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Review

The life and death of RNA across temperatures

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ABSTRACT

Temperature is an environmental condition that has a pervasive effect on cells along with all the molecules and reactions in them. The mechanisms by which prototypical RNA molecules sense and withstand heat have been identified mostly in bacteria and archaea. The relevance of these phenomena is, however, broader, and similar mechanisms have been recently found throughout the tree of life, from sex determination in reptiles to adaptation of viral RNA polymerases, to genetic disorders in humans. We illustrate the temperature dependence of RNA metabolism with examples from the synthesis to the degradation of mRNAs, and review recently emerged questions. Are cells exposed to greater temperature variations and gradients than previously surmised? How do cells reconcile the conflicting thermal stability requirements of primary and tertiary structures of RNAs? To what extent do enzymes contribute to the temperature compensation of the reaction rates in mRNA turnover by lowering the energy barrier of the catalyzed reactions? We conclude with the ecological, forensic applications of the temperature-dependence of RNA degradation and the biotechnological aspects of mRNA vaccine production.

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1. Introduction

The information RNA molecules transmit from each gene varies depending on the cell type, time and environmental conditions, such as temperature. All types of macromolecules, proteins, RNAs, lipids and sugars, play a role in the sensing and adaptation to temperature; however, the physicochemical constraints and the molecular mechanisms differ for each class of molecules. The sensing of temperature by RNA molecules and their heat resistance has mostly been studied in bacteria [1,2], but more and more cases have been identified in higher organisms.

The range of temperature to which microorganisms, plants and poikilothermic animals can adapt is quite broad, but even mammals face temperature gradients larger than often assumed. As an introduction, we review the relevant ranges of temperatures cells face. Subsequently, we focus on how these temperatures affect the integrity, folding and structure of RNAs and the strategies how organisms maintain RNA integrity and folding at extreme temperatures.

In the following sections, we will describe the physical principles underlying the temperature-dependence of enzyme reactions that help to understand how specific steps of RNA synthesis, processing and degradation are coordinated. Instead of a comprehensive overview, we present a few examples in particular model organisms at each step between the birth and death of RNA molecules (Sections 6-10). Since all reactions in RNA metabolism – transcription, splicing, RNA degradation etc. – are catalyzed by enzymes, we will discuss the Arrhenius relation to understand how the temperature dependence of enzymatic activities affects RNA abundances (Section 5). Some of these processes, such as transcription, are deeply intertwined with protein-based heat- and cold-shock regulation, which have been extensively reviewed. Therefore, we do not cover upstream transcriptional regulation and will focus on general processes related to mRNA synthesis, the temperature dependence of transcriptional initiation and elongation.

We highlight new developments in areas that await resolution, in the hope that the interactions of these areas will contribute to a better understanding of RNA-mediated temperature adaptation. We conclude the review with the biotechnological applications of the temperature control of RNA-based processes (Section 11).

2. The relevant range of temperatures

2.1. Temperature ranges compatible with cellular growth

The growth range of some extremophiles overlaps with the freezing or boiling points of water. The cold adapted (psychrophilic) microorganism *Methanococcoides burtonii,* an archaeon isolated from a lake in Antarctica, grows between −2 °C and 28 °C [3,4]. On the other end, *Pyrococcus furiosus* has an optimum growth temperature at around 100 °C. Discovered on the Volcano island in Italy, this hyperthermophilic archaeon can multiply rapidly even at the boiling point of water, with a doubling time of 37 min [5].

Whereas special forms of organisms, such as spores, can survive freezing and boiling temperatures [6], the temperature range in which individual microorganisms can grow, and not just survive, span at most 45 °C. Cells mount a cold or heat shock response when exposed to temperatures at the lower or upper limit of their growth range, respectively. Importantly, the temperature that evokes stress responses is not uniform across the tree of life. In fact, the cold shock temperature of a microorganism can be deadly hot for another. For example, a lukewarm environment (23 °C) evokes heat stress in *M. burtonii*, [3], whereas *P. furiosus* experiences cold shock at 75 °C [7].

Interestingly, the heat shock response of *M. burtonii* at 23 °C coincides with the optimal growth temperature, indicating that growth at optimal temperature is stressful for this cold-adapted organism. However, such an overlap is not specific to psychrophilic (cold-loving) organisms. In *Saccharomyces cerevisiae* (yeast), a mesophilic model organism, transcription driven by heat shock response elements is activated already at optimal growth temperature (30 °C). The intensity of this response depends on how cold the prior environment was and how long the temperature was raised [8,9]. Furthermore, the heat shock response is transient at 37 °C but long lasting at 42 °C [10]. Thus, no single temperature threshold can be defined for cold or heat shock responses. Similarly, the temperature optimum can vary depending on the particular trait or function, as exemplified by the model organism *Drosophila*, which can develop from egg to adult fruit fly in the range between 12 and 32 °C. Maximum values are observed at 22 °C for ovariole number but at 16 °C for wing lengths of females [11], indicating that the particular optimum temperature can differ as much as 6 °C. The response can even become binary: the temperature of eggs determines the sex of the developing reptiles [12].

In summary, the temperature optimum of growth or specific organismic activity may not coincide with the temperature at which cold and heat shock responses are minimal, and a certain degree of stress response may be part of normal physiology.

2.2. Basal metabolism in mammals and the contribution of RNA turnover to it

All homeotherms, which regulate their body temperatures, such as birds and mammals, maintain high body temperatures in the range of 36 to 42 °C [13]. When low ambient temperatures threaten to overcool the body, muscles generate heat through shivering as do mitochondria in brown adipose tissue or beige cells through proton leak [14]. For example, the body temperature of men with brown adipose tissue raises in response to cold environment by 0.4 °C more than of those without [15]. At intermediate temperatures, also known as the thermoneutral zone, energy expenditure is the lowest and corresponds to the basal metabolic rate. Counterintuitively, the metabolic rate increases again above the thermoneutral zone, which is attributed to the acceleration of reactions with temperature (see 5.1.). Interestingly, several mammals, such as mice, react biphasically. Mice exposed to high ambient temperatures (39.5 °C) increase their core body temperature to 42-43 °C. Upon returning mice to 25 °C, the core temperature declines rapidly to around 28-30 °C [16], which is clearly below the temperature that evokes cold shock (less than 32–34 °C) [17,18].

Thus, cells experience both heat and cold shock in close succession before returning to the thermoneutral zone.

The major source of heat generated by basal metabolic rate, which is typically measured by oxygen consumption, is the proton leak in mitochondrial respiration. In addition, other ATP consuming processes, especially ion pumps (Na^+/K^+ ATPase and $Ca2^+$ pump), contribute significantly. Similarly, transcription consumes ATP, CTP GTP and UTP as RNA is synthesized, which is likely to contribute between 1 % and 5 % of basal metabolism and hence heat generation in some cells, particularly in the liver [19].

2.3. Temperature gradients in the cells and the body, and their potential impact on RNA

In homeotherms, even small temperature variations can be lethal. Athletes can tolerate core temperatures as high as 41.7 °C without adverse effects. The critical thermal maximum in humans is a body temperature of 42 °C lasting for few hours. Due to the ongoing climate change, the mortality due to heat strokes is expected to increase [20]. At extreme temperatures (49 °C to 50 °C), all cellular structures are destroyed and cellular necrosis occurs in less than five minutes. At lower temperatures, cell death is largely due to apoptosis. Apoptosis affects dividing cells in the developing brain but not in postmitotic neurons [21].

Despite the relative constancy of core body temperatures, gradients are common in the bodies of most homeotherms. For example, the skin, has lower temperature (33–35 °C), especially on the extremities [22], which can further decrease to 29 °C when the ambient temperature drops from 25 °C to 15 °C [23]. Thus, cells in the skin and most cells in the extremities experience lower temperatures than the standard 37 °C.

In addition to gradients within the body, there are also gradients at the cellular scale. In particular, the nuclei and the mitochondria may have higher temperatures than the rest of the cells [15,24]. Some studies suggest that mitochondria can be as hot as close to 50 °C based on measurements with a fluorescent protein thermosensor, whose fluorescence emission changes in response to temperature [25]. If mitochondria are so hot, the intramitochondrial transcription, ribosomes and other RNA mediated processes will be affected by the high temperature. There is evidence that another feature of mitochondria, the strong oxidizing environment in the mitochondrial matrix, affects RNAs. For example, oxidatively damaged RNA is nearly exclusively observed in the mitochondria of retinal ganglion cells [26]. Furthermore, guanine, which is the most sensitive base to oxidative damage, are predominantly localized in solvent inaccessible parts of ribosomal RNA in mitochondrial ribosomes, a sign of RNA adaptation to the mitochondrial environment [27]. It remains to be determined whether RNAs in the mitochondria show also signs of adaptation to higher temperature. An example has been recently suggested: the mitochondrial isoform of an RNase in *Arabidopis*, AtPRORP1, has a 12 °C higher melting temperature than the nuclear isoforms [28].

This raises the question of how steep the intracellular temperature gradients can be. Steep gradients do not easily arise because water is an excellent heat conductor. Computational models based on Fourier's law of heat conduction suggest that at the scale of a cell, temperature gradients of most 10^{-5}C can arise, which is considerably surpassed by the 1 to 10 °C difference detected in the above studies. This discrepancy may be explained by the spatial and temporal averaging through which fluorescent reporters reflect the proton spikes near the ATP synthase [29,30]. In this case, the proteins would nonlinearly overestimate the actual temperature in the mitochondrial matrix. On the other hand, the heat conduction models should incorporate endothermic reactions in the cell, which can steepen the gradients because they absorb heat [24]. Consequently, the high temperature may be limited to the respiratory chain or the immediate vicinity of the inner mitochondrial membrane. This hypothesis is compatible with the results of a systematic study on the melting temperature of proteins; this study found that mitochondrial proteins were not substantially more stable than others were, but the members of the mitochondrial respiratory chain have robustly higher melting temperatures [31].

Nuclei also appear to have higher temperature than the cytoplasm [24], possibly due to the strongly exothermic RNA synthesis (2.2). The faster the RNA turnover, the larger the heat production.

3. RNA folding and temperature

The characteristics of RNA folding is easy to understand when contrasted to protein folding. The secondary structure motifs (e.g. alpha-helices, beta-sheets) of proteins are contextual, i.e. they are stable in the context of the rest of the protein, but they may not form when they are isolated [32]. Such a strong dependence on tertiary structures is typically absent in RNAs, because secondary structures (e.g. stem-loops, see Fig. 1) are highly autonomous, largely due to the stronger interactions between the base pairs. Magnesium promotes the appearance of tertiary structure. This dominance of secondary structures in RNAs may explain the relatively high accuracy of structure prediction models based on physical rules, which reaches around 80 % (the fraction of correctly predicted base pairs in all predicted base pairs) [33–35].

Two questions arise regarding the temperature dependence of RNA folding, which will be addressed in the next subsections. How can RNAs sense changes in ambient temperatures? How can RNAs maintain folded structure at extreme temperatures?

3.1. Temperature sensing by RNA thermosensors

RNAs, just like proteins, contain a very large number of secondary bonds, mostly hydrogen bonds [36], which are sensitive to changes in ambient temperature. Consequently, both proteins and mRNAs can undergo substantial conformational changes with temperature. When a conformational change alters the protein output, such as DNA binding, ion channel activity or inhibition of translation, we talk about protein thermosensors [37]. Many protein thermosensors have been described in both in prokaryotes and eukaryotes. In comparison, RNA thermosensors affect a narrower range of processes, typically splicing and translation, because they act within the same RNA molecule. For example, an RNA stem-loop close to the translation start site inhibits translation of the same RNA (in cis) [1,38]. As always in biology, there are exceptions to the rule: a recent study unveiled that the pairing of two different mRNA molecules controls the expression of a bacterial cold-shock protein (in trans) [39].

Even in non-bacterial organisms, stem-loops can effectively tune translation. A stem consisting of 6 G-C pairs inserted upstream of a start codon decreases translation by roughly 100-fold in yeast cells. When the stem is shortened to 5 base pairs, consisting of three A-T the two G-C pairs, the number of possible hydrogen bonds is reduced from 18 to 12, and consequently translation is inhibited much less, only fourfold [40]. Analogously, a temperature-induced reduction of base pairing is expected to alleviate the inhibition. Indeed, increasing temperatures shift the equilibrium from the folded to the unfolded state of RNA stem loops [41]. The shift between folded and unfolded structures provides a simple intuitive view, but there may be many more structures in equilibrium [42].

Most biophysical studies on RNA structures are performed in vitro, raising the question of how they reflect in vivo conditions. For example, a comparative study did not find a significant differ-

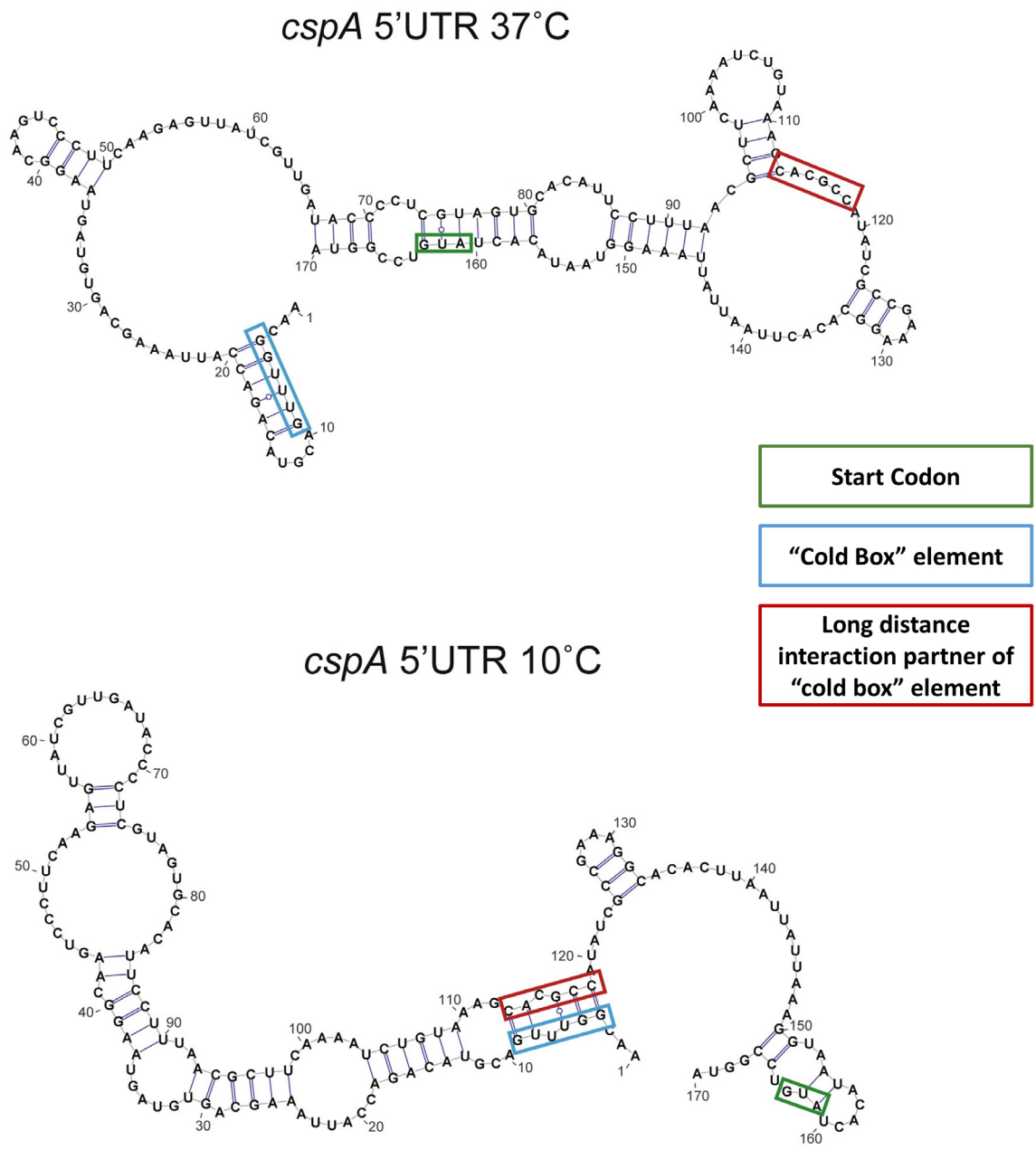

Fig. 1. The structure of the 5′UTR of the *cspA* mRNA in E.coli at 37 and 10 °C. Reproduced from Zhang et al [45].

ence in the computed melting temperature between the bacterial thermosensor and non-thermosensor RNAs [43]. Can this surprising observation be explained by differences between in vivo and in vitro structures? This question can be answered using methods capable of detecting in vivo structures, such as DMS-seq. Dimethyl sulfate (DMS) labels unpaired C and this modification can be detected with RNA sequencing (DMS-seq) [44]. Much fewer in vivo RNA structures were found in yeast cells (around 4 % of the mRNA regions) with DMS-seq in comparison to isolated RNA in vitro (24 %) This reduction of structures is energy-dependent, as evidenced by ATP depletion experiments. The scarcity of RNA structures is not restricted to the coding regions and extend to the untranslated regions (UTRs). Structures appear only much below the optimal growth temperature of yeast cells.

With the same method (DMS-seq), a similar increase in RNA folding was found at low temperatures in *E. coli* [45]. At 10 °C, RNA translation decreases due to the increased RNA folding; however, the abundance of the cold shock proteins (csp) increases after the cold shock. This is possible in part due to the major rearrangement of the structure of the 5′UTR of the RNA chaperone cspA (Fig. 1). While it is easy to understand how high temperatures can increase abundance of protein by disinhibiting translation that is under the control of a stem-loop, it is less clear how cold can increase protein abundance via RNA structures. At 37 °C, there is a 6 bp stem loop the 5′end of the cspA 5′UTR, which contains the cold box sequence. At 10 °C, the cold box sequence pairs with a more distant downstream sequence, resulting in a long tandem of stems, which is likely to prevent the degradation of the mRNA. The cspA mRNA has a much longer half-life at 10 °C than at 37 °C, resulting in higher cspA RNA abundance after cold shock [46]. At the same time, the structure around the start codon becomes looser, which facilitates the translation of Csp. Thus, a real-life RNA thermosensor can undergo complex rearrangements, affecting multiple functions. Well-designed experiments tare

required to decipher the structure-function relationship in these molecules. The increased abundance of the cspA protein leads to a genome-wide reduction of RNA structures due to its RNA chaperone activity in the later phases of the adaptation to the cold shock.

The strong folding of RNAs in the cold, observed in prokaryotes and eukaryotes, and their ATP dependent, active unfolding may explain why RNA helicases, RNA chaperones and nucleases play a prominent role in the cold shock in both bacteria and plants [2,47].

Bacterial RNA thermosensors can detect not only cold, but also warmth [48]. Bacteria often respond when temperature reaches or exceeds 37 °C, as this threshold signals them they are likely to have entered the host organism. A lead (Pb)-based method was used to monitor RNA structures in Yersinia bacteria when temperature was shifted from 25 °C to 37 °C. Slightly more unfolding was observed in the UTRs than in the coding regions. Confirming some well-known riboswitches, this study also identified new mRNAs that contain structures in the UTR that can alter translation in a temperature dependent way [49].

3.2. RNA folding at extreme temperatures

The structural stabilization of ribonucleic acids in hyperthermophiles is particularly important in transfer RNAs (tRNAs), which must maintain a complex three-dimensional structure. Covalent modification of nucleotides seem to provide the answer [50]. The analysis of ribonucleosides from *P. furiosus* grown at 70, 85 and 100 °C revealed that the relative abundance of the nucleoside 5-methyl-2-thiouridine and several other acetylated and methylated nucleotides increased as a function of cell culture temperature [51]. In addition to covalent modification of nucleotides, macromolecular associations can promote tRNA folding. Polyamines are cations that bind to nucleic acids, which were shown to increase the melting temperature of tRNAs. The number of polyamine species is higher in hyperthermophilic microorganisms, exceeding the three standard polyamines: spermin, spermidine and putrescine [52]. The polyamines specific to hyperthermophilic organisms have complex, branched structures.

On the other hand, tRNAs in the psychrophilic archaea, *M. burtonii*, have very few modifications. One of them is dihydrouridine, molecule associated with maintenance of polynucleotide flexibility at low temperatures [53].

4. Integrity of RNA molecules at high temperatures

High temperatures do not only unfold RNAs but can also affect their primary bonds by hydrolyzing the RNA, irreversibly damaging RNA integrity. RNA is more susceptible to hydrolysis than DNA since the 2′OH group, present only in RNAs, can interact with the phosphate group, which results in breaking up of the bonds through transesterification. Whereas magnesium promotes tertiary structure of RNA, it does also efficiently catalyze transesterification, especially at high pH. Thus, the primary and tertiary structures have conflicting requirements for magnesium. In contrast to magnesium, monovalent cations oppose the transesterification. Taking into account realistic solvent properties, the expected half-life of an mRNAs consisting of 1000 or more nucleotides (nt) is expected to be ~1 min or less at 100 °C due to the chemical instability of RNA at such high temperatures [54]. Even smaller RNAs such as tRNA would have a half-life of only ~10 min at 100 °C.

The above in vitro studies on RNA hydrolysis raise the question of how RNAs withstand the high temperatures in hyperthermophilic organisms. Interestingly, recent studies indicate that the intracellular milieu can resolve the opposing effect of magnesium: the detrimental effect on RNA hydrolysis and stabilizing effect on tertiary structure promotion. The free amino acids in the cell weakly chelate magnesium. The amino acid-chelated Mg2 + retains its capacity to enhance RNA folding, by shifting the equilibrium distribution toward the folded structure, but at the same time prevents RNA degradation [55]. Other chelators, such as citrate may further decrease the free magnesium, which may explain how RNA integrity is maintained at high temperatures [56]. Furthermore, the concentration of potassium, a monovalent cation, is often higher in thermophilic than in mesophilic organisms [57], which further enhances the integrity of RNAs.

Potassium may play a role also in the thermotolerance of mesophilic organisms. Intracellular potassium concentration as well as thermotolerance increase in proportion to extracellular osmolality [58–60]. It remains to be determined if the enhanced thermotolerance is mediated by potassium through the stabilization of RNA primary structure.

5. Temperature effect on reactions

5.1. The Arrhenius equation and the catalysis of RNA turnover

Changes in RNA abundance in response to temperature are determined by both the RNA structures and the reactions catalyzed by the enzymes in RNA metabolism. Since these two factors can have different contributions, the response of the reactions has to be quantified separately. Several empirical relations were suggested by the end of 19th century for the temperature dependence of reaction rate constants. van't Hoff observed that the rate constants (k) of most reactions increase by a factor Q_{10} = 2 to 3 when the temperature is increased by 10 °C in the range of ambient temperatures [61–64]. Arrhenius provided a physical explanation for this relation, which is similar to but not identical with the van't Hoff's functional form (Fig. 2A), stating that a reaction can occur only if the preceding molecular collision has a sufficient energy (activation energy. E_a):

$$k = Ae^{-E_a/RT} \tag{1}$$

Measurement of the reaction rate constants at two (or more) temperatures permits the determination of E_a from equation 1:

$$E_a = R \ln\left(\frac{k_2}{k_1}\right)\frac{T_1 T_2}{T_2 - T_1} \tag{2}$$

The Arrhenius relation (equation 1) closely approximates the observations with most elementary uni- and bimolecular reactions, but deviations occur when the relation is examined over a broad temperature range [65].

Most biochemical reactions are not elementary reactions and the reaction rates do not increase monotonically but reach a maximum because the enzymes that catalyze the reaction undergo a phase transition (i.e. denature) at high temperatures (Fig. 2B). Consequently, the temperature at which the reaction rate reaches a maximum has been often termed optimal temperature [66]. It is difficult to determine this value because enzyme denaturation is often slow and thus the temperature optimum depends on the duration of temperature shift before starting the kinetic experiment [67]. Therefore, the optimum temperature is now rarely used but it can be helpful when similar enzymes are directly compared, as illustrated by the following example. It has been shown that a miniaturized ribozymes can be assembled from the fragments of a parent ribozyme. The smaller, fragmented ribozyme did not only have lower optimal temperature but also a higher catalytic activity at lower temperatures in comparison to the parent ribozyme [68]. This shift in the optimal temperature and the improved activity enables ribozymes to function at lower temperatures, which is relevant in a a hypothetical primordial RNA world.

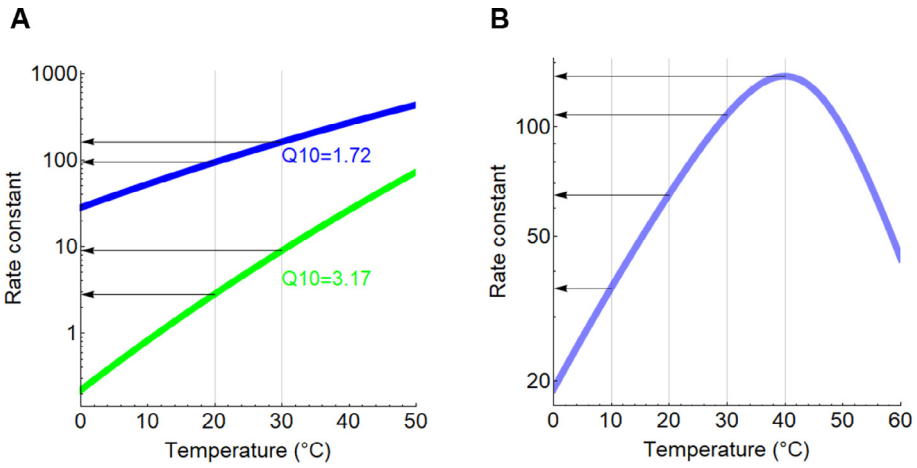

Fig. 2. Temperature dependence of reaction rates (A) Reaction rates calculated according to Arrhenius formula. The blue curve represents an enzymatic reaction (E_a = 40-kJ/mol [9.5 kcal/mol]), whereas the green curve denotes the uncatalyzed counterpart with higher activation energy (E_a = 85 kJ/mol [20.3 kcal/mol]). The corresponding Q_{10} values are shown for a temperature shift between 20 and 30 °C. (B) Temperature dependence of an imaginary enzymatic reaction rate based on Peterson et al that takes enzyme denaturation into account, with a melting temperature of 45 °C, in equilibrium condition [66]. The Q_{10} values indicate a gradual decrease with temperature: Q_{10}(10/20 °C) = 1.81, Q_{10}(20/30 °C) = 1.65 and Q_{10} (30/40 °C) = 1.27. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A systematic analysis showed that the mean Q_{10} of mesophilic enzymes is 1.8 [69]. This value is equivalent to E_a = 10.3 kcal / mol (assuming temperature is shifted between 25 and 35 °C, equation (2)). At the same time, Q_{10} for the same reactions without enzymatic catalysis was found to be 3.4 (n = 9 reactions), i.e. E_a = 20.7 kcal / mol. In other words, when an enzyme lowers the energy barrier of the reaction, the temperature dependence curve of the reaction rate becomes flattened (see also 5.2).

Interestingly, the Q_{10} of thermophilic and psychrophilic enzymes is similar to that of mesophilic enzymes [69]. This also means that compared to the non-catalyzed reaction, the enzymatic reaction rate increases less with temperature (Fig. 2A). Nevertheless, the absolute rates are very high even at high temperatures, which allows fast kinetics of enzymatic reactions.

In order to assess the activation energies in mRNA metabolism, we collected E_a for RNA hydrolysis (degradation). For the enzymatic reactions E_a = 13.1–16 kcal/mol (hammerhead ribozyme, ribonuclease T1) [54,70], whereas E_a = 23.9, 28.5 and 29 kcal/mol for the non-enzymatic reactions [54,71,72]. The ~ 10 kcal reduction of the activation energies by ribonucleases and ribozymes is comparable to the recent observations with other mesophilic enzymes [69]. It also underscores the observation the Q_{10} has a lower value for enzymatic reactions than for the uncatalyzed counterparts, a distinction not made until the second half of the 20th century [64,69]. The determination of E_a of RNA hydrolysis can help also the improvement of RNA vaccine formulation (see 11.2).

Enzymes can reduce the activation energy by multiple mechanisms, among which the stabilization of the transient state stands out. The transient state has an intermediate configuration between the substrate and product. The pre-organized polar environment of the enzyme stabilizes the transition state by electrostatic interactions much more than the corresponding environment in water [73]. Metal cations are key contributors to electrostatic interactions in many enzymes. The importance of metals in DNA and RNA processing enzymes is underscored by the observation that metal cations are coordinated by acidic amino acids to relatively fixed positions within the active site in a large number of enzymes [74]. Interestingly, no major rearrangement was observed in the RNase H1during the transition state formation; instead, cation trafficking was found: magnesium and potassium ions enter and exit the active site at specific reaction steps [75].

5.2. Temperature compensation in reaction networks

In biochemical networks, the primary source of temperature compensation is simply catalysis by enzymes, which lower the activation energy of the reaction, thereby making the temperature dependence of the reaction rate less steep (Fig. 2A, section 5.1).

If the individual reactions in RNA metabolism had the same Q_{10} values, the RNA would be expressed at a constant level under temperature fluctuations, since the faster RNA synthesis rate is compensated by the equally faster rate of degradation.

Q_{10} was measured in *Arabidopis* upon shifting the temperature from 17 °C to 27 °C. The mean (and median) Q_{10} calculated from transcriptome-wide measurement of RNA synthesis rate was 3.6 (3.4). For the degradation rate, Q_{10} = 3.3 (2.8). These Q_{10} values were relatively high in comparison to average Q_{10} of enzymes (see sections 5.1, 6). Since the Q_{10} of RNA synthesis and degradation are similar, only a small average (10 %) change is expected in the steady-state expression upon the temperature shift, implying the two processes are reasonably well compensated.

Even if the Q_{10} values differ among the reactions in a reaction network, gene or biochemical networks can display adaptation, compensating the differences in the Q_{10} values by various network motifs [76,77]. Time-varying expression, such as oscillations are particularly sensitive to temperature-dependent changes in reactions [78,79]. They are more dependent on active temperature compensation by tuning of activation energies and network motifs, such as negative feedback [79].

6. Transcription

In this section, we review the temperature dependence of transcription (6.1) and transcriptional fluctuations (6.2), and the relevant genetic diseases (6.3).

6.1. Effect of temperature on transcriptional initiation and elongation

RNA synthesis occurs in three main steps: transcriptional initiation, elongation, and termination. Elongation can be interrupted by backtracking of RNAP, a phenomenon when the 3′ end of RNA disengages from the template DNA and the polymerase shifts backward along the DNA. Backtracking can occur due to misincorpora-

tion of nucleotides. The resulting pause ends mostly with the correction of the mutant RNA sequence. Longer pauses, on the other hand, are usually caused by RNA secondary structures, such as hairpins [80,81].

The effect of temperature on transcriptional elongation can be monitored in vitro by heating single molecules with infrared laser. In such an assay, the rate of elongation of the *E. coli* RNA polymerase increases with temperature. Conversely, little change was found in the frequency and the lifetime of the paused states [82]. In a related study, which examined the RNA polymerase over broader temperature range, both elongation and pausing was found to be temperature dependent [83]. The (pause-free) velocity increased from 3 nt/s at 7 °C to 22 nt/s at 45 °C, implying E_a = 9. 7 kcal/mol. However, E_a of the entry into the paused state was only 1.3 kcal/mol, which is much smaller than the activation energy of the elongation, suggesting that the RNA polymerase is prone to pause, which may actually be beneficial for the correction of misincorporated nucleotides.

In yeast cells, the analysis of RNA polymerase II with a genomic run-on assay yielded a somewhat lower activation energy of the elongation (6.6 kcal/mol), with velocities from 11.6 nt/s (23 °C) to 21.8 nt/s (37 °C). The faster elongation, however, did not result in larger transcription rate because the polymerase density across the genes reduced, possibly due to the lower initiation rate at higher temperatures [84]. Transcription initiation rate displays also a very complex temperature dependence in *E.coli* [85], suggesting that the reaction is not elementary enough to follow the Arrhenius relation.

Different viral RNA-dependent RNA polymerases show strikingly different temperature dependence [86]. Whereas some human viruses, the poliovirus and the human rhinovirus C, show temperature dependence similar to that of the *E. coli* polymerase, the RNA polymerase of the phage Φ6 has nearly constant elongation speed of ∼ 20 nt/s with minimal increase between 25 and 45 °C, implying a low activation energy (4.1 kcal/mol). This unusual property may be related to the bacterial host of this phage, a plant pathogen, which grows over a broad range of temperatures.

Fever-like temperatures have been shown to reduce transcription of SARS-CoV-2 and hence their replication in respiratory epithelium. This phenomenon was specifically due to inhibition of transcription since the virus entry into the cells was intact [87]. The mechanism of the temperature dependence remains to be determined.

6.2. Temperature dependence of transcriptional fluctuations

Messenger RNAs are typically present at low copy numbers [88], which results in substantial fluctuations in molecule numbers. Consequently, RNA copy numbers are distributed in a cell population. A typical distribution is the Poisson distribution, with a characteristic an inverse relationship between the coefficient of variation (CV) and the mean copy number ($CV^2 = 1/\mu$). These fluctuations due to the low discrete number of molecules can be supplemented by fluctuations in epigenetic changes, gene regulation, chromosomal neighborhood, RNA synthesis and RNA degradation [89–93], which can increase noise above the Poisson level. Such an increase is prominent when transitions between active and inactive states of a promoter occur rarely and active promoter initiates the transcription of multiple mRNAs in bursts, resulting in a large burst size. While most additional regulation increases noise, it can be also reduced: a posttranscriptional RNA negative feedback in HIV reduces noise and stabilizes the viral decision [94].

The temperature results in a velocity distribution of molecular velocities, the Maxwell-Boltzmann distribution, the effect of which can be observed in liquid water, as well [95]. The velocity distribution is taken into account by widely used stochastic simulation algorithm (SSA) [96]. Thus, temperature-related distribution is inherent in this algorithm.

In mammalian cells, the burst size of reporter gene expression was inversely proportional with temperature [97]. Some heat shock genes associate with the nuclear speckle compartment after heat shock, which is associated with a higher transcription rate, which may result in a burst-like gene expression [98]. These two studies indicate that heat shock genes and other genes may react differently to temperature shifts in their stochastic behavior.

6.3. Genetic disorders due to temperature sensitive alleles of general transcription factors

Temperature can play a role in diseases by an inverse mechanism when certain gene alleles render a protein temperature sensitive. Temperature-sensitive alleles have played an important role in molecular biology as tools to inhibit a process when temperature is raised. For example, such methods can be used to inhibit transcription to study mRNA half-lives. A similar mechanism was found to underlie genetically inherited diseases, affecting specific components of transcriptional initiation. Specific mutations of the xeroderma pigmentosum group D, XPD, one of the subunits of the transcription/DNA repair complex *TFIIH,* affect transcription and DNA repair in a temperature-dependent way. These mutations lead to a fever-dependent reversible deterioration of the disease symptom brittle hair. This symptom (also known as trichothiodystrophy) arises due to the transient reduction of cross-linking proteins during the febrile periods, which introduces a fragile point at the base at the hair, resulting in complete hair loss [99,100]. Whereas transcription is similar at 37 °C and 40 °C in WT cells, cells expressing the relevant mutation have less than half of the original transcription upon this temperature shift.

Mutations have been described that can deteriorate cell function already at physiological temperatures, causing a permanent dysfunctionality in the BRCA1 protein. BCRA1 combines the functions transcription and DNA repair, just like XPD [101,102]. Carriers of BRCA1 mutations are predisposed to breast and ovarian cancer. One of these mutants yields a functional protein at 30 °C but fails to do so at 37 °C [103] making it constitutively inactive at the normal body temperature.

7. Splicing

Thermosensitive alternative splicing can alter the distribution of protein isoforms or suppress the expression of some mRNA isoforms. The simplest form of alternative splicing is intron retention. mRNAs with retained introns contain a premature stop codon which targets the mRNA for rapid degradation. An intron retention based mechanism was found to be associated with the temperature-dependent sex determination in reptiles [104]. Thermosensitive alternative splicing has been recently identified even in mouse, a homoeothermic animal. A protein thermosensor, the clock kinase protein (CLK1/4) phosphorylates the serine arginine rich SR proteins, which executes the actual alternative splicing event [105]. The mRNA encoding the SR proteins is itself subject to temperature-dependent alternative splicing in both mice and plants, indicating a conservation of the process [106]. This process may sense smaller, diurnal variations in body temperature.

Heat shock typically acts instantaneously but it also elicits a longer-term memory. Priming of plants with a non-lethal heat stress results in de-repression of splicing after a second exposure to heat stress. In contrast, non-primed plants showed significant repression of splicing. This "splicing memory" helps plants to survive subsequent and otherwise lethal heat stress [107].

8. RNAi-mediated gene silencing

RNAi-mediated gene silencing acts as an antiviral mechanism in plants, which is triggered by double-stranded viral RNA (dsRNA). Viral dsRNA is cleaved into virus-derived small interfering RNAs (vsiRNAs) with lengths of 21–24 nucleotides by the dsRNA-specific DICER-LIKE (DCL) endonuclease. The short length siRNAs are then loaded into ARGONAUTE (AGO), which silences the target RNAs, including viral RNA by cleaving target viral RNAs or by repressing their translation.

Several studies suggested that vsiRNAs amounts and silencing increased in strength with temperature in some systems, resulting in a better antiviral defense at higher temperature [108,109]. In some systems, however, increases in temperature was associated with larger viral titers [110]. A strong temperature dependence of AGO activity was observed also in animals [111]. Furthermore, base pairing strength may affect the functionality of the miRNA-target duplex. The temperature-dependence of this pairing is also reflected in the significant correlation between the average GC content of miRNAs and the physiological temperature of a given organism [112].

9. RNA degradation and its measurement across temperatures

9.1. Nonlinear temperature dependence in RNA degradation in yeast cells

Being a first order reaction, RNA degradation is well suited to study the van't Hoff law. A shift of temperature from 20 °C to 30 °C increases mRNAs degradation rate in yeast cells Q_{10}(20/30 °C) = 1.82 times on average [113]. This value is in close agreement with the mean Q_{10} = 1.8 calculated for different mesophilic enzymes (section 5.1). The half-life of mRNAs is further shortened when the cells are exposed to heat shock at 42 °C. However, the extent of this shortening is smaller, Q(30/42 °C) = 1.58. This indicates a plateauing of decay rates possibly due to the partial denaturing of the RNA degradation enzymes at higher temperatures. Indeed, the melting temperature of Xrn1, which degrades mRNAs in the 5′ to 3′ direction, is 44 °C, implying that 50 % of this protein is denatured at this temperature [31].

The median mRNA half-lives determined by a gene control method are 3.4 and 1.5 min at 20 and 42 °C, respectively, which indicates that the mRNA turnover is very rapid throughout temperature range that permits cell growth. Short half-lives are important because they allow oscillatory transcription to propagate between the stages of the cell-cycle [114], which would be impossible with longer mRNA half-lives, as suggested by earlier studies [115]. This brings us to the next topic, how the methods affect the measurement of mRNA half-life, which will be discussed in the next section.

9.2. Impact of the methods on the measurement of RNA degradation at high temperatures

For many years, different methods and sometimes even different studies using the very same method had yielded uncorrelated mRNA half-lives [116]. However, datasets obtained with different methods were published in the past decade that displayed higher correlation, putting an end to the inconsistencies at the standard growth temperature (30 °C). On the other hand, exposing the cells to heat shock resulted in uncorrelated half-lives even between methods that otherwise show a high correlation at the standard temperature [113]. Thus, the measurement of half-lives under heat shock is particularly challenging, and displays strong method-dependence, which is partly caused by the interference from the different methods [116].

This raises the question of whether the heat shock results described in the previous section reflect the degradation rates faithfully. The reasoning applied at the standard temperature gives the way out: if two different methods yield consistent results, it is likely that the half-lives will be reflected with little distortion or completely faithfully. The gene control method used in the above study correlates highly (r_s = 0.75) with one of the datasets that use transcriptional inhibition with the temperature sensitive allele of RNA polymerase II, rbp1-1 at 37 °C [117], which suggests that consistent results can be indeed achieved even at heat shock (Fig. 3). Since the rbp1-1 allele can be used to measure half-lives only at high temperatures, Q_{10} cannot be measured by this method.

Q_{10} was higher in *Arabidopsis* than in yeast (3.3, see section 5.1) when the temperature was shifted from 17 to 27 °C. It remains to be determined if the large value is due to a molecular mechanism, such as temperature induced unfolding of RNA structures in the UTR, or due to method-dependence. In *Arabidopsis,* various protocols of transcriptional inhibition and metabolic labelling have been used and the correlations between different the various studies are low to intermediate (Fig. 4). Datasets with good correlations (>0.7) are yet to come. The application of a third, independent method can also help the verification of the findings.

10. Regulation of translation by RNAs

In mammals, the cold shock response is triggered at and below 32 °C. A major mediator of this response is the cold inducible RNA-binding protein (CIRP or CIRBP). CIRBP is upregulated in various organs upon mild hypothermia or cold stress, in different species from amphibians to humans. It typically binds to the 5′UTRs of mRNAs and can affect their stability and translation [118]. In mouse fibroblasts, CIRP selectively binds to the 5′ untranslated region of the CDK (cyclin dependent kinase) inhibitor p27Kip1 mRNA and enhances its translation, which in turn reduces cell division [119]. p27Kip1 is central for the decreased proliferation at lower temperature, since mouse embryonic fibroblasts lacking this protein did not reduce their doubling time in hypothermic conditions.

In keratinocytes, the induction of CIRP at 32 °C is dependent on a protein thermosensor, the transient receptor potential vanilloid 4 (TRPV4) channel protein [120]. Since keratinocytes in the skin are frequently exposed to lower temperatures (2.3), cold shock proteins may affect skin physiology [121]. Interestingly, most experiments with keratinocytes were performed at 37 °C despite the fact that dermis regenerates and keratinocytes differentiate better at 31–32 °C than at 37 °C [122,123].

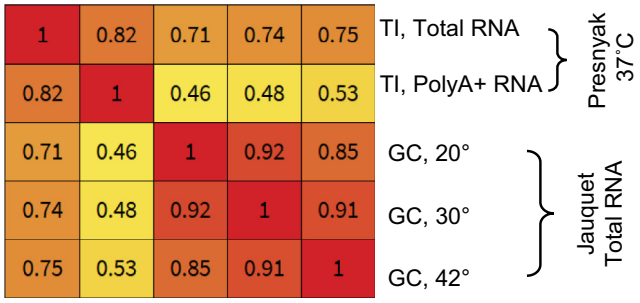

Fig. 3. Correlation of RNA half-lives at high temperatures in budding yeast measured with gene control (GC) and transcriptional inhibition (TI) methods. The Spearman rank correlation (r_s) is calculated from half-lives of mRNAs common in all indicated half-life datasets (n = 75). The correlation between the GC (Jaquet el, 42 °C) [113] and TI (Presnyak et al, 37 °C) [117] datasets is 0.75. The respective median half-lives are 1.5 and 6.4 min.

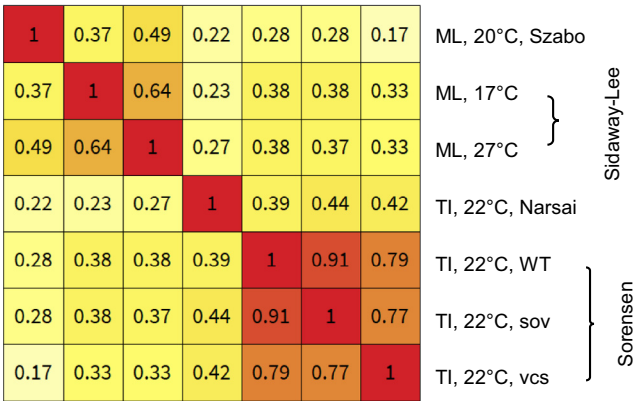

Fig. 4. Correlation of RNA half-lives in *Arabidopis thaliana* measured with metabolic labelling (ML) or transcriptional inhibition (TI). The Spearman rank correlation is calculated between datasets for genes common in all studies (n = 5049). The following median half-lives were obtained for these common genes: 1.61 h in Szabo et al [147], 3.99 h (17 °C) and 1.35 h (27 °C) in Sidaway-Lee et al [148], 3.70 h in Narsai et al [149], and 1.83 (WT), 1. 64 (sov) and 2.42 (vcs) in Sorensent et al [150].

In turtles, the expression of CIRBP is allele specific and temperature dependent: one of the alleles is induced in embryos exposed to a female-producing temperature, while expression of the other allele does not differ between female- and male-producing temperatures [124]. The SNP underlying the alleles is associated with temperature-dependent sex determination. Thus, thermosensitive splicing and CIRBP may cooperate in temperature-dependent sex determination [104,124].

11. Biotechnological applications of temperature control

11.1. Ribozymes, riboswitches and RNA thermosensors

An outstanding achievement in combining temperature control with the synthesis of nucleic acids polymers was the discovery of PCR. The DNA conformational alterations induced by temperature cycling (i.e. denaturation and hybridization) are coupled with alternating DNA synthesis rates, leading to an amplification of DNA. Temperature cycling has been also applied to RNA synthesis [125]. A ribozyme catalyzed ligation process was developed to understand RNA amplification in a possible primordial RNA world in the early terrestrial history. The biotechnological application was not the primary aim of RNA amplification in this study, since RNA can be transcribed from DNA. It is important, however, to note that temperature cycling is not required for RNA replication per se [126]. However the temperature alterations may enhance ribozyme reactions [125,127], with possible applications in biotechnology.

RNA folding is dominated by secondary structures with less contextual effects; this modularity permits thermosensing stem-loops to be easily inserted to the RNAs of interests [128]. RNA thermometers can be coupled to riboswitches, which bind and sense small molecules, and this combination permits the sensing of small molecules at different temperatures. However, some riboswitches, for example the add riboswitch, which senses both adenine and temperature, requires the interaction with a specific protein subunit of the ribosome, which may limit the transmission between different species [129]. Additional engineering of RNA sequence may be required to facilitate their use in different species. This riboswitch illustrates that many RNA structures cannot be analyzed in isolation. As an alternative to pure RNA modules, RNA and protein thermosensors can be combined to amplify the the temperature response. For this purpose, the temperature-sensitive coiled-coil domain of the Tlpa protein can be fused to the protein of interest [130,131].

11.2. Role of temperature in the manufacturing and delivery of mRNA vaccines

Whereas mRNA vaccines have only been developed recently, they have proven to be the most effective vaccines against the coronavirus in the Covid pandemics (2019–2022) [132,133]. An important step in this development was the incorporation of modified nucleosides, such as pseudouridine, in the mRNA, which reduces the undesired immune response and enhances translation [134–136]. One of the main causes of the undesired immune response is the double stranded RNA, which can arise as a byproduct of the vitro transcription. An extra HLPC purification of the in vitro transcribed RNA was shown to reduce this immune response [137]. An alternative method based on the thermostable T7 RNA polymerase has been developed recently. This thermostable polymerase permits the synthesis of the mRNA at high temperature (50 °C), preventing the formation of double stranded RNAs [138]. The mutation rate in the transcription by the T7 RNA polymerase does not increase when the temperature is raised from 30 to 42 °C [139]. Whether this finding can be extended to the transcription at 50 °C by the thermostable T7 RNA polymerase variants remains to be determined.

The degradation of the mRNA is an important practical aspect of the vaccine since RNAs require storage at very low temperatures. The activation energy of 17.9 kcal/mol (74.8 kJ/mol) has been found for mRNA degradation in lipid particles, which is less than the previous estimates for nonenzymatic hydrolysis (see section 5.1). These data suggest that the degradation of mRNAs encapsulated in lipid nanoparticles requires low activation energy, making it susceptible to degradation [140]. Using different materials for encapsulation has been shown to change the activation energies for degradation of different compounds [141]. Thus, the modification of encapsulation technology may permit mRNA vaccines to be stored at higher temperatures by raising the activation energy of degradation.

11.3. Forensic and ecological applications of the temperature dependence of RNA degradation

RNA degradation plays an important role in diagnostic and forensic science [142]. Evidence of degradation of some mRNAs suggest that the 5′ end of an mRNA transcript degrades in dried stains faster than the 3′ end. Statistical analysis of degradation kinetics suggests, depending upon the age of the sample, the age of bloodstains can be accurately estimated to within 2–4 weeks for stains less than 6 months of age and 4–6 weeks for stains 6 months to 1 year old [143]. The RNA half-life is dependent on temperature and air humidity [144].

RNAs can be also used to assess the past microbial communities in environmental samples [145]. The temperature dependence of RNA degradation affects the interpretation of microbiome since RNA persists much longer in low temperature soil [146].

12. Conclusions

Our review highlighted recent developments in temperature control of RNA biology. There are many phenomena that are more closely connected than commonly assumed, whose understanding requires both empirical observations and computational modeling, summarized briefly below.

Due to the biphasic temperature response observed in mouse, a succession of high and low temperature pulses may be more appropriate to study cell adaptation instead of monophasic heat or cold shock. In human biology, it remains to be seen how often SNPs encode temperature-sensitive enzymes, from RNA polymerases to processing enzymes, that display temperature-dependent symptoms.

New imaging results revealed the existence of intracellular temperature gradients, the magnitude of which is a subject of a lively debate at the interface of physical modelling and experimental observations. RNA turnover may contribute to these gradients through basal metabolism, and vice versa, RNA may experience temperature-induced changes in the nuclei and mitochondria.

RNA is prone to hydrolysis at high temperatures a typical hyperthermophile is exposed to, but the intracellular milieu seems to employ tricks to mitigate the detrimental effect of magnesium. If RNA is sufficiently stabilized by proper cation composition and chelation, the main source of degradation is likely to be enzymatic just like in most other organism. This hypothesis remains to be tested. The Arrhenius plot can help distinguishing enzymatic degradation from non-enzymatic hydrolysis since enzymes lower the activation energy of a reaction (Fig. 2A).

On the other hand, the appearance of strong RNA structures in cold environment may reduce the relevant reaction rates more than expected from a typical Q_{10} value of around two, which may contribute to a cessation of microorganism growth at low temperatures above the freezing point. Measurement of activation energies in these processes can help to understand the complex interplay between RNA structures, RNA degradation and translation.

CRediT authorship contribution statement

Attila Becskei: Writing – original draft, Writing – review & editing, Conceptualization, Supervision, Visualization. **Sayanur Rahaman:** Writing – original draft, Writing – review & editing, Conceptualization, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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