Transfer Ribonucleic Acid Biosynthesis

SUBSTRATE SPECIFICITY OF RIBONUCLEASE P*

(Received for publication, September 22, 1975)

FRANCIS J. SCHMIDT,‡ J. G. SEIDMAN,§ AND ROBERT M. BOCK

From the Department of Bacteriology and Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706

Bacteriophage T4 synthesizes proline and serine tRNA species which are derived from a common precursor RNA. The processing of this precursor RNA involves the replacement of a U-A-A terminus in serine tRNA by C-C-A prior to precursor cleavage. In the present work we have examined in detail the cleavage of T4 proline-serine precursor RNA by the previously identified ribonuclease P.

Ribonuclease P accurately cleaves precursor RNA terminating in either C-C-A or U-A-A to generate the 5' termini characteristic of both mature tRNA species. These cleavages do not depend solely on the nucleotide sequence of the precursor RNA since isolated oligonucleotides spanning the cleavage sites are not substrates for the enzyme.

Two types of experiments show that RNase P kinetically favors precursor RNA ending C-C-A over that ending U-A-A. Isolated preparations of precursor RNA containing the C-C-A sequence were cleaved more rapidly by RNase P than precursor RNA ending U-A-A. In addition, the serine tRNA generated by limited cleavage of a mixed population of precursor RNA ending C-C-A or U-A-A was enriched 3-fold in the C-C-A sequence relative to the starting material.

Bacteriophage T4 proline-serine precursor RNA, in contrast to other tRNA precursors, accumulates in measurable amounts in wild type cells. This accumulation would appear to be a consequence of the requirement for the generation of the C-C-A sequence prior to RNase P cleavage. The enzymic specificity of RNase P in vitro therefore reflects the in vivo pathway for serine tRNA biosynthesis, where the C-C-A sequence is synthesized while the serine tRNA sequence is still a part of the large precursor RNA.
Fig. 1. Nucleotide sequence of phage T4 proline-serine precursor RNA (12). Proline tRNA is the 5' portion of the molecule. This sequence is the longest sequence available from the data; the sequence of precursors RNA as isolated is heterogeneous in length at the 5' and 3' termini. The 3' end of the serine tRNA sequence depends on its stage of biosynthesis (6); in the present experiments, precursor RNA ending by nucleotide sequence. We present evidence of a kinetic preference for T4 proline serine precursor RNA ending C-C-A which may help to account for some of the unusual aspects of its metabolism.

**EXPERIMENTAL PROCEDURES**

**Bacterial and Phage Strains—**Phage strains T4D and T2L and strains T4 psu, R1e-L, H12 have been described previously (6). Escherichia coli strains A49 and PP113 (3), strain BN (8, 10), and wild type strain MREG00 were from the collection of W. H. McClain.

Preparation of Precursor RNA—Proline-serine precursor RNA was routinely prepared from phage-infected cultures labeled with 1 mCi ml−1 of [32P]phosphate. Precursor RNA was prepared as described elsewhere (6) by successive steps of electrophoresis in 10% and 20% polyacrylamide gels.

Carrier RNA used was either E. coli transfer RNA, RNA from phage φ2 (a gift of Dr. H. D. Robertson) or polyuridylic acid. These last two were used because transfer RNA has been shown to substantially inhibit RNase P activity.1

Sequence Analysis of RNA—Analysis of [32P]RNA was by the methods of Sanger and associates as described by Barrel1 (11). Sequences of 3'-terminal oligonucleotides were confirmed by two-dimensional chromatography of RNase T2 or venom phosphodiesterase digests of the oligonucleotides (12). The sequences of the 5' termini were established by further digestion with RNase A and RNase U3 (12).

Growth of Cells and Purification of RNase P—E. coli strains A49, PP113, and MREG00 were grown in C broth (13) at 30°. Cell-free extracts (830 fraction) were prepared by grinding with alumina as described by Robertson et al. (2). The extracts could be frozen and stored at −20° for several months with no apparent loss of RNase P activity.

RNase P was purified from E. coli MREG00 cell paste by the procedure of Robertson et al. (2), except that the batch elution of the enzyme from DEAE-Sephadex A-50 was from 0.30 to 0.50 M NH4Cl. Throughout the purification, the fractions were assayed for concurrent cleavage of 480 psu,1 tyrosine precursor RNA and phage T4 proline-serine precursor RNA. This preparation of RNase P could be stored at −70° for several months with no apparent loss of activity. For some experiments RNase P was further purified by chromatography on phosphocellulose (2) or on Sephadex G-200.1 Sephadex chromatography also served to remove some of the nucleic acid present in the RNase P preparation.1 Enzyme at this further stage of purification appeared to be somewhat less stable to storage on ice than the preceding fraction.

**Rate Measurements of RNase P Cleavage—**Rate measurements of RNase P cleavage were done at 37°. At the indicated times, aliquots U-A-A or C-C-A was used as a substrate for RNase P. Phage T2 synthesizes identical proline and serine tRNAs, but the precursor RNA contains the 3'-terminal sequences C-C-A or C-C-A-G (12). The positions of the RNase P cleavages of proline-serine precursor RNA are indicated by arrows.

When sample volumes greater than 0.1 ml were involved or where the reactions contained a large amount of protein (as in reactions using crude cell-free extracts), the reactions were terminated by phenol extraction (9). RNA was then precipitated from the aqueous phase, redissolved, and applied to the gel.

When comparisons were made of the rates of two substrates, care was taken to ensure that different reactions contained equivalent amounts of substrate and enzyme. Equal numbers of counts per min of precursor RNA prepared at the same time from cells at the same density were used. This precaution was essential since measurements are not expressed in molar terms. The total amount of RNA counted from the gel was not constant from sample to sample; therefore, all quantitations are expressed as percentages relative to the total amount of RNA in the gel lane.

**Measurement of Enzyme Preference for Precursor Ending C-C-A over Precursor Ending U-A-A—**To 2.5 ml of cleavage buffer (0.01 M Tris-CI, pH 8.0/0.1 M NH4CI/0.005 M MgCl2/10−4 M EDTA/10−4 M 2-mercaptoethanol) were added 0.2 ml of enzyme buffer (0.02 M Tris-CI, pH 7.6/0.02 M NH4CI/0.016 M MgCl2/0.006 M 2-mercaptoethanol), 5 × 105 cpm of precursor RNA in 25 μl of H2O, and 25 μl of RNase P. The RNase P, purified through the DEAEx-Sephadex step of Robertson et al. (2), was calibrated to cleave the precursor to the extent of 25% under the experimental conditions. The cleavage was allowed to proceed at 37° for 20 min and terminated by the addition of 0.5 ml of phenol and 2 A260 units E. coli tRNA. RNA was precipitated from the aqueous phase by the addition of 1 ml of 5 M potassium acetate, pH 5.0, and 10 ml of cold absolute ethanol. The RNA was reprecipitated from 1.0 ml of 0.9 M potassium acetate, pH 5.0, and analyzed by gel electrophoresis.

The serine tRNA was fingerprinted and the molar yields of C-C-A and U-A-A were determined by counting the paper in toluene scintillator. The oligonucleotide C-A-C-G was used as the standard (12).

**Attempted Cleavage of Isolated Oligonucleotides Spanning Cleavage Site in Precursor RNA—**Since oligonucleotides derived from the 5' end of precursor RNA are poorly resolved in standard fingerprints, they were isolated from fingerprints using homochromatography in the
The 5' oligonucleotides a, pU-U-U-A-A-U-U-U-A-C-U-C-C-Gp (3 x 10^3 cpm), b, pU-U-A-A-U-U-U-A-C-U-C-C-Gp (8 x 10^2 cpm), c, pU-U-A-U-A-U-C-U-C-C-Gp (8 x 10^3 cpm), d, pU-U-A-U-A-U-C-U-C-C-Gp (8 x 10^4 cpm), and the interstitial oligonucleotide e, A-C-U-Gp (1.5 x 10^3 cpm) were treated with 25 µl of an RNase P fraction isolated after Sephadex G-200 chromatography. After 1.5 hours of incubation at 37°C, the reactions were quenched by adding them to 10 µl of 0.1 M Na,EDTA, pH 7.0. The samples were dried, redissolved in 10 µl of H2O, and applied to DEAE-cellulose paper. The paper was washed with ethanol to remove salt. Electrophoresis was for 1 hr at 70 volts in 7% formic acid. The autoradiograph is shown in Fig. 4. The streaking in samples a through d results from the presence of salt in the samples. All these bands were cut out and eluted from the paper. Their identities were confirmed by digestion with RNase A and RNase U1. All the oligonucleotides characteristic of the original fragments were obtained. Separate aliquots of the fragments were rerun on DEAE-paper in 7% formic acid. In this experiment the oligonucleotides were single, narrow bands at the origin except for A-C-U-Gp, which migrated as shown in Fig. 4. In the position of untreated A-C-U-Gp. We conclude from this that the oligonucleotides were not cleaved by RNase P. An amount of precursor RNA equimolar to sample 1 was treated as above and applied to a 10% polyacrylamide gel.

RESULTS

RNase P Cleaves Proline-Serine Precursor RNA in Vitro—Guthrie et al. (14) reported that cell-free extracts of uninfected Escherichia coli were able to cleave proline-serine precursor RNA. We therefore purified the cleavage activity from E. coli MRE6000 cell paste, using the procedure of Robertson et al. (2).

The activities responsible for cleavage of proline-serine precursor RNA and 32P-labeled tyrosine precursor RNA were not separated by gel filtration through the DEAE-Sephadex chromatography step described by Robertson et al. (2). Further purification by chromatography on phosphocellulose as described by these authors resulted in proline-serine precursor RNA cleavage activity eluting with the buffer front, as was reported for RNase P. We therefore conclude that the proline-serine precursor RNA is a substrate for RNase P, although we have not demonstrated this unequivocally, since the purity of the RNase P preparation is not known.

Further indication that RNase P catalyzes the cleavage of proline-serine precursor RNA is provided by mutant strain E. coli A49, which is temperature-sensitive for RNase P activity (3). In vitro, heat-treated extracts of strain A49 are unable to carry out the cleavage of T2 or T4 proline-serine precursor RNA (data not shown). Extracts derived from E. coli strain PP113, which contains wild type RNase P activity, cleave the precursor RNA into two products with the electrophoretic mobility near that expected for serine and proline tRNAs.

Precursor RNA Ending Either C-C-A or U-A-A Is Cleaved Twice by RNase P to Generate 5' Sequences of Mature tRNAs—RNase P purified through the phosphocellulose step (2) was used to cleave precursor RNA isolated from T4 infection of E. coli strain BN. This strain lacks the nuclease activity responsible for removal of the U-A-A residues at the 3' end of the precursor RNA prior to C-C-A synthesis; therefore, proline-serine precursor RNA with 3' terminus U-A-A or U-A accumulates on this strain (8). The products of the reaction (Fig. 2a) were proline tRNA ending A-C-U (Fig. 2b) and serine tRNA ending U-A or U-A-A (Fig. 2c). The 5' sequences of these RNAs pC-U-C-C-Gp and pGp, respectively, were identical with those of the mature tRNA species. The positions of these 3'- and 5'-terminal oligonucleotides are indicated in Fig. 2.

Bacteriophage T4 proline-serine precursor RNA isolated after infection of strain A49 is also a substrate for purified RNase P. The results of the limited cleavage of this RNA are shown in Fig. 3a. Serine tRNA is a product of the reaction. Its fingerprint is shown in Fig. 3b. The proline tRNA sequence appears in three positions, depending on the 5' sequence. The 5' termini of Bands 1 and 2 in Fig. 3a were determined by fingerprinting using homochromatography in the second dimension (12). Band 1 (Fig. 3a) proline tRNA has the 5' sequence pU-U-U-A-A-U-U-A-C-U-C-C-Gp or pU-U-U-A-U-U-A-C-U-C-C-Gp. Band 2 in Fig. 3a is proline tRNA with the shorter 5' sequences pU-U-U-A-U-U-A-C-C-Gp or pU-U-U-A-U-U-A-C-U-C-C-Gp. In addition to the above intermediate products, proline tRNA with the mature 5' end is generated. All the proline tRNA species have the 3' terminus A-C-U. Thus the two RNase P cleavages required to generate the 5' ends of mature proline and serine tRNAs need not occur simultaneously. Guthrie (7) has noted analogous intermediate species after limited cleavage of phage T4 glutamine-leucine precursor RNA by RNase P, i.e., immature glutamine tRNA containing the 5' sequence of the precursor RNA was a product of limited RNase P cleavage of glutamine-leucine precursor RNA.

We conclude that proline-serine precursor RNA ending either C-C-A or U-A-A is a substrate for RNase P. Therefore, those structural features governing the accuracy of RNase P cleavage are shared by the two precursor forms.

RNase P Cleavage Is Not Directed Solely by Nucleotide Sequence—The involvement of RNase P in the biosynthesis of a large number of tRNA species has been documented by two criteria: the accumulation of precursor RNA species in strains of E. coli temperature-sensitive for RNase P activity (3-7;15, 16) and by the ability of partially purified preparations of RNase P to carry out the accurate cleavage of these precursor RNA species in vitro (2, 7, 16). Since the nucleotide sequences of these precursor RNAs include at least four dinucleotide sequences, it is not clear whether the nucleotide sequence(s) of RNase P cleavage is sufficient to direct RNase P cleavage. The oligonucleotide sequences spanning the RNase P cleavage site were isolated by homochromatography after RNase T1 digestion. Since the preparation of precursor RNA was heterogeneous at the 5' end, the oligonucleotides pU-U-U-A-U-U-U-A-C-C-Gp, pU-U-U-A-U-U-A-C-U-C-C-Gp, pU-U-A-U-A-U-A-C-C-Gp, and pU-U-A-U-A-U-A-C-U-C-C-Gp, as well as the interstitial oligonucleotide A-C-U-Gp, were tested as possible substrates for RNase P. Fig. 1 shows the nucleotide sequence of precursor RNA which includes these sequences.

Under conditions where 40% of the precursor RNA was cleaved to serine tRNA, no cleavage of the oligonucleotides was detected (Fig. 4). The lower limit of detection is approximately 2% cleavage of the oligonucleotides; therefore, RNase P exhibits at least a 20-fold preference for the precursor RNA structure over that of the isolated oligonucleotides. We infer from this result that precursor RNA secondary and/or tertiary structure is required for RNase P cleavage. The possibility of a kinetic preference for some few dinucleotide sequences at the cleavage site in an intact tRNA precursor has not been tested here.

RNase P Cleavage of Proline-Serine Precursor RNA Is
Specificity of RNase P

FIG. 2 (upper). Proline-serine precursor RNA ending U-A-A is a substrate for RNase P. a, separation of proline and serine tRNA species by electrophoresis. The input precursor RNA, 95%, was cleaved to RNA products with mobilities close to the component tRNA species. b, fingerprint of immature proline tRNA generated by RNase P cleavage. The 5' end of this RNA is pC-U-C-C-Gp and the 3' end is A-C-U. The positions of these oligonucleotides are indicated on the fingerprint. c, fingerprint of serine tRNA sequence generated by RNase P cleavage. The 5' end of this RNA is pGp; and the 3' end is U-A-A or U-A.

FIG. 3 (lower). Limited cleavage of proline-serine precursor RNA generates serine tRNA and several species of immature proline tRNA. a, separation of the products of RNase P cleavage by gel electrophoresis. b, fingerprint of serine tRNA. The 5' terminus is pGp; the 3' end is enriched for C-C-A but contains some U-A-A. See Table I for the proportions of these termini. RNase P purified through the DEAE-Sephadex step was used in this experiment.

Accelerated by Presence of 3'-Terminal C-C-A Sequence of Serine tRNA—Since precursor RNAs ending either U-A-A or C-C-A are substrates for accurate cleavage in vitro by RNase P, the requirement in vivo for 3'-terminal processing of precursor RNA (6, 8) prior to precursor cleavage cannot be based on an absolute discrimination between the two precursor forms by RNase P. Several in vivo lines of evidence, however, indicated a possible kinetic preference of RNase P for proline-serine precursor RNA containing the C-C-A sequence.

In wild type strains of E. coli, phage T2 proline-serine precursor RNA does not accumulate to a measurable extent. The terminal C-C-A sequence of this precursor RNA is thought to be derived by transcription (12). Similarly, phage T2 proline-serine precursor RNA does not accumulate in mutant strains of E. coli lacking the enzymes required for 3'-terminal maturation of T4 proline-serine precursor RNA (8, 12).

While T4 proline-serine precursor RNA accumulates in the wild type strain E. coli B/5, the predominant 3'-terminal sequences observed are U-A-A or U-A, with C-C-A not being detected, even though precursor RNA ending C-C-A is presumably being continually synthesized (12). Experiments were therefore performed to measure the relative rates of cleavage of precursor RNA containing the C-C-A or U-A-A sequence.

Two types of experiments correlate the presence of the C-C-A sequence with an increased rate of precursor cleavage in vitro. In the first experiment, isolated precursor RNAs containing the C-C-A and U-A-A sequences were subjected to hydrolysis by RNase P purified through the DEAE-Sephadex step of Robertson et al. (2). As shown in Fig. 5, precursor RNAs containing the C-C-A sequence were cleaved more rapidly by RNase P in vitro. Although the nucleotide sequences of these precursor RNAs were identical except at the 3' end, the specific
Fig. 4. RNase P did not cleave the T$_1$ oligonucleotides spanning the cleavage sites in precursor RNA. We attempted to cleave the 5' oligonucleotides pU-U-A-A-U-U-U-C-U-C-C-Gp (a), pU-U-A-A-U-U-A-C-U-C-C-Gp (b), pU-U-A-C-U-C-C-Gp (c), pU-U-A-C-U-C-C-Gp (d), and the interstitial oligonucleotide A-C-U-Gp (e) with purified RNase P. The positions of the product oligonucleotides, pC-U-C-C-Gp in lanes a to d, A-C-U and pGp in lane e, are indicated on the figure. In lanes a to d, the other oligonucleotide generated would remain at or near the origin throughout electrophoresis.

activities of the [32P]phosphate could have varied in a noncontrolled manner. Further, initial velocities of precursor RNA cleavage will depend on the enzyme:substrate ratio; therefore, this experiment should be interpreted only in a qualitative sense.

A second measurement of the enzymatic preference for precursor RNA ending C-C-A is provided by an analysis of the relative amounts of C-C-A and U-A-A in serine tRNA derived from a mixed population of precursor RNA containing both species. Precursor RNA from T4 infection of strain A49 containing both species (Table I) was cleaved by RNase P and analyzed by gel electrophoresis (Fig. 3a). In order to obtain information regarding the relative rates of cleavage at nearly initial velocity conditions, the products were examined after only 25% of the precursor RNA had been cleaved. The enzymatic preference for precursor RNA ending C-C-A is given by the following formula:

Preference for precursor ending C-C-A = (C-C-A) product / (U-A-A) starting

This ratio measurement is equivalent to the ratio between the $V_{max}/K_m$ values of the two competing substrates (17). The fingerprint of the product serine tRNA is shown in Fig. 3b. The data in Table I show that the C-C-A sequence was nearly 3-fold enriched in the product serine tRNA relative to precursor RNA before cleavage; thus, there is a 3-fold enzymatic preference for the C-C-A form of precursor RNA. The sum of the molar yields of C-C-A and U-A-A is less than unity for each of the RNAs in Table I. Routinely, we obtain submolar

TABLE I

<table>
<thead>
<tr>
<th>Molar yields of 3'-terminal oligonucleotide</th>
<th>Before cleavage</th>
<th>After cleavage</th>
<th>Preference for precursor RNA containing the C-C-A sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C-A</td>
<td>0.56 ± 0.02</td>
<td>0.68 ± 0.02</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>U-A-A</td>
<td>0.17 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. RNase P cleavage is accelerated by the presence of the 3' C-C-A sequence. Proline-serine precursor RNAs isolated from T4 labeling of strain BN, strain A49, or from T2 labeling of strain A49 were cleaved with RNase P. The phage strains from which the precursors are derived and their 3' sequences are: ●, T4 precursor with 3' end U-A-A; ▲, T4 precursor with 3' end C-C-A and U-A-A. The ratio of C-C-A to U-A-A in this sample was approximately 4:1. We assume that the presence of residual precursor RNA ending U-A-A caused the higher leveling off of the curve relative to the curve for T2 precursor RNA. △, T2 precursor with 3' end C-C-A or C-C-A-G.

DISCUSSION

Ribonuclease P from Escherichia coli has been shown to cleave a number of precursor RNA molecules, both in vivo and in vitro (2-7, 14, 15). Several lines of evidence point to the requirement for some secondary or tertiary structure, rather than nucleotide sequence in determining the site of this cleavage. First, although a number of tRNA precursor substrates of the enzyme have been sequenced, there exist no obvious sequence similarities which might serve as recognition sites (7). Second, the existence of tRNA base-change mutants causing both precursor accumulation in vivo (18, 19) and reduced activity as RNase P substrates in vitro (15) points to the requirement for a structure that can be disrupted by

*We are grateful to Professor W. W. Cleland for this observation.
mutation; the base sequence at the RNase P cleavage site is identical with the wild type sequence in these mutants. Finally, in the present work, we have shown that the base sequence immediately adjoining the cleavage site is insufficient to direct RNase P cleavage. This last observation is of interest because one might hope to reconstruct partial molecules which are substrates for RNase P, thereby gaining insight into the processes governing precursor-enzyme interaction.

In phage T4, enzymatic processing to synthesize the 3' terminal C-C-A sequence of serine tRNA occurs at the tRNA precursor level prior to cleavage by RNase P (6, 8, 9, 12). One determinant of the specific order of this processing may lie with the observation reported here that the presence of the C-C-A sequence accelerates the cleavage of precursor RNA by RNase P. Thus, the lack of detectable C-C-A in T4 proline-serine precursor RNA obtained from wild type cells (12) may reflect the more facile RNase P cleavage of precursor RNA ending C-C-A. Although the discrimination in vivo between precursor RNA ending C-C-A and U-A-A appears to be complete (i.e., greater than 30-fold), the kinetic parameters determined by in vitro measurement yield a 3-fold difference in rate constants ($V_{\text{max}}/K_m$) for cleavage of the two tRNA precursor forms. While substantial, this kinetic parameter cannot by simple extrapolation yield the somewhat greater specificity which apparently exists in the steady-state situation in the cell. The quantitative in vivo specificity may depend on the presence of cofactors, other proteins, precursor concentration, subcellular organization, etc. Qualitative observations agreeing with those shown in Fig. 5 and Table 1 have been obtained with RNase P preparations at several stages of purification, including crude S30 extracts as well as the most purified (Sephadex G-200) fraction. We therefore surmise that this discrimination is an intrinsic property of RNase P.

Those tRNA precursors whose 3' C-C-A sequences are derived by transcription accumulation in vivo only under specialized conditions, e.g. in mutants lacking RNase P (3, 4, 15) or when the RNA sequence is itself mutated (18). This may be an indication that the substrate discrimination for the C-C-A sequence observed here applies to tRNA precursors other than proline-serine precursor RNA. Altman et al. have observed that chemical removal of some nucleotides from the 3' terminus of φ80 psu* tyrosine precursor RNA decreases the extent of precursor cleavage by RNase P (15). A similar case may be made for phage T4 glutamine-leucine precursor RNA which also does not accumulate in wild type cells (7, 14). Phage T4 leucine tRNA does not require wild type amounts of host tRNA nucleotidyltransferase for its biosynthesis and so presumably contains the C-C-A sequence encoded in the genome. The low level of this precursor RNA in vivo may reflect the lack of a requirement for 3' terminal processing prior to RNase P cleavage.

The mechanism of enzymatic recognition of the C-C-A containing precursor RNA is unclear, but we note that the double cloverleaf structure proposed by McClain and Seidman (20) would permit the simultaneous interaction of the enzyme with the precursor cleavage site as well as the nearby C-C-A sequence. While other (e.g. conformational) differences between the two forms of precursor RNA could account for the differential rates of cleavage, specific interaction of RNase P with the C-C-A sequence of tRNA precursors is an attractive possibility.

Acknowledgements—We thank Dr. W. H. McClain, in whose laboratory much of this work was done, for helpful discussions, support, and encouragement. We thank B. Stark and S. Altman, Yale University, for generous gifts of φ80 psu* tyrosine tRNA precursor used in the early phases of this work and for communication of unpublished results. We thank Mrs. P. Krie and Ms. K. Foss for technical assistance.

REFERENCES
Transfer ribonucleic acid biosynthesis. Substrate specificity of ribonuclease P.
F J Schmidt, J G Seidman and R M Bock