Point mutations in the middle of 16S ribosomal RNA of *E. coli* produced by deletion loop mutagenesis

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ABSTRACT

Using the plasmid pKK3535, which contains the *rrnB* operon of *Escherichia coli* in pBR322, a deletion mutation was constructed which lacks bases 822 to 874 in the middle of the 16S ribosomal RNA. This results in an "amputation" of a very distinct stem and loop structure in the RNA. By forming a heteroduplex between the deletion plasmid and the original pKK3535 and by modifying the single-stranded deletion loops with bisulfite, we produced plasmids containing one or two base changes at positions 839, 840, 841, 867 or 876. The clustering of the mutations near the top of the stem, and the inability to get base changes at other positions, suggests that single alterations at particular positions severely affect the formation of a functional ribosome. The ability to recover mutations at these positions is not determined by the secondary structure of the DNA during bisulfite mutagenesis. Restriction enzyme analysis of 12 revertants from a slow growing mutant (altered at positions 839 and 876) shows that they did not compensate for the mutation by re-establishing the original wild type sequence.

INTRODUCTION

The production and isolation of mutants in ribosomal RNA is made difficult by the fact that the genome of *E. coli* contains seven different operons coding for ribosomal RNA (1,2). The availability of a multicopy plasmid containing a single copy of the *rrnB* operon (3,4) has allowed for the application of *in vitro* mutagenic techniques to the plasmid coded rDNA. This provides a way of investigating the expression and regulation of ribosomal RNA synthesis and of examining the structural and functional features of rRNA in the ribosome (see 5 for review).

Since the entire sequence of pKK3535 is known, deletion mutations ranging from one to several hundred bases can be constructed at specific restriction sites in both 16S rDNA and 23S rDNA (6). In a modified maxicell system (7,8) plasmid coded

rRNA is synthesized in the absence of host coded rRNA synthesis. Ribosomal proteins and rRNA processing enzymes continue to be synthesized from the host chromosome. Thus plasmid coded rRNA can be processed and fully assembled into ribosomes in the maxicell. This makes it possible to study the effects of rRNA mutations on ribosome structure, function and assembly.

Our initial studies on rRNA mutants described deletion mutations produced by exonuclease digestion of the rDNA cleaved at specific restriction sites. By forming a heteroduplex between DNA from a plasmid containing a deletion and DNA from an unaltered plasmid, a single stranded region opposing the site of the deletion is produced. Point mutations can be introduced into this region by modifying the single stranded loop with bisulfite. Because both DNA strands of each plasmid form heteroduplexes, G to A as well as C to U base changes are to be expected in the rRNA. This approach has been used successfully with derivatives of pBR322 containing genes coding for specific proteins (9,10). Here we report on the first base changes produced in rRNA by bisulfite mutagenesis using a 53 base deletion mutant in 16S rDNA.

MATERIALS AND METHODS

Bacterial Strains

E. coli HB 101; BD 817 (ung⁻) was kindly provided by Dr. B. Duncan (Institute for Cancer Research, Philadelphia). All strains were stored in 50% glycerol at -80°C.

Plasmid

pKK3535 containing the rrnB operon of E. coli (4) was kindly provided by Dr. H. Noller. Large scale preparations of this plasmid and the plasmid SalI-72 were carried out according to standard procedures.

Enzymes

Polynucleotide kinase, T4 DNA polymerase and restriction enzymes were purchased from New England Biolabs. Reactions were carried out as recommended by the company, but leaving out the BSA. Calf alkaline phosphatase was from Boehringer, Mannheim; ribonucleases T₁ and A from Sigma.

Gel Electrophoresis

DNA fragments were analyzed on 0.8 to 2% agarose gels containing Tris-acetate, EDTA (11) and 1 µg/ml ethidium bromide.

Separation of smaller fragments for sequencing was carried out on 7% acrylamide gels containing Tris-borate-EDTA buffer (11).

Construction of the Deletion Mutant Sall-72

pKK3535 was partially digested with the restriction endonuclease Sall. After phenol extraction and religation, the DNA was added to competent (12) cells of *E. coli* HB101. Transformants were grown at 37°C on LB agarose plates containing 50 µg/ml ampicillin (6) and after 1 to 3 days single colonies were picked and grown on LB agarose plates containing 200 µg/ml ampicillin. Plasmids from these cells were isolated and the DNA was sized on a 0.8% agarose gel. Plasmids of approximately wild type size were then digested with BglI. The appropriate deletion mutant within the 16S rRNA gene was readily detected by the appearance of a new, large (8 kb) fragment due to the loss of a BglI restriction site located between the two Sal I sites in 16S rDNA (Fig. 1).

Heteroduplex Formation and Bisulfite Modification

Point mutations were produced by the procedure described by Kalderon et al. (9). Approximately 5 µg of plasmid DNA was linearized by digestion with the appropriate restriction endonuclease. EcoRV and HpaI were used with pKK3535 and the Sall-72 mutant, respectively (see Fig. 2). The samples were phenol extracted and then 3.5 µg aliquots of each DNA were combined, ethanol precipitated, washed twice with 95% ethanol, vacuum dried and finally dissolved in 23 µl of water. The DNA was denatured by adding 4.8 µl of 1 M NaOH and incubating at 20°C for 10 min. Reannealing was then accomplished by adding 280 µl water, 40 µl of 0.5 M Tris-HCl pH 8.0 and 4.8 µl of 100 mM HCl and incubating at 63°C for 2 hrs. The DNA was precipitated three times with ethanol and finally dissolved in 100 µl water. Deamination was carried out in the dark under paraffin oil for 4 hrs at 37°C after addition of 1 ml fresh 3 M sodium bisulfite, 2 mM hydroquinone. DNA was dialyzed against 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, overnight at 4°C. After ethanol precipitation

Fig. 1 Construction of the deletion mutant Sall-72.

the pellet was dissolved in 40 μ l dialysis buffer. Transformation of competent BD 817 cells, isolation of single colonies and plasmid miniprepations were carried out as described above for the construction of the deletion mutant Sall-72.

Sequence Analysis of the Mutants

An EcoR1 DNA fragment of 2191 basepairs, covering the region of bisulfite mutagenesis, was isolated from a 2% agarose gel by electroelution. The DNA was end labeled with α -P³²ATP using polynucleotide kinase and digested with Hha1, resulting in a 432 basepair fragment labeled on one end only. This was sequenced by the chemical method of Maxam and Gilbert (13).

RESULTS

Isolation of Point Mutations

To study the effects of single base changes in ribosomal RNA, bisulfite mutagenesis of cytosine residues in single strand regions of DNA was performed as described in Materials and Methods. The deletion mutant, Sall-72, was constructed as shown

Fig. 2 Scheme outlining site specific, deletion loop mutagenesis using pKK3535 and Sall-72. Linear homoduplexes have a much lower efficiency of transformation than the circular heteroduplex.

in Fig. 1, and results in a clean "amputation" of a stem and loop structure (bases 822 to 874) in 16S RNA. This mutation increased the doubling time of BD817 cells to 95 min from a normal doubling time of 45 min when wild-type pKK3535 is present. In this study the mutant plasmid DNA was used as the starting material to introduce point mutations into this particular region. After bisulfite treatment of the heteroduplexes and transformation (see Fig. 2), colonies were picked daily over a period of four days. While rapidly grown colonies appeared after the first day, many slow growing colonies became apparent only after three days. Plasmids were isolated from the transformed cells and analyzed by digestion with the restriction enzyme XmnI, which creates a fragment of 960 basepairs if no sequences are deleted from the

Fig. 3 Autoradiogram of a 10% sequencing gel showing the sequence of the wild-type plasmid and bisulfite mutants. Solid arrows indicate sites of mutations. Open arrows are corresponding sites in wild-type. BS36 is identical to the sequence of pKK3535.

pKK3535, and a fragment of 907 basepairs in the SalI72 mutant. All of the faster growing colonies were found to contain the 960 bp fragment while the very slow growing colonies contained the SalI-72 mutant. Plasmids containing the larger, 960 basepair fragment were then subjected to sequence analysis using the G and C reactions of the Maxam-Gilbert procedure (13).

The sequence data of three mutant plasmids are given in Fig. 3. In the case of the transformant BS18, all four reactions were used but only the expected C to U changes were observed. In analyzing many of the plasmids we sequenced about 150 bases, including the 53 bases subjected to bisulfite modification. Mutations were detected within the region of the SalI-72 deletion

	SAL1 820 ↓ 830	BGL1 840 ↓ 850	HHA1 860 ↓ 870	SAL1 880 ↓	Doubling time of BD 817 (Min.)
pKK 3535	AT GTCGACTTCG	AGGTTGTGCC CTTGAGGCGT	GGCTTCGGGA GCTAACGCCGT	TAAGTCGACC	
BS 17,21,22,36,49	no changes				46
BS 3,27	T				46
BS 4	TT				49
BS 18	T T				56
BS 25,30,31,37	T				49
BS 35	T				48
BS 32	A				46

Fig. 4 Summary of the point mutations obtained by bisulfite treatment. In addition to sequence data all changes were confirmed by the use of restriction enzymes as shown (on top). The doubling time of the different mutants is shown on the right side of the figure. The growth rate of cells containing pKK3535 is identical to mutants BS17, 21, 22, 36 and 49.

from position 822 to 874, in all but one case. The only exception was the mutant BS18 which contained a base change at position 876 (see Fig. 4). This can be explained by the fact that the deletion was created by cutting with Sal1 at two sites with identical sequences and religating, thus replacing the two Sal 1 sites with a single site. As a result either of the two Sal1 sites in pKK3535 could base pair to the newly constructed Sal1 site in the deletion mutant. The second site remains in the single stranded loop-out and is thus accessible to bisulfite mutagenesis.

The results of the sequence analysis of 15 transformants are listed in Fig. 4. Also listed are the doubling times of cells containing the mutant plasmids. Base changes were found in five positions: C-839 (BS3, BS4, BS18, BS27), C-840 (BS4, BS25, BS30, BS31, BS37), C-841 (BS35), G-867 (BS32) and C-876 (BS18). In addition, five transformants showed no altered sequence, (BS17, BS21, BS22, BS36, BS49), grew at normal rates and are therefore presumed to be wild-type pKK3535.

Assuming that all of the cytosine-residues within the single stranded region of the heteroduplex were equally accessible to bisulfite treatment, 33 possible positions are available for modification. However, only five positions were found to be altered and these are clustered in positions 839, 840 and 841 in nine of ten transformants. In addition, only one G to A

transition was observed, yet it was clear from the analysis of 65 transformants that both strands of the heteroduplex were replicated.

The C to U transition at position 839 did not affect the growth rate of cells containing this particular plasmid, as shown in BS3 and BS27 in Fig. 4, while a small effect on growth rate was detected for alterations at positions 840 and 841, as shown by BS25 and BS35. The mutant BS18 contains changes at position 839 and 876. While the mutation at position 839 is silent in the transformants BS3 and BS27, we speculate that it is also silent in BS18 and that the altered growth rate of BS18 is the result of the mutation at position 876.

Secondary Structure Analysis of the Single-Stranded Regions of the Heteroduplexes

It was clear from the analysis of the growth rates of the isolated mutants that a single base change could have a profound effect on the growth rate (BS18). It was not obvious, however, whether there was a strong selection against most of the potential base changes by the cell or if only a few bases were modified by bisulfite in the first place, due to secondary structure in the heteroduplex. To explore this possibility we examined possible base pairing of the single-stranded regions in the heteroduplexes. The stability of potential interactions was calculated according to Tinoco et al. (15), using relative stability designations of 2, 1 and 0 for G-C, A-T and G-T base pairs, respectively. Only helix formations with a stability of 5 or greater are taken into account. Fig. 5 (a) shows the matrix for possible structures of the sequence which is responsible for C to U transitions in the RNA. Fig. 5 (b) shows the same basepairing scheme for the complementary strand which is responsible for G to A transitions.

It is obvious from Fig. 5 (a) that almost all bases in the region 822 to 874 are, theoretically, able to pair with each other and establish several different alternative structures. All of the C to U transition mutations we observed occurred in bases which could pair in one or more secondary structures. Thus, the altered bases were not especially exposed and there is no reason why they should have been modified preferentially by

Fig. 5 Possible secondary structures of the single stranded DNA region during bisulphite mutagenesis. a) Structure of the strand responsible for C to U changes; b) structure of the complementary strand responsible for G to A changes. Possible secondary structures are indicated by rows of stars. Black diamonds point to bases which were found to be modified by bisulfite. Circled stars give the positions of these bases in the hypothetical secondary structure.

bisulfite. Thus, secondary structure was not a likely reason for the clustering of the mutations.

In the case of A to G transitions (Fig. 5 b), the matrix showed very little potential for basepairing. The greatest stability that could be calculated did not exceed 6. Therefore it is even less likely that secondary structure played a role in limiting the selection of A to G transitions to only one mutant plasmid.

Isolation of revertants from BS 18

During the growth of strain BD 817 containing plasmid BS 18 on LB plates (200 µg/ml ampicillin), it was noticed that somewhat larger colonies appeared at a frequency of about 0.2% of viable colonies. Plasmids were isolated from 12 different colonies in order to answer the question of whether these plasmids had somehow reestablished the original pKK3535 sequence. The DNA was digested with Sall and SmaI and compared to DNA from pKK3535 and BS18, also digested with both enzymes,

Fig. 6 Restriction enzyme analysis of plasmid DNA from 12 revertants of the double point mutation BS 18. DNA from revertants, mutant BS28 and pKK3535 was digested with Sall and SmaI and electrophoresed into a 7% acrylamide gel (11).

with the results shown in Fig. 6. In all 12 cases fragments identical to those of BS18, not pKK3535, were produced. In addition, this experiment ruled out the possibility that G-824, normally base paired with C-876 in the rRNA structure (Fig. 7), had changed to an A to complement the C to U transition at 876. This would have destroyed the second Sall site and given rise to a new 770 base pair fragment, which was not observed in Fig. 6. Thus this experiment shows that strains BS18r1 to r12 are actually pseudorevertants which have undergone an alteration elsewhere to compensate for the harmful C to U transition at position 876.

DISCUSSION

We have demonstrated that it is possible to introduce point mutations in rRNA by deletion loop mutagenesis in an ung⁻ strain. Mutants were produced by bisulfite treatment of single stranded regions of rDNA in a heteroduplex formed between a deletion-mutant plasmid and a plasmid containing the undeleted

Fig. 7

Location of the altered bases in the secondary structure of 16S ribosomal RNA.

Solid arrows point to new bases in mutants.

The shaded area defines the region where

bases are conserved between small rRNA's of

E. coli, Zea mays chloroplasts, Saccharomyces cerevisiae and Xenopus laevis (17).

operon. The point mutations appeared to be limited to the area defined by the deletion. We cannot, however, exclude the possibility that base changes did occur elsewhere without sequencing the entire operon. This possibility seems unlikely, however, because sequence analysis of approximately 50 bases on each side of the 53 base deletion revealed no alterations and the growth rates of mutants with the same base change were identical. Revertants that were detected were shown to have retained the original point mutations, and thus base substitutions, once introduced into the rRNA, did not revert to reestablish the original sequence.

The region of 16S rRNA we have studied has a well defined stem and loop structure (see Fig. 7) as determined primarily by phylogenetic comparison of rDNA sequences from different organisms (16,17,18,19). Contained within the structure are conserved as well as variable regions. The area at the base of the stem is conserved among E. coli, Zea mays chloroplast, Saccharomyces cerevisiae and Xenopus laevis small rRNA's (16). As might be expected, a base change in this conserved area (position 876 in BS18) had the most severe effect on the growth rate of cells containing this particular plasmid (Fig. 4).

Almost all of the other base changes are clustered near the top of the stem (positions 839, 840, 841) and they produced little or no change in the growth rates. Interestingly this region of the stem and loop structure varies considerably (base changes and/or deletions) among different organisms underlining the apparent reduced importance of this area for the function of the E. coli ribosome. This is also the only region of this structure (bases 840-843) not protected from ribonuclease digestion in the RNP complex composed of S6, S8, S15, S18 and 16S rRNA (20). A protected region of quite variable sequence among different organisms, at positions 852 to 857 within the stem structure, did not contain any point mutations.

Analysis of possible interactions within the single stranded region of the DNA heteroduplex suggested that no secondary structure effects exist during bisulfite modification. This is apparent when analyzing the DNA strand where deamination of C residues creates G to A changes in the RNA (compare Fig. 5a and b). The highest stability of any possible structure is 6 and it is unlikely that such a weak interaction can exist during bisulfite modification. However, we cannot exclude the possibility that non -Watson-Crick base pairs contribute to the structure, which might then prevent modification of certain bases. Nevertheless it is remarkable that only one G to A transition (position 867) was found among the mutants isolated. The C to U transition always weakens the interaction between two RNA strands by bolishing the three hydrogen bonds of the G-C pair, but the new G-U pair does not disrupt and destabilize the secondary structure (16). This is not the case for a G to A transition. Basepairing between A and C should not occur at all. On the other hand a stabilization of secondary structure should take place if a G involved in a G-U "base pair" is converted to an A. In the upper part of the rRNA stem (832-837 paired with 849-854) there exists a row of five G-U base pairs interrupted by one G-C interaction. One might expect to see a G to A mutation in this region which would produce an A-U base pair and stabilize the stem structure. This was not observed and therefore we must suspect that the G-U "pairs" are at these positions for a particular reason. It could well be that they do

not exist in a stem structure in the ribosome but instead interact with other regions of the RNA or are opened up by a ribosomal protein, possibly S6. Data that protein S6 is close to or even binding to this region of the RNA come from protection experiments using isolated ribosomal proteins and 16S rRNA (20) as well as mutant rRNA containing various deletions affecting this central region of 16S rRNA (21).

The mutagenesis approach we have described here has the advantage of providing information about the significance of selected bases in the RNA in an in vivo environment. The transformed cells are provided with a whole series of different point mutations contained within a defined region of rRNA, of which, we assume, only some are viable and can be isolated. A disadvantage of this approach clearly lies in the fact that not every theoretically possible base change in the plasmid rDNA can be isolated, particularly changes at certain positions so detrimental to ribosome function and the translation process that the cells cannot survive or they grow very slowly. The isolation of mutant plasmids from very slow growing cells is made difficult by the fact that the deletion mutant Sall-72, used in the bisulfite mutagenesis, is expressed at high rates (about 50%) and these transformed cells grow very slowly. Thus, the likelihood of selecting a slow growing point mutation in a vast excess of slow growing Sall-72 colonies is low. Furthermore, there is no simple procedure for selecting point mutants. More advanced techniques such as site-directed mutagenesis using synthetic oligonucleotides are currently being applied in our laboratory to circumvent these problems and determine if base changes at certain positions actually do have a detrimental effect. In summary, bisulfite mutagenesis of rDNA should prove to be a very useful approach to study rRNA in regions where little is known about its structural or functional role. This is the case for most of the regions of ribosomal RNA at the present time.

It is interesting that a relatively large amount of revertants could be isolated from one particular point mutation (BS18). Analysis of 12 independent revertants (fast growing cells containing the mutant plasmid) from BS18 showed that they were actually pseudorevertants. In preliminary experiments we

find no evidence for a reduced or enhanced copy number of the plasmid (data not shown) and it is likely that the reversions represent one or more types of alteration in the host genome, possibly a change in a ribosomal protein or some other factor involved in rRNA processing, assembly or function. Studies are now under way to determine the nature of these changes. This will provide a new approach to the study of the ribosome.

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