MATERIALS

Cell Extract

The cell extract used in this study is a crude cytoplasmic extract from *E. coli*. The liquid part of the cell (cytoplasm) is extracted by breaking the cells. Preparation procedures are described in [*S1*]. We review its content here.

Enzymes

The extract contains the soluble proteins of *E. coli* (above 10 kDa molecular weight cut-off), with concentrations of 28-32 mg/ml proteins in the crude extract and 10 mg/ml in the final reaction which is the optimum concentration for expression. Membranes and insoluble debris are removed by centrifugation. During extract preparation, the endogenous information is erased from the cytoplasm (DNA and messengers are removed). The cell extract provides the transcription and the translation machineries necessary to run synthetic DNA programs. The transcription is driven by the endogenous *E. coli* RNA polymerase. It is the only major difference with standard extracts, which use bacteriophage RNA polymerases. In addition, the cell extract contains active proteases and ribonucleases. In this work we refer to the ClpXP complex which is part of the AAA+ proteolysis pathway [*S2*, *S3*]. The AAA+ pathway is the main proteolytic pathway in *E. coli*, and has the advantage of being tag specific. The protein to be degraded needs to have a 11 amino acid tag either in N or C terminal [*S4*]. Most of the other important proteases are found at the membrane, and are therefore removed during extract preparation. The main mRNA degradation pathway is the degradosome whose activity is thought to begin with cleavage at internal sites by RNAse E, also considered to be the rate limiting step [*S5*–*S7*]. It should be noted that a spectrum of ribonucleases exists besides the degradasome.

Nutrients

Nutrients and building blocks (rNTPs, amino acids, tRNA) are essential reagents in the background of cell-free reactions and are added to the crude cell-extract. Shin and Noireaux have

demonstrated that protein expression is not sensitive to small changes in these components [*S1*]. For tRNA, the total protein yield is identical in the range $200 < \text{tRNA} < 350\,\mu\text{g}\,\text{ml}^{-1}$. While amino acid concentration needs to be adjusted around 1 mM for optimum expression, the effect on protein production is less than a factor of 2 when the concentration of amino acids is changed within 0.5 mM to 2 mM. Similar results were obtained with ribonucleotides where a wide range of concentrations ($1.5 < \text{ATP} < 2.2\,\text{mM}$, $0.55 < \text{GTP} < 2\,\text{mM}$, $0.55 < \text{UTP/CTP} < 0.9\,\text{mM}$) had no effect on protein expression dynamics. In conclusion, the concentrations of nutrients and building blocks that we have used are standard and they can be changed over a wide range without altering the expression dynamics significantly. It is important to note that when nutrients concentrations are changed, the concentration of magnesium and potassium have to be adjusted.

Extract variance

The data presented in the article have been obtained from a single extract batch except for the mRNA degradation assay which was measured in a different batch (same protocol, different preparation). Both extracts have been produced by Shin and Noireaux in their laboratory. The maximal protein yield systematically varies within a 5% error bar between different extracts while the synthesis kinetics is identical in the first hour (Fig. S1). The synthesis rate dependence on DNA varies within 15% indicating a small change in the enzyme concentration (Fig. S1) consistent with the crude extract concentration variance (28 - 32 mg/ml).

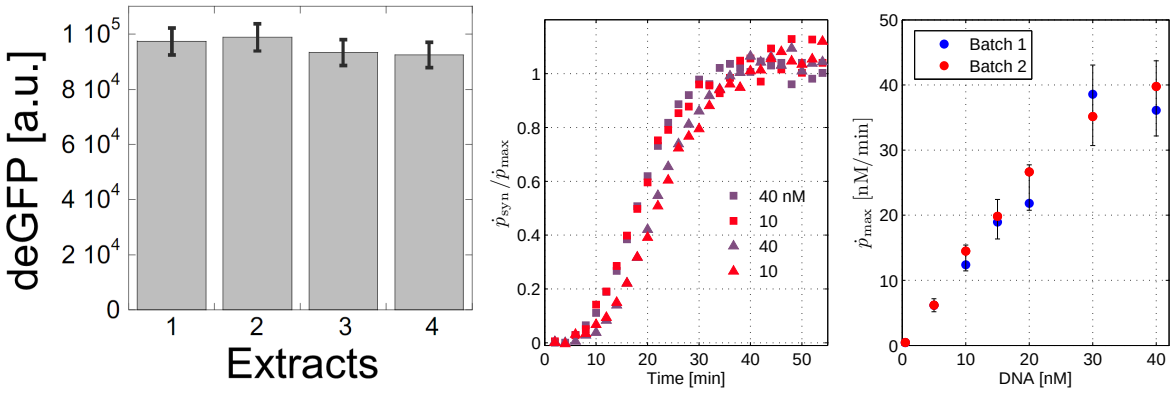

Figure S1: Expression variance for deGFP: (left) maximum synthesized deGFP in four different extracts at their optimal conditions, prepared in the past ten months. The protein yield of each batch differs in 5% error range. (middle) Normalized synthesis rate for two different extracts (triangle and square) at 40 and 10 nM DNA exhibit identical kinetics. (right) Maximal synthesis rate as a function of DNA for two different extracts, with 15 % variance bar.

Plasmids

Expression experiments were carried out using two plasmids coding for enhanced green fluorescent protein (eGFP): pBEST-OR2-OR1-Pr-UTR1-eGFP-Del6-229-T500-ssrA (denoted deGFP-ssrA) coding for a degradable protein and pBEST-OR2-OR1-Pr-UTR1-eGFP-Del6-229-T500-ssrA-DD (denoted deGFP-ssrA-DD) coding for a non-degradable protein. The list and sequences of the different regulatory parts are reported in [*S8*]. Essentially, eGFP-Del6-229 (denoted deGFP) is eGFP truncated and modified in N- and C-terminal [*S1*, *S8*]. The modification were performed in order to optimize translation efficiency. The deGFP protein is 3 to 4 times more translatable than eGFP in our cell-free system and has the same fluorescent properties [*S8*]. This is an experimental observation, also confirmed on SDS PAGE. Both plasmids carry a strong λ promoter Pr (σ_{70} specific). The ssrA and ssrA-DD are C-terminal 11-residue peptide tags. The ssrA tag (AANDENYALAA) is recognized by the ClpXP complex leading to protein degradation, and ssrA-DD is a non-degradable variant (AANDENYALDD) [*S9*].

mRNA

The radioactively labeled mRNA template used in the mRNA degradation assay was produced in a T7 RNA polymerase based transcription reaction in the presence of radioactive ribonucleotides (^{32}P-α-rUTP, Perkin Elmer) as described in [*S10*]. No further purification steps were taken. The DNA template for the reaction was a PCR product of plasmid pIVEX 2.3d (ROCHE) with primers GTTGGCCGCAGTGTTATCAC and TACGCAAACCGCCTCT. This 2100-bp-long linear template contains a T7 promoter and terminator. The reaction results in two mRNA transcripts: 280 base-long (transcription terminated at the T7 terminator) and 960 base-log (transcription reaching the PCR end) both carrying a ribosome binding site (Fig. S2).

Proteins

The plasmid pET21a(+) (Novagen) was used for recombinant protein expression. The proteins His-eGFP-ssrA (6Histag in N-terminal and ssrA tag in C-terminal) and His-eGFP-ssrA-DD (6Histag in N-terminal and ssrA-DD tag in C-terminal) were over-expressed in *E. coli* BL21 (DE3) and purified by affinity chromatography on agarose nickel beads according to the manufacturer protocol (Adar Biotech). The proteins were desalted against a storage buffer (50 mM Tris HCl, pH 7.5) and stored at -80°C. The concentration of the purified proteins was measured by spectrophotometry (ND-1000, NanoDrop Technologies) based on published absorption coefficient [*S11*].

METHODS

Cell-Free Reaction

The cell-free reaction is prepared according to [*S1*] and is composed of 33 % crude extract, the other 66 % contained the reaction buffer and plasmid, mRNA or protein according to the experiment. Reactions were mixed on ice in volumes of $20-50\,\mu$l, where they are stable for at list an hour. The reaction can be started at any time by bringing the reaction to the desired temperature ($28°C$ in our study). Thus, timing is not an issue in the cell-free

experiments.

Fluorescent measurements

The fluorescence measurements were performed with a synergy-HT plate reader (Biotek, 384-well plate), in volume of 20 μl. Reactions were sealed with a cover slip to avoid evaporation. Calibration and quantification of the fluorescent signal was performed with purified His-eGFP-ssrA-DD diluted in 3-PGA buffer [*S1*], to match expression reaction conditions.

Radioactive measurements

Transcription

Radioactively labeled rUTP (^{32}P-α-rUTP, Perkin Elmer) was added to the cell-free reaction in final concentration of less than 1 μM (total rUTP concentration is $\sim$ 1 mM). The cell-free reaction was preformed in volumes of $40-50\,\mu$l, in $0.5-1.5$ ml test tubes placed in a heated bath. Samples of 2 μl were taken at different time points into 2 μl stop solution to a final concentration of 0.2 % SDS/ 20 mM EDTA, and placed immediately on ice. The samples are then mixed into $100-150\,\mu$l Formamide based loading buffer and analyzed using polyacrylamide gel electrophoresis. Polyacrylamide gel composition: $5-15$ % 19:1 Acrylamide-bis / Acrylamide (Biological Industries), 1 X TBE and 8 M urea. The polyacrylamide gel was dried and exposed to phosphorous screen (FUJI), which was then scanned by a phosphorimager (FLA-5100, FUJI) (Figure S2).

Nucleotide incorporation into mRNA was quantified using the software ImageGauge (FUJI). To derive the mRNA concentration, the fraction of incorporated nucleotides (X) was multiplied by total UTP concentration (U) and divided by the number of rUTPs in the transcript (N_{u}), $m = X \cdot U \cdot N_{\mathrm{u}}^{-1}$.

mRNA degradation

Radioactively labeled mRNA (see Materials section) was added to a 40 μl cell-free reaction, with final concentration of $\sim$ 200 nM. The mRNA carries a T7 promoter which is assumed

not to be transcribed in the cell-free system. Only the longer (960 base-long) transcript was followed. The reaction was sampled at different time points and quantified as in the translation assay.

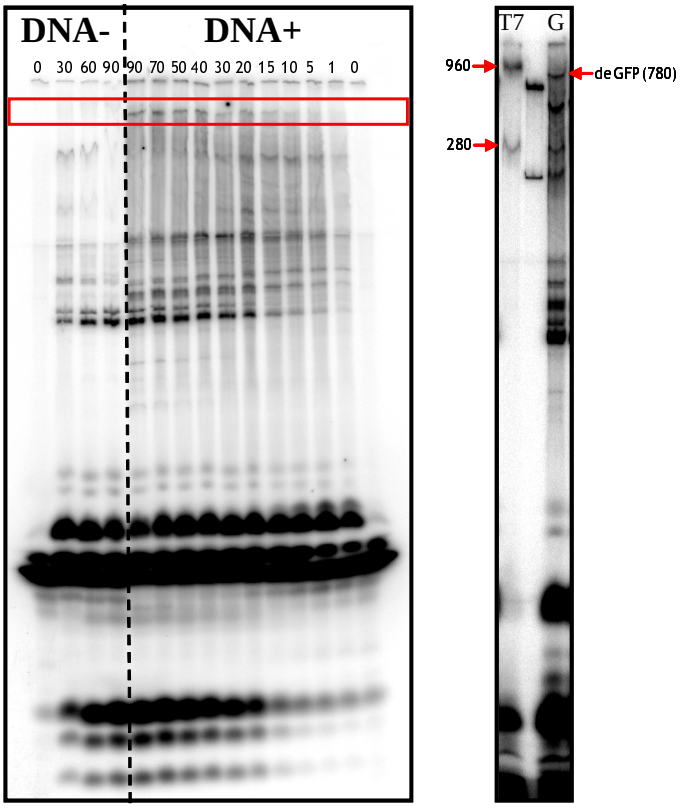

Figure S2: mRNA dynamics resolved by polyacrylamide gel. (left) mRNA transcription dynamics of deGFP-ssrA-DD. Each lane represents a different time point indicated at the top. DNA+ reaction included 30nM plasmid. DNA- reaction included no DNA template. The deGFP mRNA band is highlighted with a red frame. (right) mRNA transcripts (960 and 280 nucleotide long) result of the T7 transcription assay (lane marked T7) next to deGFP 780 nucleotides long transcript (lane marked G).

MATHEMATICAL DERIVATION

Enzyme-substrate dynamics

The Michaelis-Menten (MM) dynamics is composed of two steps: (i) Reversible binding of enzyme E and substrate S to form a complex $[ES]$. (ii) Irreversible catalysis of product P

or substrate degradation,

$$E + S \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} [ES] \xrightarrow{k_{\text{cat}}} \begin{cases} E + S + P & \textit{Biosynthesis} \\ E & \textit{Degradation}\,. \end{cases} \tag{S1}$$

The complex $[ES]$ dynamics is given by,

$$[\dot{ES}] \; = k_{\text{on}} \cdot \; E^{\text{f}} \cdot S^{\text{f}} - (k_{\text{off}} + k_{\text{cat}}) \cdot [ES], \tag{S2}$$

where $E^{\text{f}} = E - [ES]$ and $S^{\text{f}} = S - [ES]$ are the free enzyme and substrate concentrations. The product kinetics is given by,

$$\dot{P} \; = \; k_{\text{cat}} \cdot [ES]\,. \tag{S3}$$

Assuming quasi steady state and defining the MM constant $K_{\text{M}} = k_{\text{on}}^{-1} \cdot (k_{\text{off}} + k_{\text{cat}})$ we have,

$$[ES] \; = K_{\text{M}}^{-1} \cdot \; E^{\text{f}} \cdot S^{\text{f}}\,. \tag{S4}$$

In terms of the total concentrations we solve the quadratic equation and find,

$$[ES] \; = \; \frac{1}{2}\left(E + S + K_{\text{M}} - \sqrt{(E + S + K_{\text{M}})^2 - 4 \cdot E \cdot S}\right)\,. \tag{S5}$$

This expression simplifies in two limits,

$$[ES] \; \approx \; \begin{cases} \frac{E \cdot S}{S + K_{\text{M}}} & E \ll K_{\text{M}} + S \\ \frac{E \cdot S}{E + K_{\text{M}}} & S \ll K_{\text{M}} + E\,. \end{cases} \tag{S6}$$

Enzymatic degradation

Consider synthesis of a substrate S at a constant rate A and enzymatic degradation with MM constant K_{M}, catalysis rate k_{deg} and enzyme X:

$$\dot{S} \;=\; A - \frac{k_{\rm deg}}{2}\left(X + S + K_{\rm M} - \sqrt{(X+S+K_{\rm M})^2 - 4\cdot X\cdot S}\right)\,. \tag{S7}$$

Solving the equation at steady-state $\dot{S} = 0$,

$$A - \frac{k_{\rm deg}}{2}\left(X + S + K_{\rm M}\right) \;=\; -\frac{k_{\rm deg}}{2}\sqrt{(X+S+K_{\rm M})^2 - 4\cdot X\cdot S} \tag{S8}$$

We define the dimensionless parameter $\eta = A/(k_{\rm deg}\cdot E)$ and find (for $\eta < 1$)

$$S = K_M\cdot\eta\cdot(1-\eta)^{-1} + \eta\cdot X \;\approx\; \begin{cases} K_M\cdot(1-\eta)^{-1} & \eta\to 1 \\ \eta\cdot(X+K_{\rm M}) & \eta \ll 1\,. \end{cases} \tag{S9}$$

For $\eta \to 1$ the expression diverges, as no steady-state is possible when synthesis rate is greater than the maximal degradation rate $(k_{\rm deg}\cdot E)$. In the opposite limit the steady-state concentration scales with the degradation MM constant $(K_{\rm M})$ and enzyme concentration (X).

Cell-free transcription, translation and degradation

The four basic reactions of mRNA and protein biosynthesis and degradation are described in terms of four enzymes : RNA polymerase (R_p) , Ribosome (R), protease $(X_{\rm p})$ and RNAse $(X_{\rm m})$. DNA (D) and mRNA (m) are substrates for biosynthesis. mRNA and protein (p) are substrates for degradation.

$$\dot{m} \;=\; k_{\rm TX}\cdot N_{\rm m}^{-1}\cdot[R_pD] - k_{\rm deg}^{\rm m}\cdot[X_{\rm m}m] \tag{S10}$$

$$\dot{p} \;=\; k_{\rm TL}\cdot N_{\rm p}^{-1}\cdot[Rm] - k_{\rm deg}\cdot[X_{\rm p}p] \tag{S11}$$

$k_{\rm TX}(k_{\rm TL})$ are the transcription (translation) catalysis rates. $N_{\rm m}(N_{\rm p})$ is the mRNA (protein) length in nucleotides (amino acids). $k_{\rm deg}^{\rm m}(k_{\rm deg})$ are the degradation catalysis rates. $K_{\rm TX}(K_{\rm TL})$ and $K_{\rm m}(K_{\rm p})$ are the four MM constants for biosynthesis and degradation.

mRNA degradation

The mRNA degradation in the extract is exponential (Fig. 1b). This implies $[X_{\rm m}m]$ is linear in m, and thus $S \ll K_{\rm M} + E$ and $[X_{\rm m}m] = \frac{X_{\rm m}\cdot m}{X_{\rm m}+K_{\rm m}} = m/(k^{\rm m}_{\rm deg}\cdot\tau_{\rm m})$, where $\tau_{\rm m} = \frac{X_{\rm m}+K_{\rm m}}{k^{\rm m}_{\rm deg}\cdot X_{\rm m}}$.

Transcription

Transcription showed a delay τ_0 and exponential rise to a steady state (Fig. 1c) consistent with the linear degradation. The transcription rate $k_{\rm TX}$ was extracted from the delay time and we were unable to determine $[R_pD]$ (determined through the protein dynamics). Thus,

$$\dot{m}(D,t) \;=\; k_{\rm TX}\cdot N_{\rm m}^{-1}\cdot[R_pD] - m(D,t-\tau_0)/\tau_{\rm m} \qquad (\forall\; m < 10^2\,{\rm nM},\; t > \tau_0\,), \tag{S12}$$

$$[R_pD] \;=\; \frac{1}{2}\left(R_p + D + K_{\rm TX} - \sqrt{(R_p+D+K_{\rm TX})^2 - 4\cdot R_p\cdot D}\right). \tag{S13}$$

The solution is given by,

$$m(D,t) \;=\; m_{\rm ss}(D)\cdot(1-\exp[-(t-\tau_0)/\tau_{\rm m}])\,, \tag{S14}$$

$$m_{\rm ss}(D) \;=\; \tau_{\rm m}\cdot k_{\rm TX}\cdot N_{\rm m}^{-1}\cdot[R_pD]\,. \tag{S15}$$

Protein degradation

Protein degradation was not exponential, and showed zeroth-order dynamics until complete degradation (fig. 2c, main text), suggesting $X_{\rm p}, K_{\rm p} < 10\,{\rm nM}$ (our detection limit).

Translation

Protein synthesis rate, $\dot{p}_{\rm syn}(t) \propto (1-\exp[-(t-\tau_0-\tau_f)/\tau_{\rm m})] = m(t-\tau_{\rm f})$ follows the exponential mRNA dynamics for all DNA concentrations (Fig. 1c/e , main text) , suggesting first-order dynamics $[Rm] = m\cdot(1+K_{\rm TL}/R)^{-1}$ and additional delay time $\tau_{\rm f}$ (result of protein folding time),

$$\dot{p}_{\rm syn}(D,t) \;=\; k_{\rm TL}\cdot N_p^{-1}\cdot(1+K_{\rm TL}/R)^{-1}\cdot m(D,t-\tau_{\rm f}) \quad \forall {\rm t} > \tau_0+\tau_{\rm f}. \tag{S16}$$

As mRNA concentration reaches a steady state, protein synthesis rate reaches a maximum $\dot{p}_{\rm max}(D) = k_{\rm TL}\cdot N_p^{-1}\cdot(1+K_{\rm TL}/R)^{-1}\cdot m_{\rm ss}(D)$.

Protein dynamics with degradation is given by,

$$\dot{p}(D,t) \;=\; \dot{p}_{\rm syn}(D,t) - k_{\rm deg}\cdot[X_{\rm p}p(D,t)] \quad \forall {\rm t} > \tau_0+\tau_{\rm f}. \tag{S17}$$

The steady state solution is given by Eq. (S9) with $\eta(D) = \dot{p}_{\rm max}(D)/(k_{\rm deg}\cdot X_{\rm p})$. Thus, steady state is possible only for DNA concentrations where $\dot{p}_{\rm max}(D) < k_{\rm deg}\cdot X_{\rm p}$ ($\eta(D) < 1$). Protein steady-state scales with the MM constant for degradation and the protease concentration, $p \sim K_{\rm M} + X_{\rm p}$ (undetectable). For $\eta(D) > 1$ protein accumulates. However, protein accumulation begins only when synthesis is faster than degradation $\dot{p}(D,t) > 0$. This occurs at a finite lag-time T,

$$\dot{p}_{\rm syn}(D,T) \;=\; k_{\rm deg}\cdot X_{\rm p}\,. \tag{S18}$$

Using Eq. (S14-S16) we find,

$$\eta(D)\cdot(1-e^{-(T-\tau_0-\tau_{\rm f})/\tau_{\rm m}}) \;=\; 1\,. \tag{S19}$$

Finally,

$$T \;=\; \tau_0+\tau_{\rm f}-\tau_{\rm m}{\rm log}[1-\eta^{-1}(D)]. \tag{S20}$$

We note that for $\eta(D)\to 1$ the lag time diverges, indicating that protein levels are kept below detection limit.

[S1] J. Shin and V. Noireaux, J. Bio. Eng. **4**, 8 (2010).

[S2] R. Schmidt, B. Bukau, and A. Mogk, Res. Microbiol. **160**, 629 (2009).

[S3] S. Gottesman and M. Maurizi, Microbiol. Mol. Biol. Rev. **56**, 592 (1992).

[S4] J. M. Flynn, S. B. Neher, Y.-I. Kim, R. T. Sauer, and T. A. Baker, Mol. Cell **11**, 671 (2003).

[S5] A. Diwa, A. L. Bricker, C. Jain, and J. G. Belasco, Genes Dev. **14**, 1249 (2000).

[S6] L. Kelsi et al., Int. J. Microbiol. **2009** (2009).

[S7] R. Beran, A. Prud'homme-Généreux, K. Baker, X. Miao, R. Simons, and G. Mackie, in *Translation Mechanisms*, edited by J. Lapointe and L. Brakier-Gingras (New York: Plenum, 2003), pp. 148–164.

[S8] J. Shin and V. Noireaux, J. Bio. Eng. **4**, 9 (2010).

[S9] K. C. Keiler, P. R. H. Waller, and R. T. Sauer, Science **271**, 990 (1996).

[S10] S. S. Daube, D. Bracha, A. Buxboim, and R. H. Bar-Ziv, Proc. Natl. Acad. Sci. **107**, 2836 (2010).

[S11] S. McRae, C. Brown, and G. Bushell, Protein expression and purification **41**, 121 (2005).