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Rate of Translation with Different mRNA with Varying Shine-Dalgarno Sequence and Aligned Spacing

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Title (English)

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Abstract

Translation – the mRNA directed synthesis of proteins in living cills is catalysed by a large nucleic-acid protein complex called the ribosome. The ribosome binds to a specific sequence motif on mRNA, in E. coli refereed to as the Shine-Dalgarno sequence after its discoverers. In this study the effects of various Shine-Dalgarno sequence and aligned spacing on di and tri peptide formation have been measured using rapid kinetic techniques.

Keywords

Ribosome, mRNA, Protein Synthesis, Shine-Dalgarno, Kinetics

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Sammanfattning

Livet är en av de mest komplexa processer som människan känner till, varje enskild levande cell består av hundratusentals olika komponenter som samverkar med varandra på olika sätt. En av de mest grundläggande egenskaperna hos livet är dess förmåga att föröka sig och växa. För att kunna göra detta måste celler tillverka livets byggstenar – proteinerna. I cellerna, såväl mänskliga celler som de minsta bakterierna, sköts proteintillverkningen av ribosomerna. Ribosomen läser av det meddelande som bärs av ett mRNA och använder det som ritning för att tillverka ett protein. De senaste åren har mycket ny kunskap om hur ribsomen fungerar kommit i dagen men det är fortfarande mycket som är oklart. En av oklarheterna är hur olika sekvenselement i början av ett mRNA påverkar hastigheten i proteinsyntesen. I den här studien har vi försökt besvara de frågorna genom att mäta hastigheten i de här första stegen då ribosomen läser av olika mRNAn.

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Abbreviations

30S	The small ribosomal subunit
50S	The large ribosomal subunit
70S	The complete bacterial ribosome
AA-tRNA	Amino acylated transfer RNA
A-site	tRNA Acceptor site on the ribosome
ATP	Adenosine triphosphate
EF-G	Elongation Factor G
EF-Tu	Elongation Factor Tu
EF-Ts	Elongation Factor Ts
E-site	tRNA exit site on the ribosome
fMet	Formylmethionine
fMet-tRNA^{fMet}	Formylmethionine transfer RNA charged with formylmethionine
GTP	Guanosine triphosphate
IF1	Initiation Factor 1
IF2	Initiation Factor 2
IF3	Initiation Factor 3
Leu	Leucine
mRNA	Messenger RNA
PEP	Phosphoenolpyruvate
Phe	Phenylalainine
P-site	Peptidyl site on the ribosome
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SD	Shine-Dalgarno
tRNA	Transfer RNA

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1. Introduction

Protein synthesis is a central process in all living cells, it has been estimated that in growing E. coli bacteria it consumes more than 40% of all energy available to the cell. All protein synthesis in the cell is catalysed by a macromolecular complex called the ribosome. The ribosome consists of nucleic acid and protein, and is highly conserved throughout all three domains of life. Knowledge about this complex has increased rapidly in recent years, in particular since the publication of high resolution crystal structures of the bacterial ribosome[1,2] and many of its active complexes[3]. Due to the ribosome's central role in cellular metabolism and the differences between eukaryote and bacterial ribosomes it has become an important target for antibiotics. Understanding of ribosomal function and regulation also plays an important role in biotechnology; as the expression of a heterologous protein can be significantly affected by incompatibilities with the host cell's protein synthesis machinery.

1.1 Introduction to Bacterial Protein Synthesis

The ribosome directs protein synthesis by reading the nucleotide sequence of a messenger RNA (mRNA) molecule and joining together the amino acids brought to it by transfer RNA (tRNA) into a polypeptide chain. The bacterial ribosome consists of a large (50S) and a small (30S) subunit that together form the complete (70S) ribosome. Each ribosome can bind to one mRNA molecule and has three tRNA binding sites, the Acceptor, Peptidyl, and Exit sites (A, P, and E sites).

Translation is initiated by binding of mRNA to the 30S subunit together with initiation factors and initiator tRNA charged with formylated methionine (fMet-tRNA^{fMet}), this pre-initiation complex then docks with the 50S subunit to form the 70S initiation complex. Initiation of translation involves many protein factors and is in itself a field of intense study[4]. *In vivo* many ribosomes will bind sequentially to the same mRNA forming a so called polysome[5].

Once the 70S initiation complex containing mRNA and fMet-tRNA^{fMet} is formed the ribosome can transit into the elongation phase of translation (Fig. 1). Each elongation cycle begins when ternary complex, aminoacylated tRNA (AA-tRNA) in complex with elongation factor-Tu (EF-Tu) and GTP, binds to the empty ribosomal A site. If the anticodon of the tRNA does not match the codon on the ribosome bound mRNA, the tRNA will dissociate from the ribosome[6]. If the codon and the anti-codon match the tRNA will accommodate into the peptidyl transfer centre and peptide bond formation will take place. After formation of the peptide bond and transfer of the nascent

polypeptide chain to the newly arrived tRNA both the mRNA and the tRNAs will be translocated through the ribosome by elongation factor-G (EF-G). Translocation involves movement of the A and P site tRNAs to the P and E sites respectively, as well as movement of the mRNA by three nucleotides in the same direction – presenting a new codon in the A site. Shortly after translocation the E site tRNA will dissociate from the ribosome and a new ternary complex will bind to the A-site to begin the next elongation cycle[7]. This process will continue until the entire open reading frame of the mRNA has been translated into a protein.

Once translation of the mRNA is finished class I release factors (RF1 and RF2) will bind to the ribosome in a stop codon dependent way to release the newly synthesised protein. The class I release factors are removed from the ribosome by the class II release factor (RF3). The ribosome is split into subunits by the concerted action of EF-G and Ribosome Release Factor (RRF). Preparing them for another round of initiation. For a more thorough overview of translation see the review in reference[8]

1.2 Focus on Translocation

Translocation of tRNAs, from A and P sites to P and E sites is a central event in translation. While ribosomes can spontaneously translocate though the reaction is very slow. *In vivo* the reaction is catalysed by the G-protein EF-G. Once peptidyl transfer has taken place the ribosome will begin a process known as ratcheting[9]. Ratcheting involves the rapid and reversible rotation of one subunit about 3º in relation to the other[10]. In conjunction with this motion of the ribosomal subunits the tRNA will adopt hybrid binding states with the anticodon stem loops located in the A and P sites of the small subunit but the acceptor ends in the P and E sites of the large subunit[11]. EF-G binds to one of the ratcheted states of the ribosome and stabilises it, causing a massive increase in the rate of mRNA and tRNA translocation[12,13,14].

The exact sequence of events in translocation is a matter of dispute. Whether or not EF-G binds to the ribosome in its GTP or GDP bound form has been questioned[15]. There have also been conflicting reports on the timing of GTP hydrolysis by EF-G in relation to translocation. Older models suggest that GTP hydrolysis is not required for translocation itself but rather for dissociation of the factor from the post translocation ribosome[16]. However more recent studies have concluded that EF-G hydrolyses GTP before translocation, acting akin to a motor protein driving the RNA through the ribosome[17]. Further the rate of translocation has been determined using different methods by different groups[18,17,19,13] yielding results spanning from 1 s^{-1} to 25 s^{-1}. One prominent

difference in the experimental systems used by these groups is the mRNA, which varies in length as well as sequence.

1.3 Shine-Dalgarno sequences

During initiation of protein synthesis the 30S subunit binds to a specific sequence motif on the mRNA molecule. In E. coli the consensus sequence of this motif is called the Shine-Dalgarno (SD) sequence after its discoverers[20]. The SD sequence is complementary to a sequence on the 16S ribosomal RNA poetically named the anti-SD sequence. It has been known for some time that the level of complementarity of a given mRNA's SD sequence to the anti-SD sequence has an effect on total protein yield in heterologous expression[21]. Another parameter of interest, called the 'aligned spacing', is the number of bases between the start codon and the end of the matching bases in the best alignment of the mRNA to the canonical SD sequence. The aligned spacing is also thought to have an impact on protein yield[22]. It is unknown why and how the SD sequence and the aligned spacing have an effect on protein synthesis. It is of interest to know if the SD sequence and the aligned spacing affect only the rate of translation initiation or other steps in protein synthesis as well. It has been suggested that weak SD sequences might not bind stably enough to the ribosome resulting in poor translation initiation. On the other hand SD sequences that are too strong might inhibit translocation as the SD-anti-SD interaction eventually has to be broken in order to move the mRNA in relation to the ribosome. While there have been recent structural studies[23,24] on mRNA interaction with the ribosome, there has been no thorough functional characterization of the effect of different Shine Dalgarno sequences on the molecular level. Furthermore, these two mRNA motifs differ in the various mRNAs used in different studies on translocation.

1.4 Introduction to Rapid Kinetics and Quench-Flow

Investigating chemical kinetics, the rate at which chemical reactions happen, is a quantitative way to study the function of biological systems such as translation. Steady-state kinetics, determined by studying multiple substrate-product turnovers of an enzyme under substrate saturated conditions, can give information about the overall turnover rate of an enzyme-catalysed reaction. However to get information about the individual reaction steps in a complex reaction pathway such as translation it is necessary to study pre steady-state, so called single-turnover kinetics. As the name implies single turnover kinetics is concerned with the rate of events happening during a single substrate-product cycle. These events happen on very short time scales, often in the millisecond range.

Measuring chemical and structural events on such short time scales is technically challenging. One of the most versatile methods in use for the measurement of rapid chemical reactions is quench-flow[25] (Fig. 2). In quench flow the reactants are kept in two separate syringes and are then rapidly mixed to initiate the reaction. A short time after mixing (in practice 1 ms or more) the reaction is quenched by the addition of a third substance that causes the immediate cessation of the reaction of interest. The resulting mixture can then be analysed by suitable methods to quantify the reaction products. The reaction kinetics can then be determined from the amount of product formed and the reaction time.

1.5 Aim of This Study

The elongation cycle in protein synthesis is an elaborate sequence of interconnected steps many of which still require extensive research. While there are extensive studies on the kinetics of elongation[26,27,28], the effects of varying mRNA Shine-Dalgarno sequence and aligned spacing are currently unknown. This study aims to investigate *in-vitro* using quench-flow the rapid kinetics of dipeptide and tripeptide formation using nine different mRNA, systematically varying the extent of SD-anti-SD complementarity as well as the length of the aligned spacing.

2. Results

2.1 mRNA Production

Nine mRNA molecules with three different SD-sequences and three different aligned spacings, five, seven and nine bases, were designed and produced. The yield and activity varied significantly between the different mRNAs (summarised in table. 1). The three mRNAs with the weakest SD-sequences all had a very small fraction of active molecules, the reasons for this are at present unknown. mRNA activity was measured by the extent of dipeptide formation after long incubation times and expressed as a percentage of the total possible dipeptide yield. Dipeptide formation was used in place of other assays such as fMet-tRNA^{fMet} binding as the mRNA were to be used for measuring peptide formation kinetics.

2.2 Dipeptide Formation Experiments

The rate of formation of fMet-Leu dipeptide at 37º C was determined for three different mRNAs

(U5, I7, W7) with three different SD sequences (see table 1. for details). Experiments were carried out by rapidly mixing 70S initiation complex with Leu-tRNA^{Leu} • EF-Tu • GTP ternary complex using an automated Quench-flow instrument, followed by quantification of formed dipeptide using RP-HPLC. The resultant dipeptide formation data was normalised using the dipeptide yield for long incubation times and the rate of dipeptide formation estimated by fitting the data with single exponential functions. The mRNA with the weakest SD sequence (W7) had the lowest rate of dipeptide formation at approximately 10 s^{-1}, the mRNA with intermediate and strong SD sequences (I7, U5) had very similar dipeptide formation rates of approximately 16 s^{-1} and 15 s^{-1} respectively. Unfortunately there was not enough high quality experimental data to get a good estimate of the experimental error, though the rate for U5 agrees with previous results. Rate information is summarised in fig 3.

2.3 Tripeptide Formation Experiments

The rate of tripeptide formation was determined for four mRNAs; two variants of U5 (one encoding phenylalanine in the third position and one encoding leucine in the third position) as well as I7 and W7 (both encoding phenylalanine in the third position). All mRNA encoded leucine in the second position. Experiments were carried out as in the dipeptide formation experiments by rapidly mixing 70S initiation complex with Leu-tRNA^{Leu} • EF-Tu • GTP, Phe-tRNA^{Phe} • EF-Tu • GTP and EF-G. Di and tri peptides were quantified using RP-HPLC. The rate of tripeptide formation was estimated by the fitting of normalised tripeptide formation data with single exponential functions. Both variants of U5 had similar tripeptide kinetics of approximately 2,5 s^{-1}. I7 formed tripeptide at approximately 1 s^{-1} while R7 formed tripeptide slightly faster at 1,5 s^{-1}. Unfortunately there was not enough high quality data to estimate experimental errors. However the results for U5 agreed with previous thise of experiments. Peptide formation rates are summarised in fig 3.

2.4 Varying Magnesium concentration during tripeptide formation

Due to the magnesium chelating properties of PEP, ATP and GTP, components of our reaction mixture, the free magnesium concentration in our experiments is comparatively small. The K_D for Mg·PEP has been estimated as 4 mM[29]. With this dissociation constant our reaction mixture supposedly contains only 1 mM free magnesium. Magnesium is a crucially important component of the ribosome and such a small free magnesium concentration could hypothetically be the cause for the low rates of tripeptide formation. In order to test this the magnesium concentration was adjusted by addition of $MgCl_2$ in tripeptide formation experiments. Attempts to increase the free magnesium

concentration to values similar to those of other groups (7 mM) led to no tripeptide being formed. More modest additions up to free Mg concentrations of 2 and 3 mM had no effect on the rate of tripetide formation.

3. Discussion

The elongation cycle in translation has two distinct phases. An EF-Tu catalysed phase where new AA-tRNAs are brought to the ribosome and incorporated into the growing polypeptide chain. And an EF-G catalysed phase were the ribosome is translocated along the mRNA. In this study the effects of mRNA aligned spacing and Shine-Dalgarno sequence complementarity on both of these phases have been investigated using rapid kinetic techniques. Unfortunately only a subset of the different mRNAs could be characterized and then only in a limited fashion. It was suggested that the aligned spacing of an mRNA could have an effect on dipeptide formation if, as has been suggested in recent studies[26,30,31], the chemistry of peptide bond formation is rate limiting rather than other steps such as tRNA accommodation[6]. This is if the chemical step is rate limiting the exact positioning of the initiator tRNA is important, whereas this would have little or no effect on a macromolecular conformation change such as accommodation. However for an mRNA with a strong SD-sequence the rate of dipeptide formation remained unaltered when the spacing varied from five to seven bases. At the same time an mRNA with a weaker SD sequence and a seven base spacing gave a slightly lower rate.

Similarly the strength of the SD-anti-SD interaction could possibly have an effect on tripeptide formation as this interaction eventually has to be broken during translocation. Unfortunately the rates of tripeptide formation measured here were almost an order of magnitude smaller than estimates of the rate of elongation *in vivo* as well as previously published *in vitro* results on the rate of translocation. The average rate of translation *in vivo* has been estimated as 10 - 20 s^{-1}. Our dipeptide formation results are in good agreement with this number but the tripeptide formation rates are an order of magnitude smaller. There could be a multitude of reasons for this discrepancy as an *in vitro* system is vastly different from a living cell. It is important to note that the *in vivo* estimate represents an average rate of protein elongation. There could be significant kinetic differences in elongation rate between different amino acids or even different tRNA isoacceptors. This would have no effect on the average rate but would be apparent in an *in vitro* situation focusing on only one specific AA-tRNA.

Other studies *in vitro* on the rate of translocation have presented rates around 25 s^{-1} in one report[17]

and in another case a group presents a rate of 1,4 s^{-1} in one study[18] which is then upgraded to 10 s^{-1} in a later study[19] without a noticeable change in experimental methods or conditions. It should be noted that none of these studies deal directly with tripeptide formation but focus on translocation. In these studies the rate of translocation is measured using stop-flow and either fluorescence labeled mRNA[18], or fluorescence labeled tRNA in the A-site[17]. The rate of fluorescence change upon addition of EF-G is then taken as the rate of translocation. In one of the studies[17] the fluorescence measurements are complemented with a puromyocin reactivity assay to confirm that translocation has taken place. Puromyocin is a small molecule antibiotic that works as a AA-tRNA analog capable of accepting peptide from a P-site tRNA and thus in this case confirm that translocation has taken place. Neither of these methods involved formation of tripeptide and thus included only one peptide bond formation cycle.

Tripeptide formation requires two full peptidyl transfer reactions as well as translocation. In theory the second round of peptide bond formation should be similar to the first and a large reduction in the rate of peptidyl transfer would not be expected simply from moving one codon down the mRNA. In addition mRNA with leucine codons in the third position and mRNA with phenylalanine codons in the third position produce similar rates of tripeptide formation. These two amino acids produce radically different rates of dipeptide formation, varying from almost 200 s^{-1} for phenylalanine to 25 s^{-1} for leucine (Sanyal, S. unpublished results). From this one can conclude that formation of the second peptide bond is unlikely to be the rate limiting step.

This leaves the steps between EF-G binding and EF-G dissociation as the cause of the discrepancy; each of which could be slowed down for several reasons. For tripeptide to be formed ternary complex has to bind to the ribosomal A site. This means that EF-G has to dissociate from the ribosome as it binds to the same location. In neither of the previously mentioned studies is this taken into account as the only measured step is tRNA or mRNA movement while in our quench-flow based assay all steps up to and including tripeptide bond formation contribute to the rate.

The current study does not contain enough data to give a conclusive picture of the kinetic effects of different SD sequences or different aligned spacing. However, before further studies can be conducted on the effects of these mRNA sequence motifs experiments have to be designed to dissect the individual sub steps of tripeptide formation in order to isolate the rate limiting step. It is also important to identify the cause of the large reduction in the rate of elongation compared to the rate *in vivo*, and whether this is a general *in vitro* artifact or specific to the particulars of our assay system such as buffer composition, Mg^{2+} concentration or energy pump composition. A drastic

increase in the free magnesium concentration led to no tripeptide being formed, possibly because at high Mg concentrations ribosomal ratcheting is inhibited[9], smaller adjustments in Mg^{2+} concentration had little effect on trippetide kinetics. Possibly the K_D for Mg • PEP is not accurate and our buffer system contains more free Mg^{2+} than calculated. In conclusion our knowledge of this complex biological system is still tentative and much experimental work remains to be done if the *in vivo* behaviour is to be replicated in the lab.

4. Materials and Methods

4.1 mRNA Production

mRNA were prepared as following. Overlapping primers, such that the coding sequence and ribosome binding site were both on one primer and T7 promotor sequence was on the other, were ordered from Invitrogen. Primers were extended by PCR and the resultant DNA was extracted with phenol chloroform and used for in vitro transcription with T7 RNA polymerase. RNA was purified by phenol chloroform extraction and subsequent affinity chromatography using a poly-dT column (column media purchased from GE Healthcare). RNA concentration was measured using spectrophotometry and activity was estimated by dipeptide yield at long timepoints.

4.2 Quench Flow Experiments

Two reaction mixtures were prepared for quench flow. A ribosome mix containing 70S ribosomes, initiation factors IF1, IF2, IF3 in the same concentration as ribosomes, mRNA and tritium labeled fMet-$tRNA^{fMet}$ both at twice the ribosomal concentration. An elongation mix containing EF-Tu at ten times excess over the ribosomal concentration, EF-Ts at half the EF-Tu concentration, respective tRNA (3 μM), amino acid (50 μM) and amino acid synthetase (0,5 U/μl) as well as EF-G at five times excess over the ribosomal concentration. For dipeptide experiments EF-G and the second EF-Tu ternary complex were omitted from the elongation mixture.

The reactions were carried out at 37° C in 1X HEPES-Polymix buffer[32,33], containing 1 mM HEPES (pH 7.5), 95 mM KCl, 5 mM NH_4Cl, 5 mM $Mg[OAc]_2$, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, and 1 mM DTE. Both mixtures also contained 1 mM GTP, 1 mM ATP, 10 mM PEP, PK (50 μg/ml), and MK (2 μg/ml). The magnesium concentration of the buffer was adjusted by addition of $MgCl_2$ when necessary.

Both mixtures were incubated for 10 min at 37° C prior to the experiments carried out at 37° C using a quench-flow instrument (RQF-3, KinTek Corp.). Reactions were quenched using formic acid (final concentration 17%). The extent of dipeptide and tripeptide formation was analysed with RP-HPLC according to[34] for a typical HPLC profile see fig 4.

4.3 Data Analysis

Pepetide formation rates were calculated by fitting of the data using single exponential functions. Data fitting was performed using the program Simfit[35].

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6. Figures and tables

Table 1.

mRNA	Yield nmol	Activity	Sequence excerpt
U5: Strong SD 5 Base Spacing	157	58,90%	5'-UAAGGAGGUAUUAAAUGCUGUUCUAA-3'
U7: Strong SD 7 Base Spacing	140	84,74%	5'-UAAGGAGGUAUACUAAAUGCUGUUCUAA-3'
U9: Strong SD 9 Base Spacing	47,5	52,93%	5'-UAAGGAGGUAUACAUUAAAUGCUGUUCUAA-3'
I5: Intermediate SD 5 Base Spacing	100	52,62%	5'-UAAAGAGGUAUUAAAUGCUGUUCUAA-3'
I7: Intermediate SD 7 Base Spacing	150	90,75%	5'-UAAAGAGGUAUACUAAAUGCUGUUCUAA-3'
I9: Intermediate SD 9 Base Spacing	88	49,88%	5'-UAAAGAGGUAUACAUUAAAUGCUGUUCUAA-3'
W5: Weak SD 5 Base Spacing	75	23,02%	5'-UUAACAGGUAUACUAUGCUGUUCUAA-3'
W7: Weak SD 7 Base Spacing	100	44,15%	5'-UUAACAGGUAUACACUAUGCUGUUCUAA-3'
W9: Weak SD 9 Base Spacing	75	5,89%	5'-UUAACAGGUAUACAUACUAUGCUGUUCUAA-3'

Table 1. First column is mRNA name and properties, second column is the yield in nanomoles. The third column is the mRNA activity expressed as the yield of dipeptide as a percentage of the maximum possible yield in dipeptide synthesis at long time points. The fourth column shows an excerpt of the mRNA sequence, bases in the SD sequence are labeled in blue the aligned spacing is labeled in green and the start codon is labeled in red.

Fig 1.

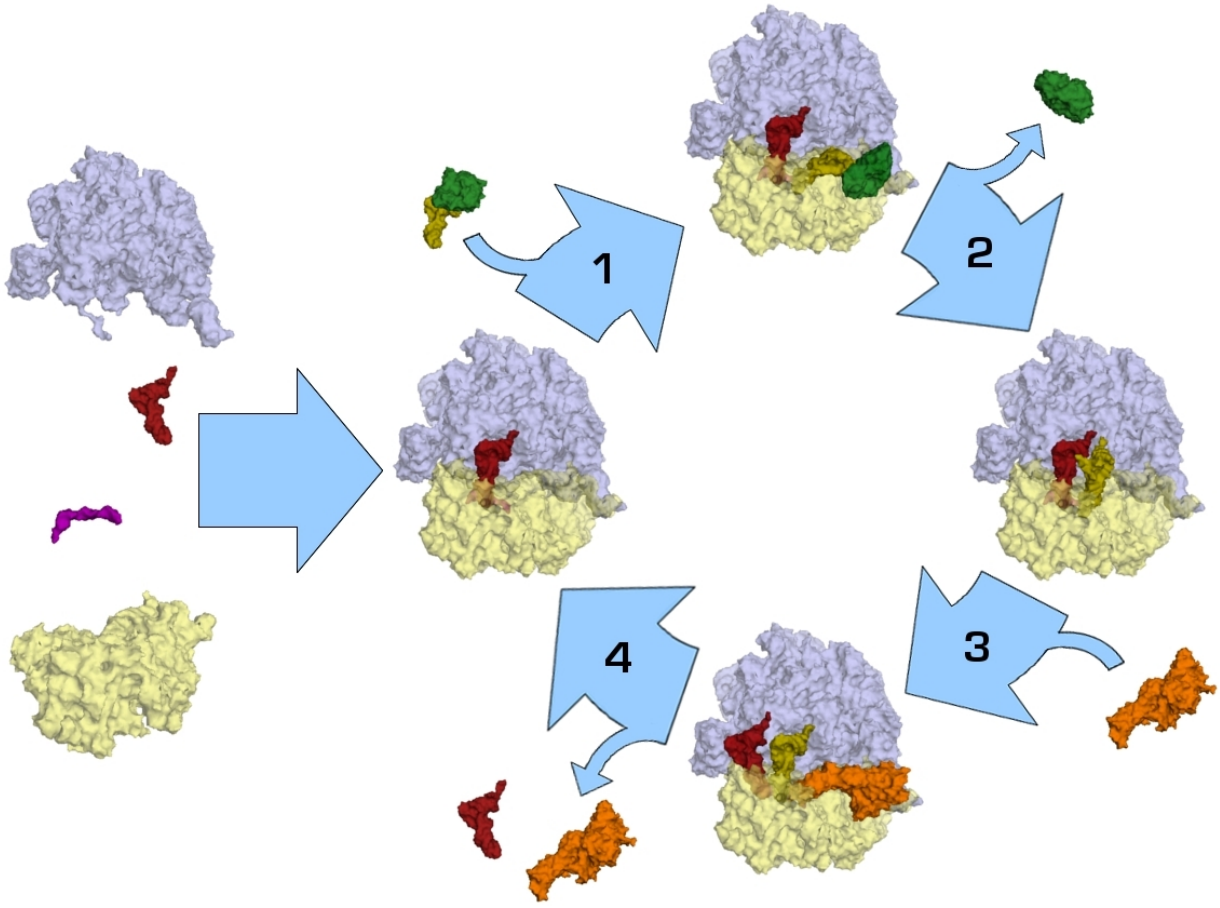


Fig 1. Overview of the elongation cycle in translation. Showing ternary complex binding (1), EF-Tu release and peptidyl transfer (2), EF-G binding and translocation (3) and release of EF-G and E-site tRNA preparing the ribosome for the next elongation cycle (4).

Fig 2.

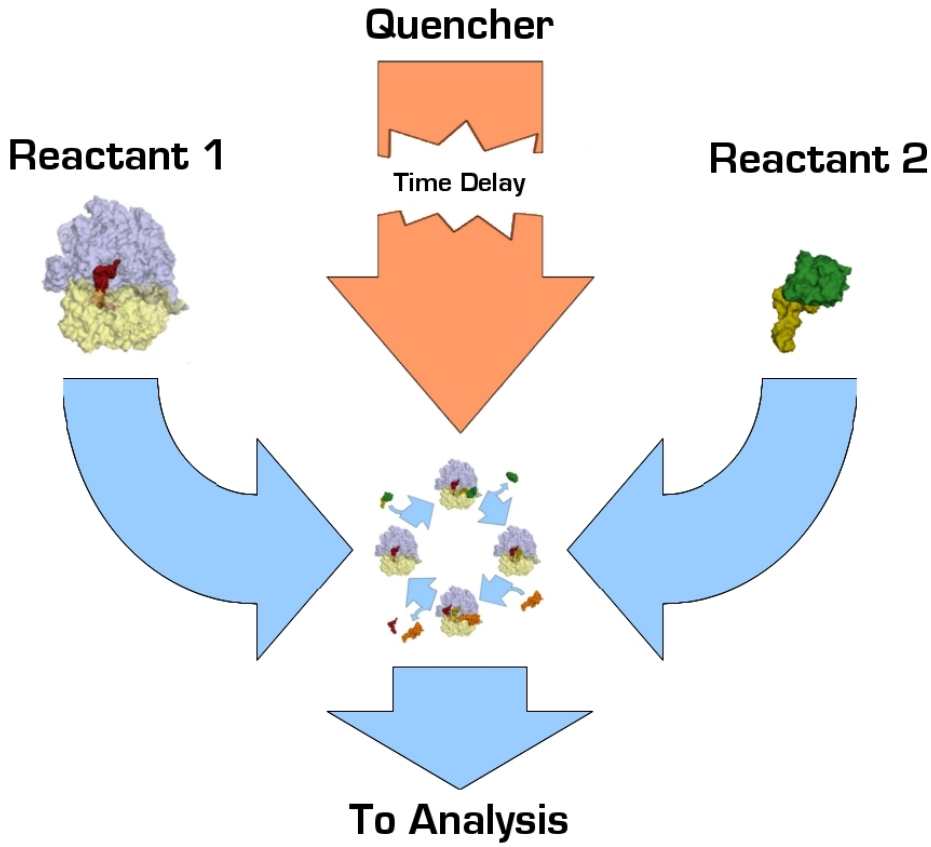
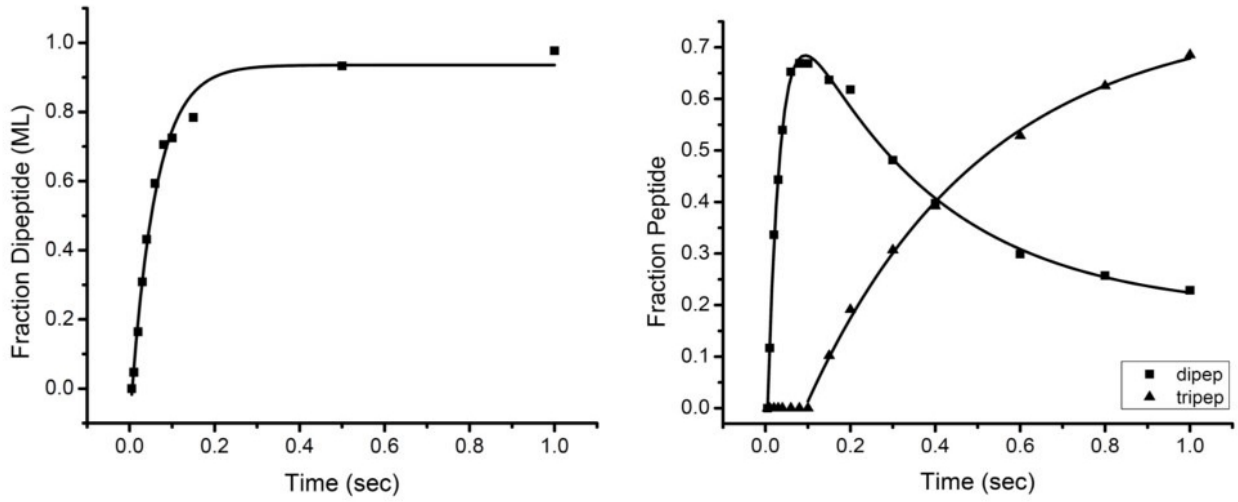


Fig 2. Overview of quench flow. Two reactants are rapidly mixed and the reaction is allowed to proceed for a short time before quencher is added to stop it. The time delay is programmable allowing for the construction of a full time course for the reaction of interest. The resulting mixture is then taken to analysis.

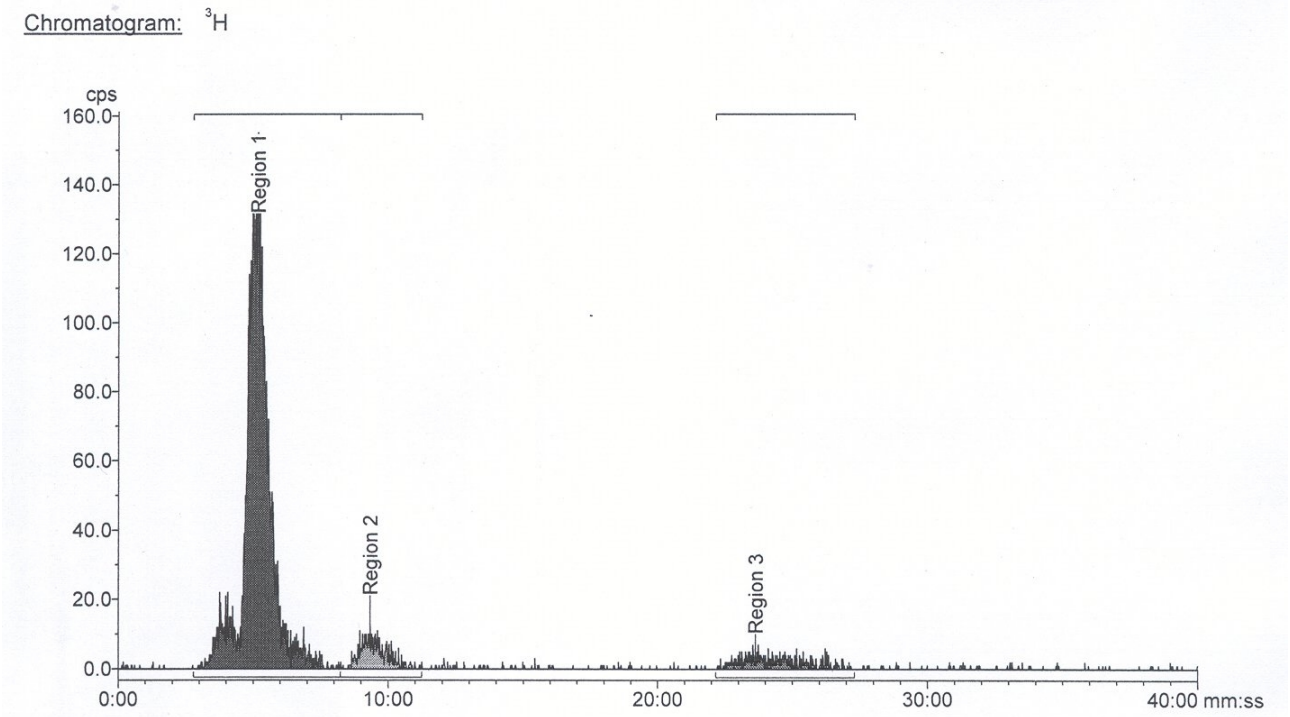
Fig 3.



mRNA	Rate of dipeptide formation	Rate of tripeptide formation
U5 (MLF)	15 s^{-1}	2,5 s^{-1}
U5 (MLL)	x	2,5 s^{-1}
I7 (MLF)	16 s^{-1}	1 s^{-1}
W7 (MLF)	10 s^{-1}	1,5 s^{-1}

Fig 3. Example datasets from dipeptide and tripeptide experiments. Squares represent fraction of dipeptide formed. Triangles represent fraction of tripeptide formed. The table summarises the rates of dipeptide and tripeptide formation for the various mRNA.

Fig 4.



Regions: ^{3}H Detector: ß-RAM

Name	Start (mm:ss)	End (mm:ss)	Retention (mm:ss)	Height (cps)	Area (cps)	%ROI (%)	%Total (%)
Region 1	2:47	8:14	5:10	155.0	7589.0	87.09	85.07
Region 2	8:14	11:15	9:20	12.0	592.0	6.79	6.64
Region 3	22:09	27:18	23:38	7.0	533.0	6.12	5.97
3 Peaks					8714.0	100.00	97.68

Net Total Counts: 8921.0 Counts
Average Background: 0.0 CPM
Unallocated Counts: 207.0 Counts

Fig 4. A typical chromatogram taken from a tripeptide experiment.