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The conformation of tRNA genes

Chemical modification studies

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It has been suggested that eukaryotic tRNA genes might adopt a higher order stem and loop structure to facilitate transcription by interaction of their variably spaced intragenic promoter blocks. Using sodium bisulphite, which reacts specifically with cytosine residues in single-stranded nucleic acids, no deamination of C in the TTCGAA sequence of the 3′ ICR of a $tRNA^{Leu}$ gene could be detected under conditions which caused 60% deamination of cytosine residues within the loop region of a synthetic cruciform cloned in the same negatively supercoiled plasmid vector. We conclude that, under these conditions, such structures occur in tRNA genes very rarely, if at all.

tRNA gene; Transfer RNA; Conformation; Bisulphite; Transcription; Cruciform

1. INTRODUCTION

Eukaryotic tRNA genes are transcribed by RNA polymerase III in conjunction with specific transcription factors, including TFIIIB and TFIIIC (reviewed in [1–3]). Although the efficiency of transcription of tRNA genes is influenced by their flanking sequences [4–6], the promoter regions are primarily within the genes themselves. By internal deletion and substitution studies of several tRNA genes, these intragenic control regions (ICRs) have been defined as two blocks [7–10]; box A, or the 5′ ICR, comprises residues 8–19 and thus corresponds to much of the D-arm of the tRNA; box B, or 3′ ICR, comprises at least eleven residues corresponding to much of the TψC arm in the tRNA. Following the standard conventional numbering adopted for tRNA [11], the 3′ ICR comprises residues 52–62. The binding of the factor TFIIIC to the two separated intragenic control regions is a prerequisite for binding of the TFIIIB upstream of the start site and subsequent assembly of RNA pol III [12]. However since tRNAs vary in the length of their extra arm the separation of the above two boxes also varies. This box A–box B separation is even greater in tRNA genes which contain introns so that, for example, it varies between 31 and 93 residues for yeast tRNA genes [13]. This observation has led to speculation as to the mechanism by which the intragenic control regions of different tRNA genes can bind the appropriate common transcription factors when their relative disposition is so variable. One attractive hypothesis is that the tRNA genes might adopt a higher order stem and loop structure which would allow interaction of the 'B block' with the 'A block' [14]. This hypothesis was supported by the results of point mutations in yeast $tRNA^{Tyr}$ gene [15] and *X. laevis* $tRNA^{Met}i$ [16]. The transcriptional activity of two poorly transcribed mutants of the latter gene was restored in a double mutant which would allow formation of a stable intrastrand hairpin. However when Newman et al. [17] tested the hypothesis, by engineering the leucine-inserting amber suppressor SUP53 tRNA gene from yeast to alter residues in the 'A block' and 'B block' but maintain potential for an intra-strand hairpin in the 'A block' and interaction of the the blocks, they found no evidence for the proposed intrastrand tertiary structure.

Reynolds and Gottesfeld [18] showed that a plasmid containing tandemly repeated units of oocyte-type 5S DNA was cleaved by S1 nuclease. This cleavage, centred at 5′ boundary of the intragenic promoter, was largely dependent on negative supercoiling. To our knowledge no comparable study has been made of eukaryotic tRNA genes. One of us has previously reported the isolation and characterisation of a human $tRNA^{Glu}$ gene, the immediate 5′-flanking sequence of which could potentially form a tRNA-like structure, the first loop of which contains a unique *Sst*I site

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(GAGCTC) [19]. Such structures appear to be common within this $tRNA^{Glu}$ gene family [20]. We have subsequently isolated a cluster of tRNA genes which includes a $tRNA^{Leu}$ gene, which contains a unique *Asu*II site (TTCGAA) in the B block, i.e. at a position corresponding to the GTψC loop in the tRNA [21]. This fortuitous location of cytidine-containing unique restriction sites within putative single-stranded regions allows an investigation of the conformation of the DNA at these sites by reaction with sodium bisulphite. This reagent is highly specific for cytosine residues within single-stranded nucleic acids [22], including those in loop regions of cruciform structures induced in negatively supercoiled DNA [23]. We have therefore sought to detect loss of these restriction sites in supercoiled recombinants which have been treated with sodium bisulphite, increasing the sensitivity of detection by further analysis of digested plasmids amplified in a repair-deficient host.

2. MATERIALS AND METHODS

2.1. *Plasmid recombinants*

The recombinant ptGlu contained residues 1–481 of a previously characterised sequence [19], including a $tRNA^{Glu}$ gene and its 5′-flanking sequence, cloned between the *Eco*RI and *Bam*HI sites of pAT153 [24]. It was constructed via the M13mp9 recombinant containing the same sequence.

The recombinant ptLeu was constructed by insertion of the 241-bp *Eco*RI–*Hin*dIII fragment, containing a characterised $tRNA^{Leu}$ gene [21], between the *Eco*RI and *Hin*dIII sites of pAT153.

The recombinant pIRekb44, kindly supplied by Dr. D.M.J. Lilley, has been described previously [25].

2.2. *Reaction with sodium bisulphite*

20 μg of DNA (ptGlu, ptLeu, pIRekb44 or pIRekb44 + ptLeu) were incubated at 37°C in the dark with 3 M sodium bisulphite, 0.5 mM hydroquinone for 0, 5 or 24 h, followed by dialyses as described by Shortle and Nathans [26]. The DNA was recovered by ethanol precipitation.

2.3. *Recombinant manipulations*

Restriction enzymes were from Pharmacia Ltd. and used in accordance with the manufacturer's recommendations. Restriction digests were analysed by electrophoresis through 1.5% agarose gels using pAT153 digested with *Hae*III and λ DNA digested with *Eco*RI and *Hin*dIII as fragment size markers.

E. coli BD 1528, a uracil glycosylase deficient (*ung*−) strain [27] kindly supplied by Dr Tom Lindahl, was transformedwith untreated or bisulphite-reacted plasmid recombinants by the method of Hanahan [28]. Transformation efficiencies were approximately 10^7 colonies/μg of DNA but when the plasmids were linearised by cleavage at a unique restriction site, these were reduced to approximately 10^5 colonies/μg of DNA.

3. RESULTS AND DISCUSSION

It has been suggested that tRNA genes may adopt a tRNA-like conformation [14]. We have used sodium bisulphite to probe for the presence of single-stranded loop regions which would occur if the higher order structure proposed for tRNA genes was induced by torsional stress by negative supercoiled DNA. This reagent, long known to specifically deaminate cytosine residues occurring in single-stranded RNA [22], has been shown to deaminate cytosines in the loop regions of cruciform structures in negative supercoiled DNAs [23].

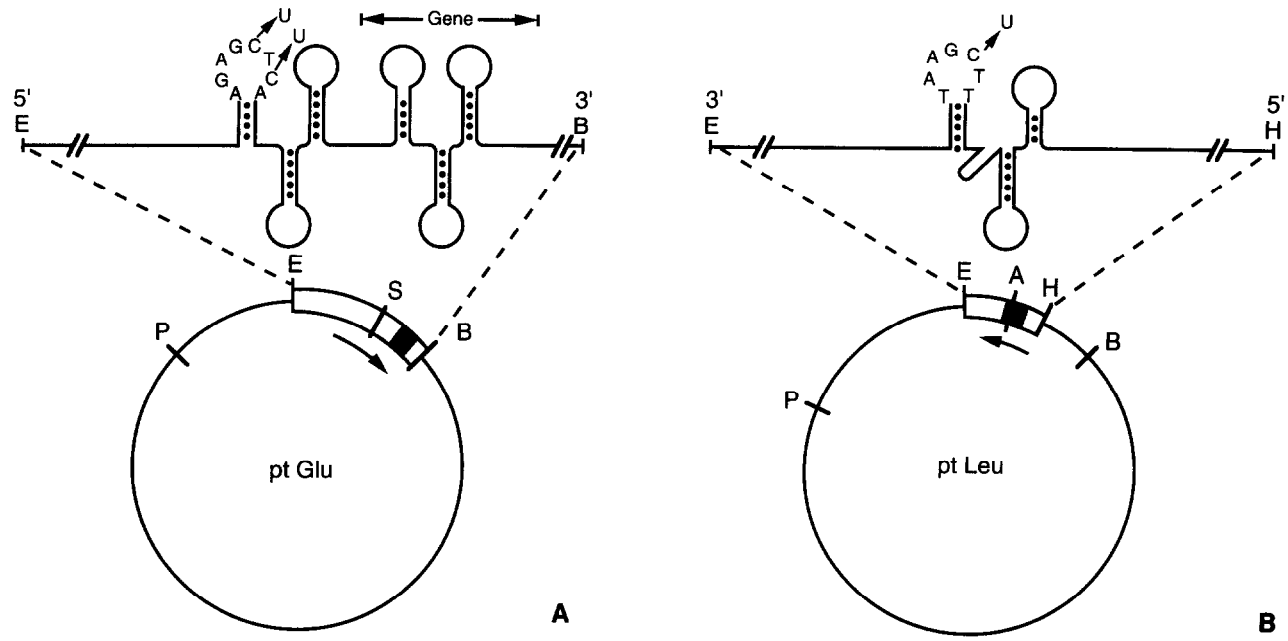

Fig. 1. Simple maps of the pAT153 recombinants ptGlu (A) and ptLeu (B). The tRNA genes are shown as filled boxes. Arrows indicate the direction of transcription. The proposed secondary structure of the RNA-like strand of each insert showing potential accessible cytosine residues within the unique *Sst*I site in the 5′ flank of the $tRNA^{Glu}$ gene (A) or the unique *Asu*II site in the 3′ ICR of the $tRNA^{Leu}$ gene (B) is also shown. (A = *Asu*II, B = *Bam*HI, E = *Eco*RI, *Hin*dIII, P = *Pst*I, S = *Sst*I, bp = base pair; restriction digest fragment sizes are P–S = 1058 bp; S–B = 184 bp (Fig. 1A) and P–A = 882 bp; A–B = 455 bp (Fig. 1B)).

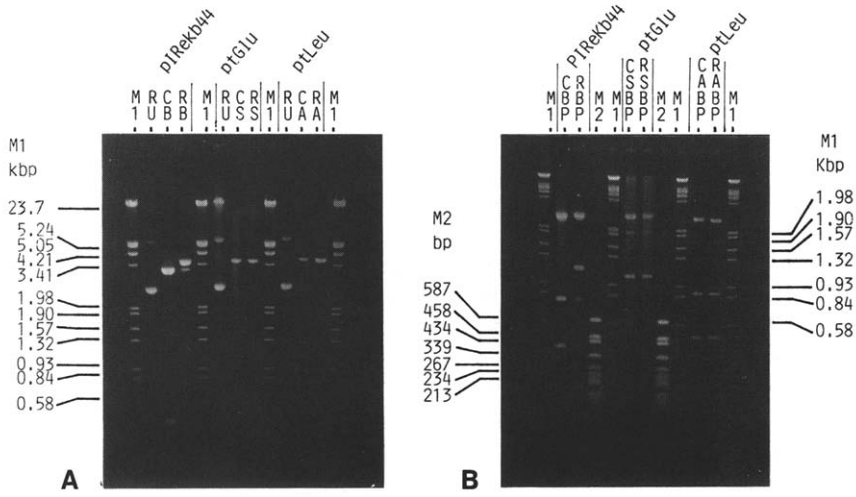

Fig. 2. (A) Analysis of bisulphite reacted [R] or untreated control [C] pIRekb44, ptGlu or ptLeu digested with *Bam*HI [B], *Sst*I [S], or *Asu*II [A], respectively, or undigested [U]. (B) Analysis of fragments released from reacted [R] or control [C] DNAs following multiple digestions with *Bam*HI [B], *Pst*I [P], *Sst*I [S] and/or *Asu*II [A]. Length of DNA size markers M1 [λ DNA/*Hin*dIII] and M2 [pAT153/*Hae*III] are shown.

One of these, pIRekb44 has a synthetic oligonucleotide, containing an inverted repeat and *Eco*RI, *Bam*HI and *Kpn*I sites, cloned into the *Eco*RI site of pAT153. It thus contains two *Bam*HI sites, one of which occurs within the loop regions of the cruciform structure adopted by an inverted repeat, so that the cytosine residues are accessible to bisulphite. However cytosines in the second *Bam*HI site, located 288 bp away in the duplex vector, are inaccessible to the reagent.

The pAT153 recombinants of tRNA genes chosen for comparison with pIRekb44 are illustrated schematically in Fig. 1. Each contained cytosine residues within a unique restriction site at a position which would be a single stranded loop region if the proposed higher order structure was adopted in the supercoiled DNA. Consequently these cytosines would be accessible to bisulphite and deaminated with consequent loss of that restriction site: recombinant ptGlu contains residues 1–481 of a previously reported sequence containing a $tRNA^{Glu}$ gene (356–427) and its 5′-flanking sequence [19]. This flanking sequence has the potential for forming a tRNA-like structure with a unique *Sst*I site in the first loop. Recombinant ptLeu contains a 241 bp *Eco*RI–*Hin*dIII fragment of human DNA containing a $tRNA^{Leu}$ gene (66–146) in which a unique *Asu*II site occurs at 129–134 within the putative TψC loop comprising the 5′ ICR [21].

These supercoiled plasmids were separately treated with sodium bisulphite under identical conditions for 24 h at 37°C. This treatment [26] caused no relaxation of the supercoiled DNA (RU in Fig. 2A). The DNAs were then digested with an excess of the appropriate restriction enzyme and analysed by gel electrophoresis. Untreated pIRekb44 yielded equimolar amounts of 3.3 kb and 388 bp fragments with no visible band at 3.7 kb. This 3.7 kb band was present in approximately 60% molar yield in the bisulphite treated pIRekb44 and the 3.3 kb and 388 bp produced were correspondingly reduced (RB cf. CB; Fig. 2A). Further digestion with *Pst*I

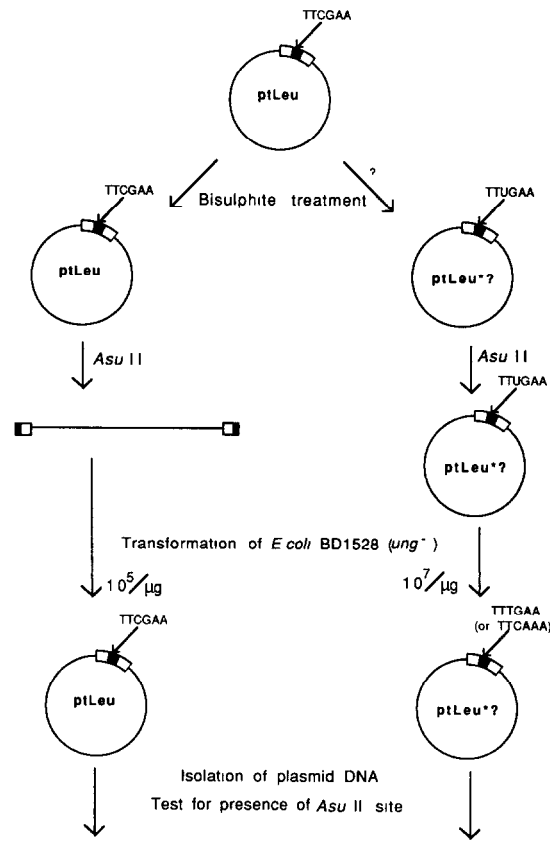

Fig. 3. Scheme for testing the accessibility of cytosine residues within the unique *Asu*II site of ptLeu. Any recombinants with a modified site (pt Leu*; right) would not be linearised by *Asu*II and thus would transform *E. coli* BD1528 far more efficiently than the unmodified *Asu*II-linearised recombinant (left). An analogous scheme is adopted for the unique *Sst*I site in ptGlu.

confirmed that all vector *Bam*HI sites had been cut and that therefore under these conditions approximately 60% of the G/GATCC sequences located in the loop of the cruciform has been modified at least once (Fig. 2B). In contrast all the bisulphite treated ptGlu or ptLeu was apparently linearised by *Sst*I or *Asu*II, respectively (Fig. 2A). This was confirmed further by digestions to release the insert fragments. No intact insert was detected on agarose gels indicating that destruction of the *Sst*I site of ptGlu or of the *Asu*II site of ptLeu by bisulphite treatment was less than 1%, the limits of detection of the intact insert fragment (Fig. 2B).

The sensitivity of detection of modified restriction sites was increased approximately 100-fold by our second analysis (Fig. 3). Untreated or bisulphite-treated plasmids before and after digestion with *Sst*I (ptGlu) or *Asu*II (ptLeu) were used to transform repair-deficient *(ung−) E. coli* BD1528. The restriction enzyme digestion reduced transformation efficiency from approximately 10^7 colonies/μg of DNA to approximately 10^5 colonies/μg of DNA in all cases. Restriction analyses of the DNA produced from colonies arising from these transformants again revealed no destruction of the *Asu*II site of the ptLeu or the *Sst*I site of the ptGlu. From these results we conclude that in supercoiled DNA where the cruciform structure of pIRekb44 is stable, the hypothetical higher order structure suggested for tRNA genes occur rarely (approximately 10^{-4}), if at all. Although such structures do not appear to be induced by supercoiling alone, their generation by factors in the formation of the initiation complex cannot be ruled out. Negative supercoiling of the gene may enhance the efficiency of this complex formation since we (E.S. Gonos, unpublished) and others [29] have noted that linearisation of negatively supercoiled recombinant tRNA genes reduces their in vitro transcriptional activity 2- to 3-fold.

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