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**Characterization of the RNaseP Sequence in
*Chlorobium Limicola***

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Abstract

RNAse P is a ribonucleoprotein involved in cutting out pieces of RNA. Specifically, it is involved in tRNA processing as it makes a single endonucleolytic cleavage in all precursor tRNA's to produce mature 5' ends. The sequence encoding the RNA subunit (rnpB gene) of RNAseP has been previously determined in one strain of Chlorobium limicola. A second strain was obtained from Carolina Biological Supply to test for conservation of sequence characteristics. This second strain was used in a PCR with the primers described in Haas. The amplified rnpB gene sequence appeared to have fewer base pairs than the Haas strain when analyzed against a template in gel electrophoresis. Restriction enzyme analysis of the amplified showed consistencies with the Haas sequence. Sequencing of the isolated gene confirmed the similarity between the the Carolina strain of RNAseP and the Haas strain. Structural differences were maintained.

Introduction: RNaseP is a bacterial enzyme involved with tRNA processing. It makes a single endonucleolytic cut in pre-cursor tRNAs to remove 5' flanking sequences and leave the 5' phosphate of mature tRNA's (3). The flanking sequences which exist in pre-cursor tRNA's are quite varied, yet RNaseP will recognize and cleave all tRNA's. This suggests that RNaseP recognizes the shape of the tRNA's rather than a specific sequence of bases on the pre-cursor tRNA's (8). Perhaps the secondary structure, or "shape", of RNaseP facilitates its shape recognition of tRNA's.

It has been shown that the RNA component (377 residues) of RNaseP harbors the catalytic capability (8). With respect to the complete enzyme, the RNA subunit of RNaseP has been shown to work on pre-cursor tRNA with comparable efficiency in recognition, binding, and cleavage in the presence of high magnesium or spermidine concentrations (6). This proves important because most enzymes use proteins as the catalytic moiety. The catalytic RNA moiety adds evidence to the idea that RNA's can be more efficient than proteins in working on some RNA's. This efficiency could be attributed to an increased substrate sequence flexibility with RNA enzymes like RNaseP (8).

This flexibility is exemplified in RNaseP, for it has a highly

conserved functionality across bacterial species while being encoded by varied sequences in different bacteria. The secondary structure of RNaseP RNA also differs between bacterial species apart from the conserved consensus structure.

Because of the variability of the RNaseP enzyme with respect to gene sequence and specifically secondary structure, much time has been devoted to the elucidation of the common catalytic core (consensus structure) of the RNA portion of RNaseP. Comparative analyses of the RNaseP structures and sequences in different bacteria have been initiated to examine this consensus structure (4,2). A better understanding of the consensus structure should lead to a better understanding of how RNaseP functions.

One bacteria that has shown much deviation in RNA subunit structure is *Chlorobium limicola*. *Chlorobium limicola* is a green bacteria that metabolizes sulfur. As an anaerobe, it flourishes in the depths of fresh water lakes and ponds.

This bacteria replaces a helix (P18) that was formerly part of the consensus structure for RNaseP RNA with a single C residue. Since this helix was part of the consensus structure, it was assumed to be important to the functioning of RNaseP. Experiments replacing the P18 helix with a

single C residue in *E. coli*, showed that function was maintained without the helix (4,7,5). The P18 helix was not essential for catalytic activity.

Because *Chlorobium limicola* demonstrated such profound structural differences, the question arose as to the conservation of these structural differences in other strains. This research examined the DNA sequence of a second strain of *Chlorobium limicola* to determine if the structural differences are maintained. Identical DNA sequences would indicate identical RNA sequences which would in turn indicate identical secondary structures.

Materials and Methods:

Culture: A culture of *Chlorobium limicola* was obtained from Carolina Biological Supply Company. The *Chlorobium limicola* was grown in Chromatium medium anaerobically under constant light.

Nucleic Acid extraction: This procedure was adapted from *Current Protocols in Molecular Biology* (1). The bacterial cells were centrifuged for 8 minutes at 10,000 rpm. The pellet was resuspended in 567ul of TE buffer. To this solution were added 30ul of 10% SDS and 3ul of 20mg/ml proteinase K. The contents were mixed thoroughly and incubated at 37°C for one hour. Next, 100ul of 5M NaCl were mixed with the solution. CTAB/NaCl solution was added in the amount of 80ul, mixed and incubated

for 10 minutes at 65 C. An equal volume of chloroform was added, mixed, and the contents were centrifuged for 10 minutes at 14,000 rpm. The aqueous supernatant was transferred to a clean microcentrifuge tube. An equal volume of phenol chloroform was added to the supernatant, mixed, and microcentrifuged for 5 minutes. The supernatant was again transferred to a new microcentrifuge tube; 0.6 volumes of isopropanol were added. The tube was inverted several times. The DNA precipitate was recovered using a ring stick and centrifugation and placed in a new microcentrifuge tube. The DNA was washed with 70% ethanol and microcentrifuged for 5 minutes. The supernatant was discarded and the pellet was left to air dry. The pellet was dissolved in 100ul of TE.

Purifying the DNA: The DNA solution was divided into two separate tubes. To one tube containing 50ul of DNA was added 0.5ul of RNase. This was incubated in a 37°C water bath for 1.5 hours. The DNA was again isolated. An equal volume of chloroform was added, mixed and microcentrifuged for 5 minutes. The aqueous supernatant was transferred to a new microcentrifuge tube. An equal volume of phenol chloroform was added, inverted and microcentrifuged for 5 minutes. The supernatant was again transferred to a new microcentrifuge tube. To this supernatant 0.1 volume of 3M 4.5 pH sodium acetate and 60ul of 95% ethanol were added.

The solution was mixed and microcentrifuged briefly. The supernatant was discarded. The pellet was washed with 70% ethanol and allowed to air dry. The pellet was then resuspended in 100ul of TE. The DNA was spectrometered and diluted to 25ng/ul.

Gel Electrophoresis: Gel electrophoresis was done on 2% NuSieve 3:1 agarose gels. The electric current ran at 100 volts, 125 mA, and 12 watts for varied times. Markers containing bands of known length were run with the samples for band length comparisons.

Primer synthesis: Primers were synthesized using the Oligo 1000® oligonucleotide synthesizer from Beckman. The primers were taken from Haas, et.al. (4).

Primer 5: TAATACGACTCACTATAGGAAACCGCAAGTGTGCAG

Primer 3: CGGATCCAAACCGAAGCTGTAAG

Primer concentrations were measured by absorbance at A_{260} . Stock concentrations of each primer were diluted to 5uM concentration for use in the polymerase chain reaction.

Polymerase Chain Reaction: The polymerase chain reaction was used to amplify the portion of the DNA that encoded for the RNaseP gene in the *Chlorobium limicola* genomic DNA. Primers are short sequences of oligonucleotide that complement the DNA near the ends of the gene. Under

conditions of DNA replication the DNA between the primers (the gene for RNaseP) was amplified (replicated many times). Polymerase chain reaction machines created cycles of temperatures that forced DNA replication.

A 10ul PCR reaction was completed using the AirThermocycler® by Idaho Technologies. This small volume PCR was used to screen for the presence of the gene. The conditions were as follows:

Slope 6, 35 cycles (Different salt concentrations were used.)

Elongation temperature: 72°C for 30 seconds

Annealing temperature: 52°C

Denaturing temperature: 94°C

Reaction mixture:

1ul 3mM MgCl₂ buffer

1ul dNTP's

1ul Primer 5 (5uM)

1ul Primer 3 (5uM)

1ul DNA (25ng/ul)

1ul Taq DNA polymerase

4ul sterile water

10 ul mixture

The Amplitron® from Thermolyne was used to perform 100ul polymerase chain reactions. This larger volume PCR produced a larger amount of the gene product for use in analytical procedures. The product of this amplification was subjected to restriction enzyme analysis and dideoxy sequencing.

Conditions:

Elongation temperature: 72°C for 3 minutes

Annealing temperature: 52°C for 1 minute

Denaturing temperature: 94°C for 1 minute

Reaction mixture:

10ul 10X medium buffer (20mM MgCl₂ at 10X)

10ul 10X dNTP's (2mM each at 10X)

10ul Primer 5 (5uM)

10ul Primer 3 (5uM)

41 ul genomic DNA (25ng/ul)

18ul sterile water

1ul Taq polymerase (2.5U/ul)

100 ul reaction mixture

Restriction enzyme analysis: The DNA strider computer program was used to locate potential restriction enzyme sites in the published sequence. These restriction sites represent short specific sequences in the DNA sequence where certain restriction enzymes will cleave the DNA. The fragments of DNA left after the digestion were separated by gel electrophoresis. Electrophoresis for the restriction enzyme products were run on 2% NuSieve 3:1 agarose gels.

Reaction Set 1: The following restriction enzyme reactions were run with 0.5ul of DNA (the 100ul PCR product), 1ul of the appropriate restriction enzyme buffer, 7ul of sterile water and 1ul of the enzyme: DraI, DraIII, HindIII, or EcoRI. These were incubated in a 37°C water bath for 1 hour and 46 minutes.

Reaction Set 2: The second set of reactions was run in three parts:

1) To each of three microcentrifuge tubes were added: 1ul DNA, 1ul buffer, 7ul sterile water and 1ul of DraIII, HindIII or EcoRI.

2) To each of three microcentrifuge tubes were added: 1ul DNA, 1ul buffer, 7.5ul sterile water and 0.5ul of DraIII, HindIII or EcoRI.

3) To each of three microcentrifuge tubes were added: 1ul DNA, 1ul buffer, 7.7ul sterile water and 0.3ul of DraIII, HindIII or EcoRI.

These reactions were incubated in a 37°C water bath for 2 hours and 30 minutes. Following these reactions, the DNA was cleaned using the Purifying DNA procedure without the RNase step.

Reaction Set 3: To each of three microcentrifuge tubes was added 1ul of the clean DNA, 1ul of the appropriate restriction enzyme buffer, 7ul sterile water and 1ul of DraIII, HindIII or EcoRI. These reactions were incubated in a 37°C water bath for 19 hours.

Reaction Set 4: To each of three microcentrifuge tubes was added 1ul of the clean DNA, 1ul of the appropriate restriction enzyme buffer, 7ul sterile water and 1ul of DraI, RsaI, HindIII or EcoRI. These reactions were incubated in a 37°C water bath for 17 hours and 45 minutes.

Dideoxy Sequencing: Half of the cleaned restriction enzyme DNA was

purified by the Purifying DNA procedure without the Rnase step. This DNA was then used in a 100ul PCR reaction. The product DNA was again cleaned and diluted to 0.018 ug/ul. Original primer products were diluted to 3pm/ul. These dilute reactants were used in the *fmol*® dideoxy sequencing kit from Promega. Sequencing gels were 8% Longranger® polyacrylamide. Gels were run at around 2000 volts for 2 to 4 hours depending on the portion of the DNA being sequenced.

The *fmol* kit was used in conjunction with the Amplitron® PCR machine. Four reactions were set up in four micorcentrifuge tubes each with a different dideoxy base (A,C,T,G) that would incorporate randomly and terminate chain elongation. Different length fragments with a specific terminal base resulted in each reaction tube. The radioactive isotope ^{32}P was incorporated into the reactions to label the fragments. The fragment products of these four reactions were separated on a polyacrylamide gel. The radioactive fragments on the gel exposed autoradiograpy film. The film was developed and the fragments appeared as black bands that could be read in order according to size to determine the base sequence of the DNA.

Results:

Polymerase Chain Reactions: The first PCR reaction gave three defined bands using the three different buffer concentrations and genomic DNA.

The 100ul reaction was run with medium buffer and genomic DNA; a clear band appeared on the gel. (See Figure 1.)

Restriction Enzyme Analysis:

Reaction Set 1: (See Figure 2.) The fragment lengths expected for each restriction enzyme for the 382 base pair (bp) PCR product (amplified fragment) are included in Table 1. Actual fragment lengths produced were recorded as read by the computer program Gelreader and by visual estimation against markers.

Table 1: Restriction enzyme reaction set 1 predicted and experimental band lengths.

Enzyme	382 expected frag length	Exper. frag Gelreader	Exper. frag visual
EcoRI	359, 23		400, 30
HindIII	259, 123		400
DraIII	197, 185		400
DraI	N/A		400

Reaction Set 2: (See Figure 3.) The fragment lengths expected for each

restriction enzyme for the 382 bp PCR product are included in Table 2.

Actual fragment lengths produced were recorded as read by the computer program Gelreader and by visual estimation against markers. The DraIII fragments were difficult to compare to the size markers because the gel was photographed crooked.

Table 2: Restriction enzyme reaction set 2 predicted and experimental band lengths.

Enzyme	382 expected frag length	Exper. frag Gelreader	Exper. frag visual
1ul DraIII	197, 185	395, 438	250, 240
0.5ul DraIII	197, 185	397,433,484	250,240,220
0.3ul DraIII	197, 185	384, 481	Not visible
1ul HindIII	259, 123	No fragments	No fragments
0.5ul HindIII	259, 123	311, 478	300, 140
0.3ul HindIII	259, 123	305, 480	300, 140
1ul EcoR1	359, 23	201	400
0.5ul EcoR1	359, 23	194, 579	380, 30
0.3ul EcoR1	359, 23	209	Not visible

Reaction Set 3: (See Figure 4.) The fragment lengths expected for each restriction enzyme for the 382 bp PCR product are included in Table 3.

Actual fragment lengths produced were recorded as read by the computer program Gelreader and by visual estimation against markers. These

reactions were run with the clean DNA.

Table 3: Restriction enzyme reaction set 3 predicted and experimental band lengths.

Enzyme	382 expected frag length	Exper. frag Gelreader	Exper. frag visual
1ul DraIII	197, 185	861, 420	750, 400
1ul HindIII	259, 123	305,156	300, 125
1ul EcoR1	359, 23	862, 426	750, 400

Reaction Set 4: (See Figure 5.) The fragment lengths expected for each restriction enzyme for the 382 bp PCR product are included in Table 4.

Actual fragment lengths produced were recorded as read by the computer program Gelreader and by visual estimation against markers. These reactions were run with the clean DNA.

Table 4: Restriction enzyme reaction set 4 predicted and experimental band lengths.

Enzyme	382 expected frag length	Actual frag Gelreader	Actual frag visual
1ul DraI	N/A	550	400
1ul HindIII	259, 123	426, 262	260, 125

1ul EcoRI	359, 23	515, 158	375
1ul RsaI	219, 163	385, 312	250, 150

DNA Sequencing: The 382 bp fragment of DNA was sequenced. The same region of the gene was sequenced three times. The sequence overlapped except for 28 bases at the end of the primer 3 sequence and 5 bases at the end of the primer 5 sequence. Of those 382 base pairs 264 were sequenced. In those 264 sequenced bases 5 differences from the published sequence were found (1.89% difference). The 5 differences were all in the regions where no overlapping occurred. One mistake was a T that did not appear on the publication. The other differences were 2 G's (these were in unclear areas of the gel) and 2 C's that were on the publication but not on the dideoxy sequence.

Discussion:

The PCR reactions worked well with the primers described in Haas, et al. (4). The well defined bands were of about 350 to 400 base pairs long; this paralleled the expected fragment length of 382 base pairs from the literature. In some of the gels there appeared to be a faint larger band of about 800 base pairs. This band was reproduced in restriction enzyme analyses. The origin of this band is unknown, perhaps the primers were

slightly specific to another portion of the genome, and amplified an extra piece. The DNA was cleaned between the second and third reaction sets to remove that band.

Restriction enzyme analysis was used to compare the published and experimental fragment sequences. If the restriction enzyme sites found in the published sequence were also in the experimental sequence, fragments produced by digestion of the amplified gene would be the same length as those predicted for the published sequence. Similar size fragments would indicate similar sequences.

Gelreader was used to analyze the fragment lengths; it appeared to misjudge lengths. Visual estimations of the band lengths made by comparisons with the markers were considerably lower than Gelreader lengths. Gelreader illustrated these problems in subsequent digests. Visual estimations were used to get a general idea of whether or not the restriction enzymes were producing fragments of length comparable to those predicted for the published sequence. Thus, fragment length comparisons were not conclusive evidence but general indicators of sequence similarity.

In reaction set one, no fragments resulted from the restriction digests, except a small faint band in the EcoRI lane. This band could have

been the small 23 bp band predicted for EcoR1; the larger 359 bp fragment probably blended with the uncut amplified fragment. The predominance of uncut DNA bands indicated that some component of the digest was insufficient. The second set of reactions varied the amount of restriction enzyme added and increased the incubation time by one hour and 45 minutes. Fragments were present. The visual estimations of fragment size were comparable to the 382 expected fragment lengths. The extra fragment in 0.5ul DraIII could have been shadowing from one of the previous bands. EcoRI 1ul did not appear to cut.

After this second reaction set the DNA was cleaned to remove the faint fragment of about 800 base pairs. The third reaction set had an increased incubation time of 19 hours. This increase was implemented to allow time for maximal digestion. Results varied. Dra III and EcoR1 showed the large fragment; it was not sufficiently removed from the DNA. Their other band appeared to be an uncut amplified fragment. The HinDIII gave bands of comparable length to the predicted 382 base pair fragment lengths.

The fourth reaction set also ran for an extended incubation time. The Dra I digestion gave an uncut amplified fragment. This was expected, for Dra I had no cut site in the published sequence for the amplified

fragment. The *HinDIII* and *RsaI* digestions gave bands of reasonable size. The *EcoRI* digestion was also of comparable size, the small 23 base pair fragment probably ran off the gel.

Overall these restriction digests gave band lengths comparable to the expected fragment lengths for the published sequence. The sequences contained similarities. Sequencing gave more conclusive evidence to support the similarity of the published and experimental sequences.

The 5 differences between the sequences were in regions where the sequencing runs did not overlap. Any other differences were corrected by one of the overlapping runs. It seems likely that if those regions had been repeated the differences would have been corrected.

The mistakes could have resulted for different reasons. The gels had regions with bands that were less clearly defined; the two G's were in such a region. Some shadow bands also appeared on the gels. These bands were not actually present, but were shadows of previous bands. The T that was not present on the publication could have been a shadow band and not actually in the experimental sequence. The two missing C's were each next to another C on the publication. The two C's in a row on the gel could have been read as only one C.

The P18 helix area of the secondary structure was sequenced. No

differences appeared in this region; the P18 helix was still missing.

The restriction enzyme analysis and sequencing done on the genes supported the published sequence for *Chlorobium limicola* RNaseP RNA. Thus, the differences in secondary structure of the RNaseP RNA subunit in *Chlorobium limicola* appear to be conserved between strains. The results of this project added support to the refined consensus structure for RNaseP RNA (4) which lacks the P18 helix.

By elucidating consensus structure for an enzyme with varied sequence but uniform function, one can begin to answer questions about the mechanism for that function. In this case the mechanism by which RNaseP recognizes, binds, and cleaves pre-cursor tRNA's can be examined. When the function of the consensus sequence is learned, roles for the varied structural elements in the different bacterial RNaseP's can be assigned.

Figure 1: Electrophoresis gel showing the amplified portion of the RNaseP RNA gene in *Chlorobium limicola*. The marker in lanes 1 and 3 contains bands of lengths 50, 150, 300, 500, 750, and 1000 base pairs. The amplified band in lane 2 reads close to 400 base pairs in length. A faint band between 750 and 800 base pairs in length is also visible in lane 2.

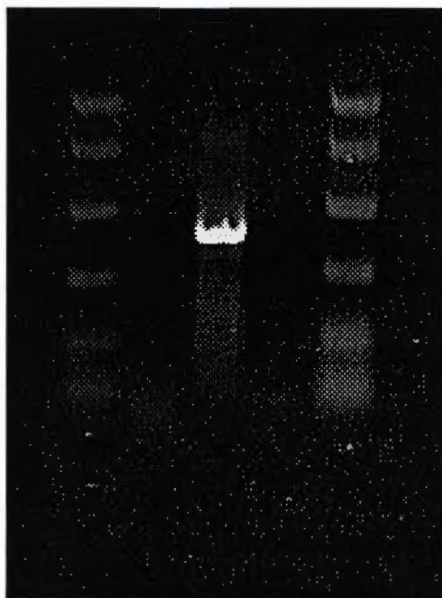


Figure 2: Electrophoresis gel for restriction enzyme reaction set 1. Lane 1 contains lambda marker. Lane 6 contains the 123 marker with band lengths increasing in multiples of 123 base pairs. Lanes 2,3,4, and 5 contain EcoRI, HindIII, DraIII and DraI, respectively.

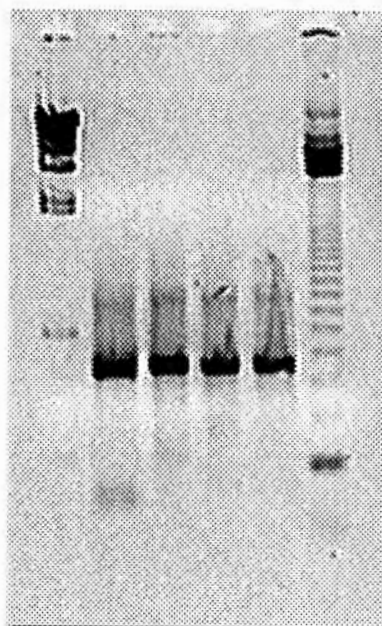


Figure 3: Electrophoresis gel for restriction enzyme reaction set 2. Lane 1 contains lambda marker. Lane 11 contains the 123 marker with band lengths increasing in multiples of 123 base pairs. Lanes 2,3, and 4 contain 1ul, 0.5ul, and 0.3ul of DraIII, respectively. Lanes 5,6, and 7 contain 1ul, 0.5ul, and 0.3ul of HindIII, respectively. Lanes 8,9, and 10 contain 1ul, 0.5ul, and 0.3ul of EcoRI, respectively.

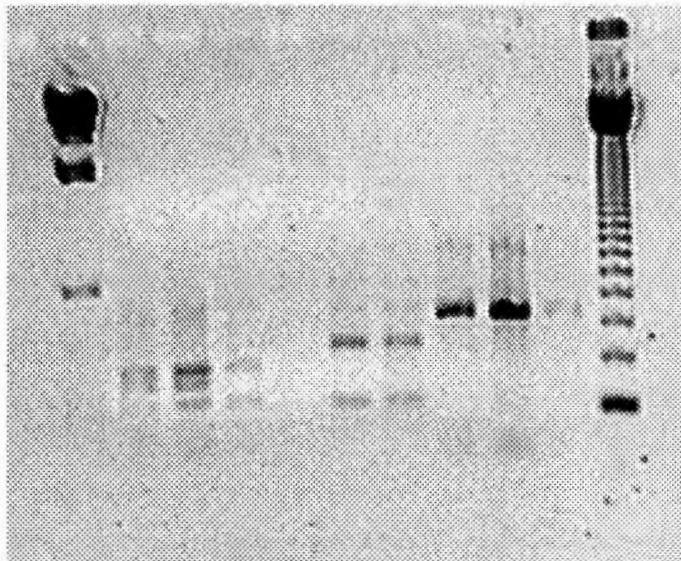


Figure 4: Electrophoresis gel for restriction enzyme reaction set 3. Lane 1 contains lambda marker. Lane 5 contains the 123 marker with band lengths increasing in multiples of 123 base pairs. Lanes 2, 3, and 4 contain DralII, HindIII, and EcoRI, respectively.

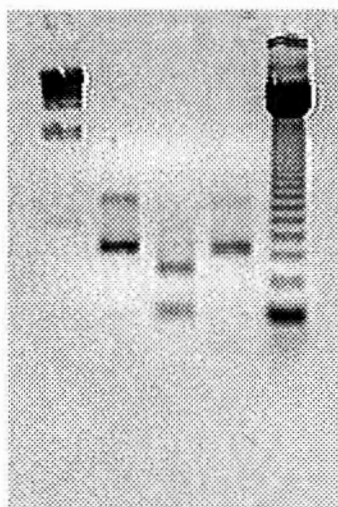
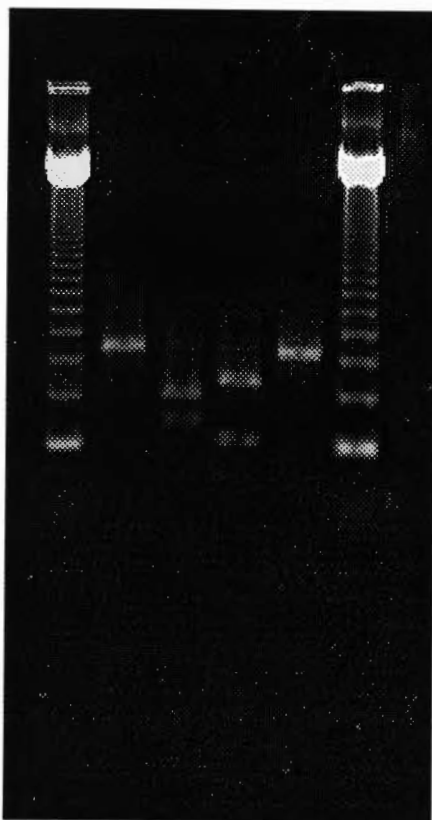


Figure 5: Electrophoresis gel for restriction enzyme reaction set 4. Lanes 1 and 6 contain 123 marker with band lengths increasing in multiples of 123 base pairs. Lanes 2,3,4, and 5 contain DraI, RsaI, HindIII, and EcoRI, respectively.



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