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Two regions in human DNA polymerase β mRNA suppress translation in *Escherichia coli*

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ABSTRACT

Although human DNA polymerase β (DNA polβ) shows 96% identity with rat DNA polβ at the amino acid level, it is weakly expressed in *Escherichia* (*E.*) *coli* relative to the rat enzyme. The mechanism of this suppression was investigated. Pulse-chase protein labeling and steady state mRNA analysis showed that mature human DNA polβ protein is relatively stable in *E.coli* and the levels of human and rat DNA polβ mRNA were comparable indicating that the human DNA pol β expression is suppressed at the translational level. By systematic expression analysis of a number of chimeric genes composed of human and rat cDNAs, two strong translational suppression regions were mapped in the human DNA polβ mRNA; one was named TSR-1, corresponding to CGG encoding arginine (arg) at postition 4 and the other, termed TSR-2, is located between codons 153 and 199. Since substitution of the rat Arg-4 codon with synonymous codons showed strong effects upon the expression level, we propose that the arg codon at the N-terminal cording region plays a role in modulating expression.

INTRODUCTION

DNA polβ is found in a wide range of animals, from sponges to humans (1), and functions in DNA repair (2) and probably recombination (3). The holoenzyme is composed of a single polypeptide of approximately 38KD and is the smallest among known prokaryotic and eukaryotic DNA polymerases. cDNAs encoding rat and human DNA polβ have been cloned and sequenced (4, 5). Rat DNA polβ has been overproduced in *E.coli* and purified to homogeneity (6), facilitating mapping of functional domains on the DNA polβ polypeptide (7–11). However, the human enzyme is expressed at a very low level relative to the rat enzyme (7), although the two cDNAs show 96% and 91% identity at the amino acid and nucleotide levels, respectively. We have initiated a study of the mechanism by which human DNA polβ expression is suppressed to learn more about the modulation of gene expression. Human DNA polβ is an ideal model for such study because comparison with the corresponding rat gene allows systematic determination of the stage when the suppression occurs and the cDNA or mRNA sequences involved.

Although many heterologous and engineered genes have been expressed in *E.coli*, some proteins were not highly synthesized even under the control of a strong promoter. Expression level is determined by the secondary structure and stability of mRNA (12–14), translational efficiency of that message, (15–18), and stability of the translated protein product (19).

In this report, we describe how the expression of human DNA polβ is suppressed and identity two strong translational suppression regions (TSR) in human DNA polβ mRNA.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *E.coli* strains used in this work were JM109 (20), and BL21 carrying a single copy of the bacteriophage T7 RNA polymerase gene in the chromosome under control of the *lac* UV5 promoter (21). Expression vector pJHpβ was constructed from pUC118 (22) by replacement of the *lac* Z gene with human polβ coding sequence as previously described for construction of the rat DNA polβ expression vector pJMpβ5 (6). Expression vector pTHpβ was constructed from pJHpβ by replacement of the *lac* promoter with a T7 promoter by one-step mutagenesis (11). For mRNA analysis, a transcription termination signal from the *trp* operon was added downstream of the muti-cloning site (MCS) in pJHpβ and pJMpβ5 (23). Nucleotides were subsequently deleted between the stop codon and termination signal to distinguish rat and human mRNAs from each other and 16S ribosomal RNA. Sequences removed were from *Stu* I to*Hind* III (just downstream of the human cDNA stop codon to the MCS) in pJHpβ and *Sac* I to*Hind* III (both in MCS) in pJMpβ5. Resultant vectors were named pJHpβ-t and pJMpβ-t, respectively.

Expression analyses of DNA polβ

A single colony was used to inoculate 20 ml 2× YT medium (1.6% polypeptone/1.0% yeast extract/0.5% NaCl) containing 50 μg/l ampicillin and grown at 37°C. When the absorbance at

600 nm reached 0.2, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and growth was continued for 4 hr. Cells were collected and disrupted by sonicator as described preivously (10). Crude lysates were analyzed by 12.5% $NaDodSO_4$-polyacrylamide gel electrophoresis and gels were stained with Coomassie brilliant blue R250. DNA polβ activity was detected by the activity gel method as described by Spanos *et al* (24).

Pulse-chase experiment

BL21 cells harboring pTHpβ or pTMpβ were grown at 37°C in 2.5 ml methionine assay medium (Difco) supplemented with 0.1% glucose. When the absorbance at 600 nm reached at 0.2, IPTG was added to a final concentration of 1 mM. After a 1 hr induction, cells were labeled with $[^{35}S]$methionine (1.0 Mbq/ml) for 30 sec and chased with unlabeled methionine at 0.5 mg/ml for the indicated times. Cells were treated with trichloroacetic acid and the cell extracts were subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis followed by fluorography (25).

Preparation and analysis of mRNA

E.coli JM109 carring pJHpβ-t or pJMpβ-t was used for in vivo RNA preparations. After a 4 hr induction of DNA polβ synthesis with IPTG, cells were collected from a 2 ml culture (2× YT medium) and mRNAs were prepared by the hot phenol method (26). One-tenth of each sample was treated with glyoxal and then electrophoresed through a 0.7% agarose gel. RNA was blotted to a nylon membrane and visualized by staining with 0.04% methyleneblue. Hybridization was carried out overnight at 37°C in 30% formamide/2×Denhardt's solution/4×SSC/1% SDS/ denatured salmon sperm DNA (100 μg/ml)/$[^{32}P]$cDNA encoding human DNA polβ (0.25 MBq/ml). Blot was washed two times in 1×SSC/0.1% SDS at 42°C for 20 min and once in 0.2×SSC/0.1% SDS at 55°C for 20 min.

Site-directed mutagenesis

Changes in the nucleotide sequence were made by oligonucleotide-primed synthesis on single-stranded circular plasmids (6). Oligonucleotide sequences are as follows; 5′-TCCTGCGGCGCCTTCC-3′ for introduction of *Bbe* I; 5′-TTTG(GC)TTGATTCACTAGTGAAGC-3′ for *Spe* I introduction, 5′-GG(GC)GCCTTACGTTTAGT-3, 5′-GG(GC)GCCTTGCG-TTTAGT-3′, 5′-GG(GC)GCCTTTCGTTTAGT-3′, 5′-GG(GC)GCCTTCCG-TTTAGT-3′, 5′-GCGG(GC)GCCTTCCTTTTAGTCAT-3′, and 5′-GCGG(GC)GCCTTTCTTTTAGTCAT-3′ for Arg-4 codon substitution. All mutant sequences were confirmed by dideoxy sequencing.

RESULTS

Cloning and expression of human DNA polβ

A bacteriophage λgt11 human placenta cDNA library (Clontech) was screened for DNA polβ by plaque hybridization with the 700 bp *Hind* III fragment from rat polβ cDNA (6). Of the 10^6 recombinants screened, five were positive. A clone containing the longest fragment (1.3 kb) was subcloned to pUC118 and subjected to sequence analysis. The clone contained 84bp of 5′-UT and 223 bp of 3′-UT including a 71 b of poly A tail. When compared to the human DNA polβ sequence reported by SenGupta *et al.* (4), Eight nucleotides were different in the structural gene; four of the changes are silent (position 486, 777, 783 and 849) and four resulted in amino acid changes (Ile-22, Tyr-260 and Lys-287 are Leu, Ile and Leu, respectively in our data). For each discrepancy our sequence is equivalent to the rat cDNA sequence (5). On the basis of our data, human DNA polβ cDNA shows 91% and 96% identity with rat at the nucleotide and amino acid levels, respectively.

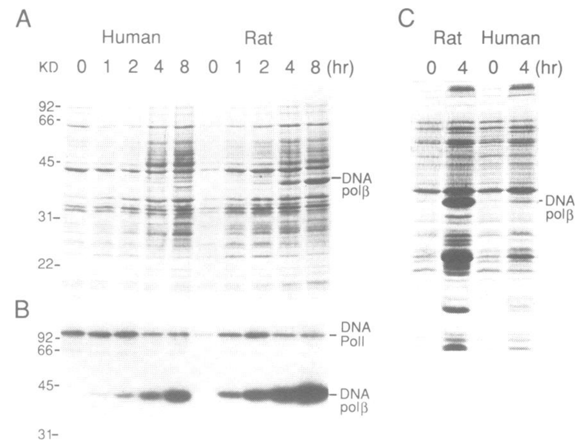

Fig. 1. Expression of human and rat DNA polβ in *E.coli.* Human and rat DNA polβ were expressed from the *lac* promoter/operator in *E.coli* JM109 and analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis (A). Cells were grown for the indicated time in the presence of IPTG. DNA polymerase activity was measured by the activity gel method (B). Human and rat DNA polβs were also produced by a T7 RNA polymerase expression vector in *E.coli* BL21 (C).

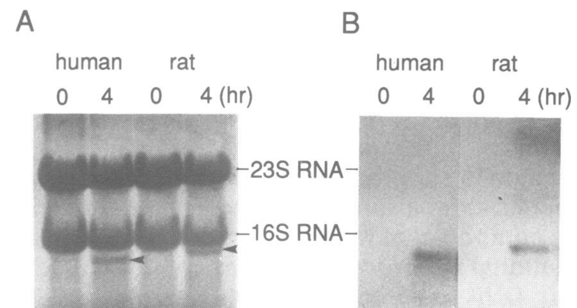

Fig. 2 Comparison of human and rat DNA polβ mRNA. After transfer of RNA to nylon memmbrane, the membrane was stained with methyleneblue (A) followed by Northern hybridization (B). Arrows indicate mRNA encoding DNA polβ.

An expression vector for human DNA polβ, pJHpβ, was constructed from pUC118 and the enzyme encoded was expressed in *E.coli* JM109. As shown in Fig.1A, human DNA polβ (38 KD) was poorly expressed relative to rat DNA polβ. The amounts of human and rat 38 KD polypeptide detected at 8 hr after induction were 0.4% and 16% of total *E.coli* protein, respectively. The 38 KD polypeptide was confirmed as DNA polymerase by the activity gel method (Fig. 1B).

Human DNA polβ was also weakly expressed in *E.coli* BL21 from the T7 RNA polymerase expression vector (Fig. 1C). After a 4 hr induction with IPTG, human DNA polβ was synthesized

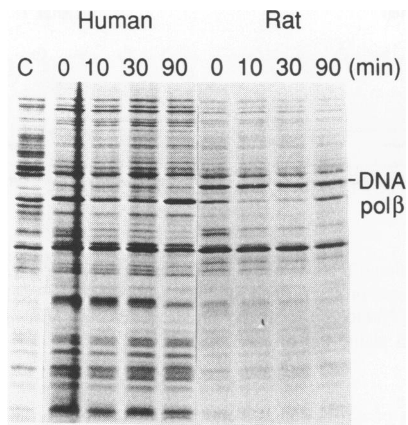

Fig. 3. Pulse-chase analysis of human and rat DNA polβ stabilities. After derepression with IPTG, cells were labeled with [^{35}S] methionine for 30 sec, chased with cold methionine, and harvested at the indicated times. The control lane (C) shows cells labeled before addition of IPTG.

at a very low level, in contrast to the corresponding rat enzyme, indicating that the expression of human DNA polβ is suppressed in *E.coli*.

Analysis of human DNA Polβ mRNA

In order to compare the amounts of mRNA encoding human and rat polβ, the expression vectors pJHpβ-t and pJMpβ-t, carrying transcription termination signals were introduced into *E.coli* JM109. These plasmids showed almost the same expression profile of 38KD protein as pJHpβ and pJMpβ5, respectively (data not shown). After 4 hr growth in the presence of 1 mM IPTG, cells induced significant amount of mRNA (Fig. 2). As expected, the sizes were 1.05 kb and 1.1 kb for human and rat DNA polβ, respectively and both mRNAs were identified as DNA polβ mRNA by hybridization using human cDNA as a probe. After normalizing to ribosomal 16S or 23S RNA, the amounts of the two DNA polβ mRNAs are almost equal, indicating that expression of human DNA polβ was not suppressed at the transcriptional level.

Stability of human DNA polβ

To determine whether the low level expression of human DNA polβ is caused by rapid protease digestion after completion of protein synthesis, a pulse-chase experiment was performed. *E.coli* BL21 harboring the pTHpβ plasmid was used since it can grow in minimal medium. After a 1 hr induction with IPTG, cells were pulse-labeled for 30 sec with [^{35}S] methionine and chased with unlabeled methionine for the indicated times (Fig. 3). Human DNA polβ was degraded with a half life of about 2 hr which is almost the same as that of rat DNA polβ. Thus, mature human DNA polβ is fairly stable after completion of translation.

Mapping of translational suppression regions

On the basis of the above findings, it is probable that expression of human DNA polβ is suppressed at the translational level due to specific sequence in the human DNA polβ mRNA. In order to map the translational suppression region (TSR), a number of human-rat chimera genes were constructed from JMpβ5 and JHpβ using the common restriction endonucleases, *Xho* I, *Eco* RI, and *Eco* RV, and expressed in *E.coli* JM109.

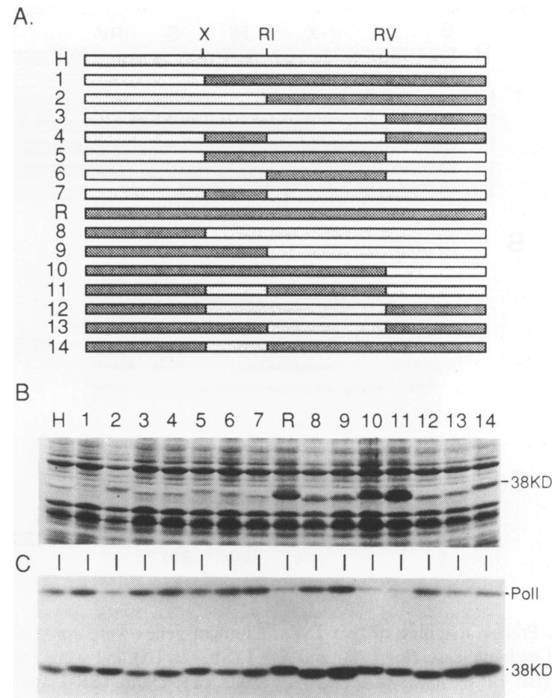

Fig. 4. Structure of chimera genes and their expression levels. Chimera genes were constructed from human and rat DNA polβ cDNAs using the shared restriction endonuclease sites, *Xho* I (X), *Eco* RI (RI), and *Eco* RV (RV). Human and rat derived sequences are shown by light and dark gray regions, respectively (A). Each chimera gene was expressed in *E. coli* JM109 and subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis (B). DNA polymerase activity of each gene product was assayed by the activity gel method (C).

Figure 4 shows 14 different constructs and their expressions levels. Since human DNA polβ contains two more basic amino acid residues than the rat while the latter contains two more acidic acid residues than the former, each chimeric protein has a different net charge and moved according to the rule that more basic proteins move faster. All recombinants containing human N-terminal coding sequence resulted in low levels of 38 KD polypeptide expression, suggesting that a strong TSR is present between the initiation codon and *Xho* I site in the human DNA polβ coding sequence. This TSR was named TSR-1. This is not the only human DNA polβ TSR, though, since the expression levels of 38 KD polypeptide produced by reciprocal chimeras which contain rat N-terminal coding sequence were higher but still variable. Recombinants containing the human cDNA fragment between *Eco* RV and the termination codon resulted in strong expression of the 38 KD polypeptide whereas recombinants containing the human fragment between *Eco* RI and *Eco* RV, such as chimeras 8, 9, 12, and 13, resulted in poor expression of the 38 KD polypeptide. These results suggest that a second TSR designated TSR-2 is present in the *Eco* RI-*Eco* RV region of the human DNA polβ coding sequence.

The activity of each chimera product was measured by the activity gel method. All products showed DNA polymerase activity (Fig. 4C) and their specific activities obtained from the ratio of incorporated [^{32}P]dCMP to staining intensity at 38 KD were similar (Data not shown). These results indicate that different amino acids between human and rat do not play an important role in protein folding and in catalysis of DNA polymerase activity.

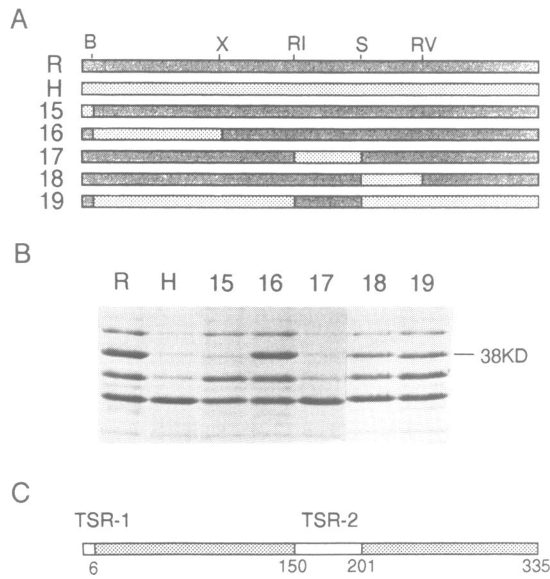

Fig. 5. Precise mapping of two TSRs. Chimera genes were constructed using created endonuclease *Bbe* I (B) and *Spe* I (S) sites (A) and were expressed in *E.coli* (B). On the basis of the above results,TSR-1 and TSR-2 are illustrated with codon positions indicated (C).

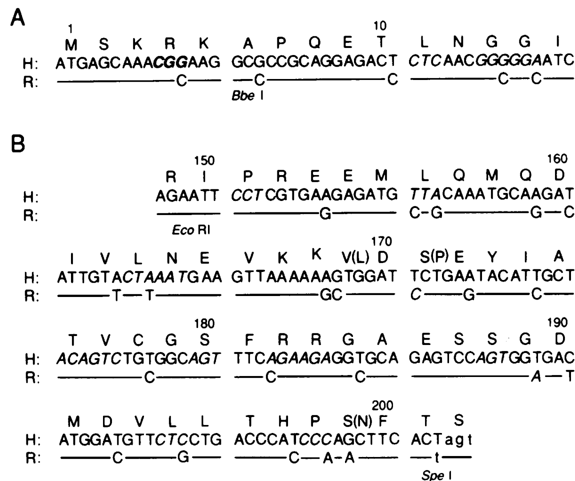

Fig. 6. The nucleotide and deduced amino acid sequences of the regions including TSR-1 (A) and TSR-2 (B) in the human DNA polβ coding sequence and comparison with the corresponding rat sequences. Amino acids are represented by one letter and numbered from the initiation codon. Differences between the human and rat cDNAs are indicated below each row for nucleotides and in parentheses for amino acid changes. Minor codons and a codon including TSR-1 are shown by italic and bold, respectively.

In order to more precisely define TSR-1 and TSR-2, further chimeras were constructed. However, no other common restriction endonuclease sites further subdivide TSR-1 and TSR-2. Thus, in the rat cDNA, we changed one nucleotide at position 18 (in 6th codon) from C to G to create a *Bbe* I site that is already present at this position in the human cDNA. Additionally, a *Spe* I site was created at nucleotide positions 601−606 (codons 201−202) in both human and rat cDNAs by changing three (in the human) and four nucleotides (in the rat cDNA) without alteration of the amino acid. Chimeras were generated with these

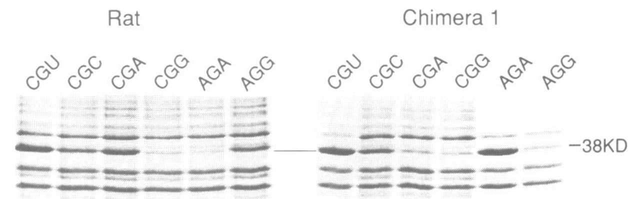

Fig. 7. Substitution effects of Arg-4 codon on the expression level of DNA polβ and Chimera 1 gene product. Each modified gene was expressed in *E.coli* JM109 and analysed by $NaDodSO_4$-polyacrylamide gel electrophoresis. The position 4 codons is shown at the top of each lane.

Table I. Effects of Arg-4 codon identity on the expression levels of rat DNA polβ and chimera 1 product.

Codon	Expression level (%)[a]		Relative codon
	Rat	Chimera 1	usagage (%) (27)
CGT	20.4	23.6	28.3
CGC	9.2	9.6	21.4
CGA	14.3	1.5	2.6
CGG	1.4	1.3	3.6
AGA	1.2	22.7	1.4
AGG	12.5	2.8	0.9

[a]The stained gels shown in Fig.7 were scanned at 540 nm from the origin to the front and the ratios of 38KD protein/total protein were obtained.

new sites and introduced into *E.coli* JM109 followed by induction of protein synthesis. The introduction of these restriction enzyme sites did not affect the expression level of DNA polβ.

As shown in Fig. 5, the chimera containing human N-terminal coding sequence resulted in very low expression of the 38 KD polypeptide. Comparison of human and rat sequence (Fig. 6A) indicates that G at position 12, the third nucleotide of the Arg-4 codon is reponsible for TSR-1 activity. On the other hand, chimeras containing the human *Eco* RI-*Spe* I region expression showed very low level expression of the 38 KD polypeptide, suggesting that TSR-2 can be further localized to codons 153−199 (Fig. 6B).

Codon dependency of Arg-4 on the expression level

In order to examine the codon dependency on the expression level, the 4th codon was substituted by synonymous codons in both the rat DNA polβ cDNA and chimera 1 gene which has the human 304 bp 5′ sequence (Fig. 4). As shown in Fig.7, the expression level of each rat polβ and chimera 1 product in *E.coli* JM109 was strongly dependent on the 4th codon identities; the statistically favored arg CGU codon enhanced expression of both and the minor codon CGG used in human DNA polβ cDNA also suppressed the expression of rat DNA polβ. However, AGA and AGG which are also minor codons for *E.coli*, showed opposite effects on the expression of the two genes. These results indicate that the translational suppression by TSR-1 is caused by neither tRNA starvation alone nor the guanine nucleotide at position 12 alone. The expression level of each modified rat DNA polβ and chimera 1 product is summarized in Table I with the values of relative codon usages for arg in *E.coli* (27). It is noteworthy that even CGC, the Arg-4 codon in unmodified rat DNA polβ mRNA, works as a weak TSR and that alteration of this codon in pJMpβ5 to CGU produced an improved expression vector for rat DNA polβ.

DISCUSSION

Human DNA polβ is expressed at a very low level in *E.coli*, in contrast to the rat enzyme. We demonstrate here that the low level expression of human DNA polβ results from inefficient translation due to *E.coli* translational suppression sequences in the protein-coding region. Systematic analysis of a number of chimeras composed of human and rat cDNA revealed that human mRNA contains two strong translational suppression regions named TSR-1 and TSR-2, which map to the Arg-4 codon and the region between codons 153 and 199, respectively.

Secondary structure of mRNA at the initiation codon is known to be responsible for translation efficiency (13, 33), i.e. the expression level of murine μ heavy chain immunoglobulin is low in *E.coli* due to the hairpin structure at the region between nucleotide positions 2 and 17. Wood *et al* overexpressed the protein by removal of this site (28). However, no such structure was detected near the initiation codon of human DNA polβ mRNA indicating that translational suppression by TSR-1 is not caused by local secondary structure of mRNA.

Human DNA polβ mRNA contains 94 codons which differ from the rat mRNA: of these only 14 encode different amino acids. In both human and rat DNA polβ coding sequences, the minor codons occur with a frequency of 28% when 22 codons are considered as minor (29). This value is remarkably higher than the average of *E.coli* genes (29−31). Codon usage patterns have been shown to be related to the relative abundance of tRNA isoacceptors (15) and genes encoding proteins of high versus low expression show differences in their codon preferences (29). The possibility that the presence of minor codons in a gene slows the rate of translation and lowers the expression level has been widely discussed, but recent experiments and compilation of gene expression data have revealed no positive correlation (17). Our results also show that weak expression of human DNA polβ in *E.coli* is not caused by the high percentage of minor codons because the expression level of the corresponding rat gene is high.

It is very interesting that TSR-1 is located in the N-terminal coding region and maps to a minor codon having a rare tRNA associated with it (15). Several experiments have demonstrated that N-terminal coding sequence plays an important role in determining the expression level in *E.coli* (17, 33, 34). On the basis of expression analysis of a *lac*Z gene having an artificial repeat of five AGA codons in its coding sequence, Chen and Inoue proposed that the minor codons occurring within the first 25 codons of a mRNA play an important role in negative regulation of expression (33). Within the first 15 codons human polβ mRNA contains three more minor codons than rat (Fig. 6A), but only one of these, CGG encoding Arg-4, is identified as TSR-1 and is also effective as a TSR in the rat mRNA. However, substitution of the 4th codon with synonymous codons, indicated that the translational suppression does not occur by starvationof tRNA associated with the 4th codon, since two minor codons, AGG and AGA, permit efficient expression. Furthermore, codon CGA, which is statistically rare codon in *E.coli* (29) but is recognized by the same tRNA for CGU and CGC (32), enhanced the expression of rat DNA polβ but not the chimera 1. The reverse effects of AGA, AGG, and CGA on the expression of the two genes is determined by nucleotide differenes within the 304 bp of the 5′ fragment, which shows 94% and 99% identity at the nucleotide and amino acid levels, respectively.

Recently, others reported that a high level of expression in *E.coli* correlates with. high adenosine and uridine content in the N-terminal sequence of the mRNA (34). The suppression of human DNA polβ expression cannot be explained by this rule due to the identical profile of adenosine and uridine nucleotides within human and rat DNA polβ N-terminal coding sequences.

Although rules for translational modulation by Arg-4 codon have not been found yet, we poropose that arg codons in the N-terminal coding sequence plays a role in modulating expression. Due to the weak correlation between codon usage and expression level, and effect of neighboring codon on the expression level, we suppose that TSR-1 in mRNA destabilizes the initiation complex by blocking access to the initiation signals (33) or stall ribosomes by slowing the rate of elongation, the latter of which is mainly caused by minor codon (16).

The further characterization of TSR-2, located between codons 153−199, is now in progress. As shown in Fig. 7, there are 21 codon differences and three of them result in amino acid changes. A computer aided search for secondary structure of this region in polβ mRNA did not show any difference between human and rat, suggesting that suppression is caused by a codon or other unknown primary sequence, possibly a protein binding site. Systematic mutagenesis of TSR-2 has yielded several recominants whose expression level are strongly modulated. The modulator sequences will be described elsewhere. The suppression mechanisms for the two TSRs should be different since the position, codon, and length of nascent polypeptide when a ribosome encounters each TSR on mRNA are different.

If TSR patterns become identifiable in underexpressed genes, our approach might provide rules for modifying codon sequences for the purpose of altering expression level in a genetically engineered expression system.

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