −9°C was measured for three pairs of infected leaf disks in 10-μl droplets from a dilution series of leaf disk homogenate (*26*). Values are the number of ice nuclei per CFU of a strain carrying p*avrB'-inaZ* minus the number generated by the same strain carrying the promoterless control plasmid, pΔ-*inaZ*. Transferring Psg0(p*avrB'-inaZ*) from KB medium to MM-mannitol caused a 20- to 60-fold increase in ice nucleation frequency [*inaZ* has been previously used as a reporter gene (*27*)].

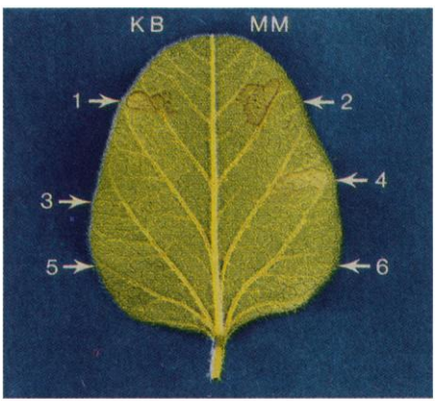

Fig. 5. Competence of *Psg*0 to induce the HR in the absence of de novo RNA synthesis. Bacteria (10^8) (*Psg*0 or *Psg*0Rif^r*avrB*::Tn5) grown in KB broth and washed as in Fig. 3 were inoculated onto agar-solidified KB medium (inoculation sites 1, 3, and 5) or MM-fructose (2, 4, and 6) and incubated at 24°C for 16 hours. Bacteria scraped from the agar surface were washed and resuspended in 10 m*M* $MgSO_4$ at OD_{600} = 0.5. Immediately after the addition of rifampicin to 200 μg/ml (prepared from a stock solution containing rifampicin at 1 mg/ml in a solution of $K_2HPO_4 \cdot 3H_2O$ at 1 mg/ml), the bacteria were inoculated into the underside of a fully expanded primary leaf of soybean cv. Harosoy with the aid of a plastic Pasteur pipette. Leaves were detached for photography after 1 day of incubation (*16*). Inoculation sites are indicated by arrows: *Psg*0 without rifampicin (1 and 2), *Psg*0 plus rifampicin (3 and 4), *Psg*0Rif^r*avrB*::Tn5 plus rifampicin (5 and 6).

TCA cycle intermediates and particular amino acids that support rapid growth rates of *Pseudomonas*.

3) The *avrB-Rpg1* interaction. Our studies reveal that the HR-inducing ability of *Psg*0 has a rapid turnover time (approximately 10 min). Since it is unlikely that *Psg*0 induces the *Rpg1* gene and then interacts with its product (or with the product of a pathway downstream from the *Rpg1* gene product) within 10 min, the *Rpg1* gene product and the mechanism for *avrB*-specific recognition probably exist in the plant before infection.

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28. We thank N. Panopoulos, P. Lindgren, A. Theologis, M. Whalen, P. Zambryski, A. Jackson, and J. Nathans for helpful discussions. T.V.H. is a Monsanto Fellow of the Life Sciences Research Foundation. Supported by U.S. Department of Agriculture grant 86-CRCR-1-2086 and U.S. Department of Energy grant DE-FG03-88 ER13917 to B.J.S.

17 January 1989; accepted 27 June 1989

The RNA Processing Enzyme RNase MRP Is Identical to the Th RNP and Related to RNase P

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Sera from patients with autoimmune diseases often contain antibodies that bind ribonucleoproteins (RNPs). Sera from 30 such patients were found to immunoprecipitate ribonuclease P (RNase P), an RNP enzyme required to process the 5′ termini of transfer RNA transcripts in nuclei and mitochondria of eukaryotic cells. All 30 sera also immunoprecipitated the nucleolar Th RNP, indicating that the two RNPs are structurally related. Nucleotide sequence analysis of the Th RNP revealed it was identical to the RNA component of the mitochondrial RNA processing enzyme known as RNase MRP. Antibodies that immunoprecipitated the Th RNP selectively depleted murine and human cell extracts of RNase MRP activity, indicating that the Th and RNase MRP RNPs are identical. Since RNase P and RNase MRP are not associated with each other during biochemical purification, we suggest that these two RNA processing enzymes share a common autoantigenic polypeptide.

PATIENTS WITH SYSTEMIC AUTOIMMUNE diseases, such as systemic lupus erythematosus (SLE), make autoantibodies, which are directed against a variety of intracellular structures. The most common targets of these autoantibodies are ribonucleoproteins (RNPs) and components of chromatin (*1*). Although the precise mechanisms leading to the genesis of autoantibodies are not known, they have served as useful probes to elucidate the structure and function of their intracellular targets. For example, autoantibodies directed against the polypeptide components of the U1 RNP and the related U-series RNPs were crucial tools in the elucidation of the structure of these molecules and their role in the splicing of pre–messenger RNA (*2*).

Sera from certain patients with systemic autoimmune diseases have recently been shown to contain antibodies to ribonuclease P (RNase P) from HeLa cells (*3*). RNase P, one of the best characterized RNA processing enzymes, is an endoribonuclease that processes all precursor tRNA transcripts to generate their mature 5′ termini (*4*). Studies of RNase P from prokaryotic sources have demonstrated that the enzyme has a catalytic RNA and a protein subunit (thus, an RNP) (*5*).

Sera that contain antibodies to RNase P from HeLa cells were found to immunoprecipitate an RNA of ~400 nucleotides from crude cell extracts and from extracts of partially purified RNase P (*3*). This RNA (called H1 RNA) is the only RNA that copurifies with RNase P activity, strongly suggesting that H1 RNA is the RNA component of RNase P (*6*). In addition, the majority of these autoimmune sera immunoprecipitated a second, smaller RNA species of approximately ~300 nucleotides named Th RNA [after the patient whose serum first immunoprecipitated it from cell extracts (*7*)]. Subsequent studies with this prototype serum and with a second serum of the same specificity demonstrated that the Th RNA was bound to at least one unidentified autoantigenic polypeptide (the RNA itself was not antigenic) and was identical to a previously described nucleolar 7-2 RNA (*8*). Indirect immunofluorescence and electron microscopy studies have confirmed the nucleolar location of the Th RNP with the antigenic polypeptide or polypeptides located in the granular region (*8, 9*).

We have now identified 30 patients with autoimmune diseases whose sera immunoprecipitate the Th RNA from crude cell extracts labeled in vivo with [^{32}P]orthophosphate. Serum samples from nine of these patients, including the prototype Th serum, have been described previously (*3, 7, 8*). All 30 sera also immunoprecipitated the H1 RNA of RNase P; representative samples are shown in Fig. 1. Twenty-five of these sera only immunoprecipitated the Th and H1 RNAs (for examples, see lanes 8 to 15 of Fig. 1), while the remaining five contained additional specificities (see lanes 7 and 16). When extracts were deproteinized with phenol or proteinase K, no RNAs were immunoprecipitated. These data strongly suggest that these two RNAs share one or more polypeptides or are physically associated.

The relationship between the RNase P and Th RNPs was examined more closely by sequencing the immunoprecipitated Th RNA. Th RNA is ~80% homologous with the RNA component of mouse mitochondrial RNA processing enzyme (RNase MRP) (*10*) and identical to the RNA component of human RNase MRP (*11*) (Fig. 2).

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