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REVIEWS

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Posttranscriptional Modification of Messenger RNAs in Eukaryotes

I. G. Laptev[a], A. Ya. Golovina[b], P. V. Sergiev[a, b], and O. A. Dontsova[a, b]

[a] *Department of Chemistry, Moscow State University, Moscow, 119991 Russia;*
e-mail: whiteswan92@gmail.com

[b] *Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119992 Russia*

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Abstract—Transcriptome-wide mapping of posttranscriptional modifications in eukaryotic RNA revealed tens of thousands of modification sites. Modified nucleotides include 6-methyladenosine, 5-methylcytidine, pseudouridine, inosine, etc. Many modification sites are conserved, and many are regulated. The function is known for a minor subset of modified nucleotides, while the role of their majority is still obscure. In view of the global character of mRNA modification, RNA epigenetics arose as a new field of molecular biology. The review considers posttranscriptional modification of eukaryotic mRNA, focusing on the major modified nucleotides, the role they play in the cell, the methods to detect them, and the enzymes responsible for modification.

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INTRODUCTION

Posttranscriptional modifications of RNA were found rather long ago, but their biological functions remain obscure apart from few exceptions. Cap modifications are the best understood in eukaryotic mRNA. Regions distant from the mRNA ends may contain N6-methyladenosine (m^6A), 5-methylcytidine (m^5C), pseudouridine (Ψ), and inosine (I), which were believed to play only a minor role because their proportion in cell RNA is extremely low as compared with the standard nucleotides. As analytical methods developed, modified nucleotides proved to occur in certain positions, rather than being spread at random, and their occurrence in such positions sometimes reaches 100% [1–6]. The review focuses on these four modifications, their occurrence, detection methods, and, when possible, the roles they play in the cell.

2'-*O*-METHYLATED NUCLEOTIDES

Higher eukaryotic mRNAs and many virus RNAs undergo 2'-O-methylation at one or two first nucleotides (methylated nucleotides are collectively designated N_m) [7, 8]. The most common are m^6A_m and other A_m, accounting for approximately 70% of all nucleotides methylated at the ribose moiety. The G_m proportion is 18%, and C_m and U_m together account for 12% [9]. Specific mRNA 2'-*O*-methyltransferases were isolated from a HeLa cell extract and characterized in 1981 [10], but it was not until 2011 that their genes were identified and cloned [11]. N_m are involved in self versus nonself RNA recognition [12]. Human and mouse coronaviruses mutated to lack 2'-*O*-methyltransferases induce high-level production of type I interferon via the MDA5 cytoplasmic protein, which is sensitive to dsRNA [13].

N6-METHYLADENOSINE

N6-methyladenosine (m^6A) is the most common mRNA modification. The first m^6A detection in mRNA dates back to the 1970s [14]. Because mRNA accounts for only a minor proportion of total cell RNA, m^6A detection in mRNA is rather problematic. By 2005, specific adenosine methylation sites were found only in two RNAs, the bovine prolactin (bPRL) mRNA [15, 16] and Rous sarcoma virus (RSV) RNA [17]. In the bPRL mRNA, methylation sites cluster in the 3'-untranslated region (3'-UTR) and in the vicinity of the polyadenylation site. The m^6A occurrence in the methylation sites is only ~20%. More than ten methylation sites were identified in the RSV RNA. Like with the bPRL mRNA, methylation of the RSV RNA is incomplete, varying from 20 to 90%. A mutation analysis of the adenosine methylation sites in vitro and in vivo established the specific site sequence, RRm^6ACH (where R is adenosine or guanosine and H is adenosine, uridine, or cytidine) [18]. Statistically, the sequence can be found in every 85 nt, so that approximately 30 adenosine methylation sites may occur in mRNA on average. Because m^6A was not detected in all of the potential sites in the bPRL mRNA and RSV RNA, their adenosine methylation was not assumed to proceed quantitatively.

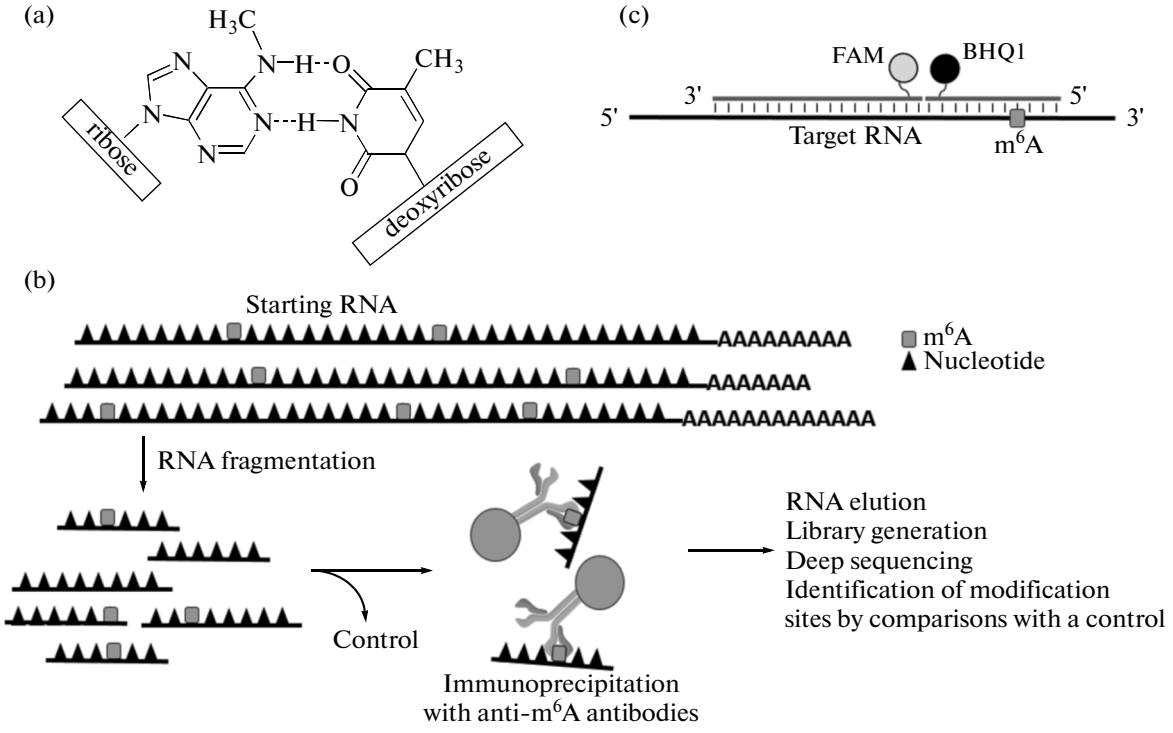

Fig. 1. (a) Adenosine methylation at N6 does not distort the Watson–Crick interactions in reverse transcription. (b) Identification of adenosine methylation sites in polyadenylated RNA by m^6A-seq and MeRIP-seq [1, 2]. (c) Primer annealing to check for the m^6A presence [22].

Methods to Study m^6A and Their Results

It is rather difficult to detect m^6A in RNA because methylation does not affect the Watson–Crick base-pairing (Fig. 1a), and reverse transcriptase does not distinguish between modified and unmodified nucleotides. In 2012, m^6A-seq [1] and MeRIP-seq [2] were developed to detect m^6A in RNA with a high sensitivity. The gist of the method is as follows (Fig. 1b). Polyadenylated RNAs are chemically cleaved into fragments of approximately 50–100 nt. Fragmented RNA is divided into two portions. One is used for immunoprecipitation with anti-m^6A antibodies to obtain a modification-enriched fraction, and the other serves as a reference. Both of the portions are subject to deep sequencing. The m^6A positions are identified by comparing the sequencing results for the two portions. Many RNAs proved to contain m^6A in the well-known context [18]. Generally, m^6A is found mostly around the stop codon of the mRNA coding region. The significance of this arrangement remains unclear. The m^6A distribution differs among different tissues and in tissues exposed to stress. A virtually unique adenosine methylation profile is therefore characteristic of each cell type.

The method reports the methylated adenosine positions with a resolution of approximately 100 nt [1, 2]. Sequencing with greater overlaps and a more stringent bioinformatics data processing were used to identify the adenosine methylation sites in the total yeast transcriptome at a better resolution [19]. RNA was fragmented into shorter segments, and a more rigorous approach was used to eliminate false positive results. RNA from cells with the inactivated gene for methyltransferase responsible for adenosine modification (which is impossible with mammalian cells because a *METTL3* knockdown leads to apoptosis) and several RNAs synthesized in vitro were used as negative controls. About half of the identified m^6A sites were considered to be false positives with these negative controls, indicating that the results reported in [1, 2] most likely need verification.

Recent single-molecule real-time sequencing showed that HIV reverse transcriptase is m^6A sensitive [20]. *Thermus thermophilus* DNA polymerase I was found to act as reverse transcriptase in the presence of Mn^{2+}, being sensitive to the m^6A presence in these conditions [21]. The enzymes might be suitable for sequencing the transcriptome with the identification of m^6A sites.

A method to verify the m^6A presence in a particular RNA site was developed in our lab [22]. The method is based on analyzing the melting curves for DNA–RNA duplexes. To detect the modification in a given RNA site, two primers with end-to-end annealing are selected (Fig. 1c). One primer contains a fluorescein (FAM) at the 5' end, and the other contains the black hole quencher BHQ1 at the 3' end. The BHQ1-containing primer is designed to hybridize with a m^6A-con-

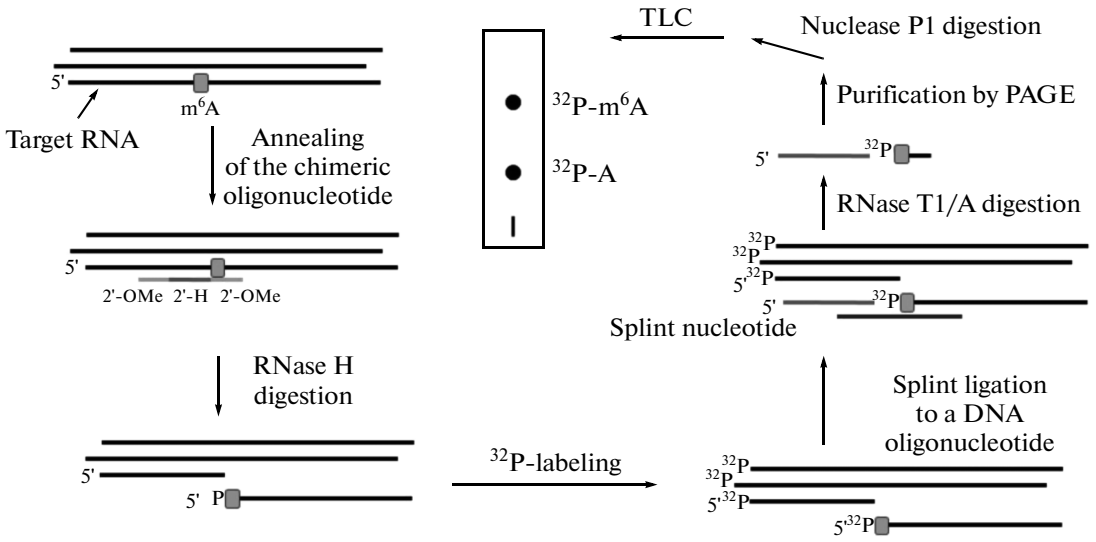

Fig. 2. Scheme of m^6A detection by SCARLET [23].

taining RNA region. A comparison of the differential melting curves for the control modification-free duplex and a test sample reports whether m^6A occurs in the given RNA site. The method can be used, for example, to identify the genes for methyltransferase that modifies a certain nucleotide. In the case of eukaryotic mRNAs, the method is suitable for probing the adenosine methylation status in a particular site of a particular RNA in various cell growth conditions.

A method known as site-specific cleavage and radioactive labeling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) [23] makes it possible to establish whether adenosine is methylated in a given position of a given molecule and to estimate the proportion of modified and unmodified nucleotides (Fig. 2). In this method, a specific chimeric 2'-O-Me/2'-H oligonucleotide is constructed and hybridized to the polyadenylated RNA fraction. The target RNA site is cleaved with RNase H to produce two RNA fragments so that the target adenosine is at the 5' end of one of the fragments. The remaining RNA is phosphorylated to add $[^{32}P]O_4^{3-}$ to the 5' ends of RNA fragments, and the fragment of interest is ligated to a long single-stranded oligodeoxyribonucleotide. The mixture is digested with RNases A and T1, which together cleave ssRNA after C, U, and G. The oligodeoxyribonucleotide with ^{32}P A/m^6A, which remains intact, is purified by denaturing electrophoresis and digested with nuclease P1, which cleaves ssDNA and RNA to 5'-monophosphates. The resulting sample is assayed for $[^{32}P]A$ and $[^{32}P]$-m^6A by thin-layer chromatography. The method is rather laborious and requires a radioactive label to be used.

Several methods were developed to detect m^6A in RNA at various resolutions. Each method has its drawbacks and advantages. High-throughput methods have insufficient resolution, while high-resolution techniques are too laborious and complex.

(Adenine N6-)-Methyltransferases

The protein product of *METTL3* was the first to be identified as eukaryotic (adenine-N6-)-methyltransferase [24] and is conserved among many organisms from yeast (*IME4*) to mammals. A knockdown or deletion of its gene exert various phenotypic effects, causing apoptosis in human cell lines, a lower survival in plants and *Drosophila melanogaster*, and sporulation defects in yeasts [25].

Recent studies identified METTL14 as another protein that catalyzes adenosine methylation in RNA and forms a heterodimeric complex with METTL3 [26]. The two proteins belong to one methyltransferase superfamily and have 43% amino acid sequence similarity. Both of them are catalytically active and methylate oligonucleotides in vitro. The findings indicate that the two proteins act as catalytic subunits of the complex.

The METTL3–METTL14 complex was shown to interact with WTAP (Wilms' tumor 1-associated protein) [26, 27], which is involved in splicing [28]. A WTAP gene knockdown considerably reduces the m^6A content, although WTAP does not display methyltransferase activity in vitro [26]. It seems that WTAP acts as a regulatory protein to facilitate methyltransferase activity or nuclear localization of methyltransferases [26, 27].

Demethylases

In addition to RNA methylation, demethylation occurs as an opposite process. It is clear that the role the process plays in the cell is probably no less important.

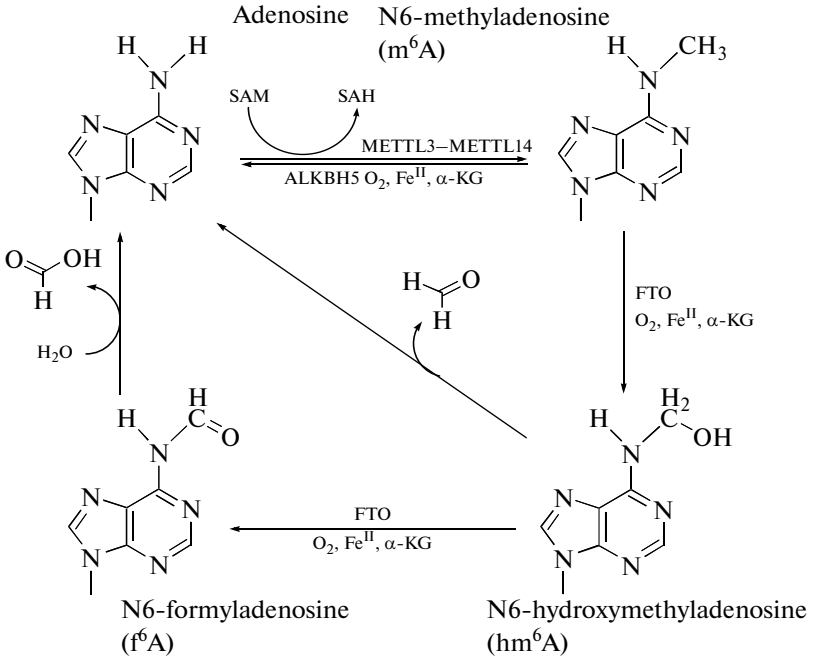

Fig. 3. Reversible adenosine methylation. α-KG, α-ketoglutarate.

FTO (fat mass and obesity-associated protein) belongs to a family of proteins homologous to Fe(II)/α-ketoglutarage-dependent dioxygenase AlkB. FTO was shown to catalyze oxidative demethylation of m^3T and m^3U in ssDNA and ssRNA [29, 30], although its activity is lower than in AlkB-family proteins [31]. Its Arg316 is essential for α-ketoglutarate binding, and the R316Q substitution abolishes FTO catalytic activity in vitro [32]. Severe growth retardation is observed in organisms carrying this mutation. The FTO preference for ssDNA and ssRNA is possibly explained by the presence of an additional loop, which covers a side of a conserved jelly roll motif and competes with the complementary stand of the duplex [33]. Surprisingly, the capability of m^6A demethylation was observed for FTO [31].

Physiological substrates of AlkB-family proteins are not limited to N1- or N3-modified purines and pyrimidines, attention was consequently attracted to m^6A as the most common mRNA modification [31]. FTO proved to convert m^6A to adenosine in synthetic ssDNA and ssRNA with an efficiency comparable with that of m^3U demethylation [31]. The m^6A modification was not detected in higher eukaryotic DNA [34], indicating that RNA acts as a physiological substrate of FTO. To check this assumption, *FTO* was silenced via RNA interference or overexpressed in HeLa and HEK293FT cells. The results confirmed that m^6A is a physiological substrate of FTO in these cell lines. FTO was observed to occur exclusively in the nucleoplasm together with the splicing factors SART1 (U4/U6.U5tri-snRNP-associated protein) and SC35 (serine/arginine-rich splicing factor 2), implicating FTO in mRNA maturation [31].

FTO demethylates m^6A via the formation of two intermediates, N6-hydroxymethyladenosine (hm^6A) and formyladenosine (f^6A) (Fig. 3) [35]. The two intermediates are detectable in vitro, indicating that their dissociation and rebinding are involved in demethylation.

FTO binds m^6A and hm^6A with comparable affinity, and the generation rate of hm^6A is higher than that of f^6A. The intermediates (hm^6A and f^6A) are found in RNA in vivo. Their content was estimated at 0.5–1% of the total m^6A content, but the intermediates might degrade during RNA isolation and fragmentation.

In 2013, ALKBH5 (alkylation repair homolog 5) was identified as another m^6A-RNA demethylase of the AlkB family [36]. Like FTO, ALKBH5 is localized in the nucleus together with the splicing factors CS35, Sm, and ASF/SF2 (alternative splicing factor/splicing factor 2). It is most likely that ALKBH5 directly interacts with RNA because a granular pattern of ALKBH5 distribution in the nucleus is almost completely eliminated by treating preparations with RNase A. An *ALKBH5* knockdown increases the mRNA content in the cytoplasm, implicating the demethylase in mRNA export [36].

Proteins Interacting with m^6A

Several proteins were found to selectively bind with synthetic oligoribonucleotides that mimic the adenosine methylation site of the RSV RNA and contain m^6A

Disorders associated with m^{6}A-related genes [25]

Gene	Function of the protein product	Disorder
METTL3	Methyltransferase	Prostatitis, Aicardi syndrome
METTL14	Methyltransferase	Alcohol addiction
WTAP	Complex targeting or localization	Wilms' tumor, hypospadias, sarcoma, malignant mesothelioma, synovial sarcoma
FTO	Demethylase	Obesity, diabetes mellitus, polycystic ovarian syndrome, infarction, cancer, alcoholism, mental disorders, cataract, hepatitis
ALKBH5	Demethylase	Hypoxia, Smith–Magenis syndrome
YTHDF1	m^{6}A binding	Pancreatic cancer, pancreatitis, dermatomyositis
YTHDF2	m^{6}A binding	Leukemia, kidney cancer, breast cancer
YTHDF3	"	No data
ELAVL1	"	Cancer, leukemia, hepatitis, oxygen starvation, Alzheimer's disease, arthritis, prostatitis, hypoxia, laryngitis, keratoconus, pancreatitis

as bait [1], ELAVL1, YTHDF2, and YTHDF3 being the best binders.

ELAVL1, which is also known as HuR (human antigen R), belongs to the ELVAL family of RNA-binding proteins and selectively binds AU-rich regions in the 3'-UTR of mRNA [37]. ELAVL1 stabilizes mRNAs that contain AU-rich elements [38, 39]. Published data on the functional relationship of ELAVL1 with mRNA methylation are discrepant. After ELAVL1 was initially reported to selectively bind m^{6}A-containing RNA [1], a more efficient binding was demonstrated for total RNA from cells lacking m^{6}A in mRNA [40].

The two other proteins, YTHDF2 and YTHDF3, belong to the YTH domain superfamily of RNA-binding proteins [41]. The YTH domain is conserved among eukaryotes and is widespread in plants. YTHDF2 recognizes m^{6}A both in vitro and in vivo [42]. YTHDF2 binding with m^{6}A-containing oligoribonucleotides is ~15 times more efficient than with nonmodified oligonucleotides in vitro. YTHDF3 and YTHDF1 similarly bind the modified nucleotide in vitro with a 5- to 20-fold higher efficiency. More than 3000 YTHDF2 targets were found in human cell lines, and the majority of the targets occur in mRNA. YTHDF2 competes with ribosomes for mRNA binding in the cytoplasm. YTHDF2-bound mRNA is committed to degradation. Thus, YTHDF2 acts as a sorter of m^{6}A-containing mRNAs. When free ribosomes are available, mRNA binds with them and is translated; otherwise, YTHDF2 binds with mRNA and relocates it to a degradation site.

Predisposition to certain disorders was associated with polymorphic variants of the genes whose products are involved in mRNA modification (table).

Role of m^{6}A in Stem Cell Proliferation and Differentiation

The effect of mRNA methylation and m^{6}A production on stem cell proliferation and differentiation has been discussed intensely in the past years. Published data are discrepant. *Mettl3* and *Mettl14* knockdowns reduce the m^{6}A content in embryonic stem cells, thus decreasing their proliferative activity and inducing a loss of pluripotency markers; i.e., the effect is promoting stem cell differentiation [40]. It is possible that methylation of the mRNAs coding for differentiation regulators facilitates their degradation in control stem cells. In contrast, a more recent study showed that complete *Mettl3* silencing in mouse stem cells increased their self-renewal potential and blocked their differentiation into cardiomyocytes and neurons [43]. Similar results were obtained when Mettl3 was inactivated via a *Mettl3* knockdown rather than RNA interference [44]. *Mettl3* knockout stem cells were viable and proliferated normally, but their differentiation was distorted. When differentiation was induced, the cells still expressed genes characteristic of pluripotent stem cells. The effect was associated with stabilization of the mRNAs coding for pluripotency markers. Thus, it is commonly accepted that methylation destabilizes mRNA, but it remains unclear what mRNAs are more prone to methylation and destabilization in early development.

5-METHYLCYTIDINE

In the 1970s, m^{5}C was found in mRNAs isolated from BHK-21 hamster cells [45] and certain virus RNAs [46, 47], but not in HeLa cell mRNA [48] and SV40 RNA [14]. Fortunately, m^{5}C proved far easier to map in RNA as compared with m^{6}A. Bisulfite treat-

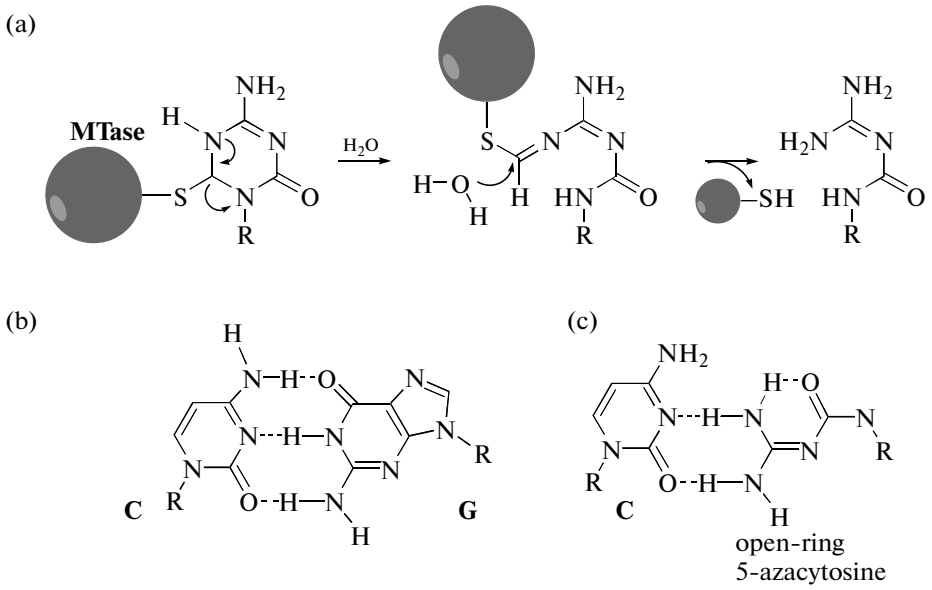

Fig. 4. (a) Hypothetical opening of the 5-azacytosine ring, (b) a G–C complementary pair, and (c) a pair presumably formed by C and open-ring 5-azacytosine. MTase, methyltransferase.

ment deaminates cytidines without affecting m^5C residues. Sequencing of bisulfite-treated RNA identifies the m^5C residues, which are not deaminated. Bisulfite sequencing first used to examine total RNA from HeLa cells showed that m^5C often occurs in mRNAs and noncoding RNAs [3], while earlier methods were not sensitive enough.

(Cytosine-C5-)-Methyltransferases

Several RNA-(cytosine-C5-)-methyltransferases were found in eukaryotic cells: the NSUN protein family (NSUN1–6 and NSUN2 homologs), DNMT2, and DNMT2 homologs. Methylating activity towards mRNA was observed only for NSUN2 as yet [3, 49].

The human NSUN2 family includes nine proteins, most of which are highly conserved among mammals [3, 50] and possess a methyltransferase domain. Activity of the domain was studied only in NSUN2 [51]. Mouse NSUN2 is a component of chromatoid body and is necessary for testicular differentiation [49, 52] and a balance between self-renewal and differentiation of skin stem cells [53]. NSUN2 is involved in tRNA methylation and modifies mRNA and noncoding RNAs as well [3, 49, 52, 54].

Methods to Study m^5C and Their Results

Studies of m^5C were initially limited to tRNA and rRNA. As for mRNA, the modification was poorly understood until bisulfite sequencing was applied to total cell RNA [3] and m^5C was identified as a modification common in mRNAs and noncoding RNAs of various, including human, cells. The method was based on conventional DNA bisulfite sequencing [55, 56] and revealed 10274 m^5C sites in mRNAs and noncoding RNAs with m^5C accounting for 0.4% of all cytidine residues. Noncoding RNAs had even a higher m^5C proportion, 1.2%. Methylated cytosine occurred mostly in untranslated regions and in the vicinity of binding sites for Argonaute-family proteins. It is possible that m^5C plays a role in the miRNA-mediated RNA degradation pathway [3].

Two new methods were recently developed to identify the cytidine methylation targets in RNA, taking advantage of the methylation mechanism [57]. Many RNA methyltransferases are known to possess two highly conserved cysteine residues, which are essential for catalysis. One forms a covalent intermediate with the target cytidine, and the other is necessary for the covalent intermediate to be resolved after methylation [57]. The Aza-IP method is based on a covalent bonding of a cytosine analog (5-azacytosine) incorporated in nascent RNA with m^5C methyltransferase (Fig. 4a). 5-Azacytidine is not methylated, and the methyltransferase cysteine residues remains linked to the heterocycle. The crosslinking is followed by immunoprecipitation and high-throughput sequencing [54]. With this method, many tRNAs and noncoding RNAs were identified as NSUN2 substrates. A high frequency of the C $\rightarrow$ G transition was observed for presumably methylated C residues, helping to recognize specifically methylated cytidines in RNA targets. It is thought that C is incorporated in place of G during replication or reverse transcription because of cycle opening [58] (Figs. 4b, 4c).

The other method is known as miCLIP (methylation iCLIP) and is based on the mechanism of NSUN2-mediated methylation. Cytidine methylation at C5 starts with a covalent bonding of Cys321 of

NSUN2 and the pyrimidine ring of cytidine. After methylation, NSUN2 Cys271 plays a role in cleaving the enzyme–RNA covalent bond. The C271A mutation of NSUN2 stabilizes the covalent RNA–protein intermediate, and a method taking advantage of this circumstance was used in place of iCLIP to identify the NSUN2 targets at a single-nucleotide resolution. With this method, NSUN2 was found to methylate tRNAs, mRNAs, and noncoding RNAs [49, 52]. The two methods will find application in studying m^5C methyltransferases.

Function of m^5C

The functional role m^5C plays in tRNA and rRNA was the subject of many studies. Occurring in the variable and anticodon loops of tRNA, m^5C stabilizes its spatial structure and the codon–anticodon duplex [60]. A double knockout in *DNMT2* and *NSUN2* totally eliminates m^5C from tRNA, thus destabilizing the tRNA structure and suppressing protein synthesis in mice [61]. The m^5C residues found in rRNA are involved in translation and tRNA recognition [62].

As already mentioned, m^5C is one of the most common modified nucleotides in mRNAs and noncoding RNAs, but its functions in these molecules are poorly understood. Bisulfite sequencing of the HeLa cell transcriptome showed that m^5C accumulates in untranslated regions and that mRNA cytidine methylation sites occur in the vicinity of binding sites for Argonaute, a major component of the miRNA/RISC complex [3]. The finding implicates m^5C in miRNA-mediated RNA degradation. The hypothesis is at variance with the fact that mRNAs identified as NSUN2 targets by miCLIP do not change in expression in the absence of NSUN2 [49, 52]; i.e., an effect of m^5C on mRNA stability is still unproven. It is of interest that noncoding vault RNA (vtRNA) is methylated by NSUN2 according to miCLIP data. When its m^5C is lost, vtRNA is abnormally processed to small vtRNAs (svRNAs), which act as miRNAs to affect expression of several genes [49, 52]. Two long noncoding RNAs, HOTAIR and XIST, were found to contain m^5C in the functional regions responsible for interactions with a complex of chromatin-associated proteins or in the vicinity of these regions. There is evidence that m^5C is capable of disrupting certain protein–protein interactions in vitro [63].

PSEUDOURIDINE

Pseudouridine (Ψ), which is also termed the "fifth nucleotide," was the first modified nucleotide discovered in RNA almost 60 years ago [64]. Pseudouridine is found in many cell RNAs, from tRNAs and rRNAs to various small nuclear RNAs [65]. Such a broad distribution in RNAs indicates that pseudouridine is important for the cell function.

Pseudouridine is an uridine isomer (5-ribosyluracil) and forms via isomerization. First, the N1–C1' glycoside bond between uracil and ribose breaks. The base thus released rotates about the N3–C6 axis and forms a new, C-glycoside bond between C5 and C1' [66]. As a result, Ψ is capable of forming a hydrogen bond, which, together with the C-glycoside, rather than N-glycoside, bond, differentiates Ψ from all other bases. Isomerization is catalyzed by enzymes of two types. The substrate is recognized by yeast Cbf5 and mammalian DKC1/Dyskerine with the aid of small nucleolar RNAs (snoRNAs) having a small region complementary to the target RNA [67–70]. Pseudouridine synthase (PUS)-family proteins directly recognize target RNAs [71, 72].

As snoRNAs complementary to mRNAs rather than to noncoding RNAs were discovered, uridine isomerization was assumed to occur in mRNA as well [73, 74]. A total of 23 human genes were predicted to code for proteins similar to known pseudouridine synthases, but their functions were not verified experimentally [75].

The RNA bases whose modification is subject to regulation are of particular interest. Isomerization of two uridines in the yeast U2 snRNA is regulated separately in a stress-depenent manner, for instance, in heat shock or nutrient deficiency [76]. Isomerization of two uridines in mammalian rRNA is regulated by the kinase mTOR [77].

A transcriptome-wide search for pseudouridine was recently reported for yeast and human cells [4–6]. Isolated RNA was treated with cyclohexyl-3-(2-morpholino-4-ethyl)carbodiimide *n*-toluenesulfonate (CMC). The CMC–uridine bond disrupts in an alkaline milieu, while modified N3-CMC-Ψ remains intact. The resulting RNAs were examined by reverse transcription (Ψ-CMC is known to terminate reverse transcription [78]) followed by deep sequencing. The method, which is known as Psi-seq, Pseudo-seq, or Ψ-seq, reported ~50–100 Ψ sites for mRNAs of yeast cells cultured in optimal conditions and ~100–400 sites in human cell lines. Noncoding RNAs were also found to contain Ψ. The number of Ψ sites mapped in different studies depended on the read depth and the criteria employed in site selection in a computer analysis. It is noteworthy that Ψ sites are regularly distributed throughout coding and noncoding regions, rather than clustering in particular regions of transcripts [4, 6].

Stress dependence of the mRNA pseudouridylation level is among the most interesting findings of transcriptome-wide Ψ mapping. The Ψ proportion in yeast mRNA considerably increases in heat shock [5, 6]. A total of 265 new pseudouridylation sites were identified, and the majority of them proved to be modified in heat shock by PUS7 pseudouridine synthase, like Ψ residues in the U2 snRNA [5]. The pseudouridylation level is approximately doubled in nutrient deficiency [4]. The majority of nucleotides subject to regulated isomerization are isomerized only in the presence of

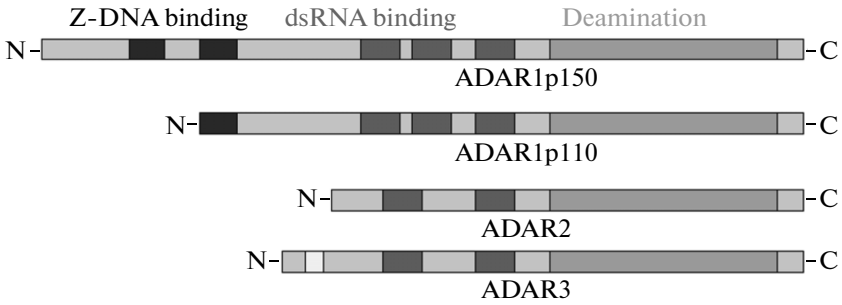

Fig. 5. Structures of ADAR-family proteins. The deaminase domain is shown light gray; the dsRNA-binding domain, moderate gray; and the Z-DNA-binding domain, dark gray.

active *PUS1* and *PUS7*. Apart from PUS-family proteins, CBF5 is responsible for pseudouridylation of certain uridine residues, and its activity is stress independent [5]. Pseudouridine synthases were found not only in yeast, but also in human cells, and transcriptome-wide mapping of pseudouridine residues in RNA was carried out for human cells growing in normal conditions [4, 5]. A total of 353 pseudouridylation sites were detected in mRNA, and the majority of them were DKC1 dependent. As for mRNA, 68 sites were observed in normal conditions and 92 sites, after 24 h serum starvation [4].

The biological role of isomerization to pseudouridine is unclear for the majority of uridine residues. Because Ψ is capable of hydrogen bonding with A, pseudouridine-containing transcripts are translated to produce a functionally active protein without changes in amino acid composition [79]. A regulatory role might be possible for uridine to pseudouridine conversion in stop codons. It was shown that a stop codon is misread as a sense codon when artificially modified with pseudouridine [80]. However, only one endogenous transcript was found to undergo pseudouridylation of the stop codon [5]; i.e., this function is hardly a main one for Ψ.

The total number of pseudouridine residues in mRNA is lower than that of other modified nucleotides. However, stress-dependent poseudouridylation suggests a regulatory function for this modification.

INOSINE

RNA editing is a type of posttranscriptional modification and involves adenosine deamination to inosine (I). Adenosine deamination is a major type of editing in the case of mammalian RNA, in contrast to *Trypanosoma* mitochondrial RNA [81]. Inosine is recognized as G, rather than A, during splicing and translation, interacting mostly with cytidine to form a complementary pair [82].

Proteins of the ADAR Family

Adenosine deamination occurs only in double-stranded RNA regions and involves proteins of the ADAR (adenosine deaminase acting on RNA) family [81, 83, 84]. Three ADAR-family proteins are encoded in the human genome: two ADAR1 isoforms (ADAR1p150, or ADAR1L, and ADAR1p110, or ADAR1S) [85], ADAR2 [86], and ADAR3 [87]. Their structures are schematically shown in Fig. 5. The proteins are highly conserved among vertebrates [88]. ADAR1 and ADAR2 occur in many tissues, while ADAR3 is found exclusively in brain tissues and is thought to be catalytically inactive [81]. All of the ADAR proteins have a dsRNA-binding domain at the N end and a conserved catalytic domain at the C end [85]. The proteins are catalytically active only as homodimers, as was shown both in vitro and in vivo [89], and are capable of specific and nonspecific editing of both noncoding and coding dsRNAs [81]. ADAR activity defects due to mutations or changes in expression are associated with various disorders, including cancer, neurology diseases, metabolic disorders, virus infections, and autoimmune diseases [90].

Methods to Identify the Adenosine Deamination Sites

Before the advent of new-generation sequencing, comparing the nucleotide sequences for cDNA and reference genomes was a basis of the majority of methods used to identify the deamination sites in RNA [91]. Only several tens of RNA editing sites were identified by this means [91]. High-throughput sequencing was first used to find the adenosine deamination sites in 2009 [92].

Several drawbacks are characteristic of the methods based exclusively on sequencing. Inosine cannot be distinguished from G appearing in cDNA as a result of a sequencing error or a single-nucleotide polymorphism. To overcome this drawback, inosine chemical erasing (ICE) was developed taking advantage of the fact that cyanoethylated inosine terminates reverse transcription [93]. Combined with high-throughput sequencing (ICE-seq), the method was used to identify the adenosine deamination site in the human brain transcriptome [94].

Inosine Function

Adenosine deamination in the pre-mRNAs of the glutamate receptor subunit B (GluR-B) and serotonin 2C receptor (5-HT2cR) are the best understood cases of RNA editing in the coding region.

Two sites where A → I deamination changes the codon were found in the GluR-B mRNA. The R/G site affects the receptor desensitization kinetics [95], and the Q/R site reduces the Ca^{2+} channel permeability [96, 97]. High-level editing at the Q/R site is of immense importance in mammals. A decrease in editing level is associated with malignant glioma and lateral amyotrophic sclerosis in humans and causes death almost immediately after birth in mice [98–102]. A low Q/R substitution rate increases the channel permeability to Ca^{2+} and Zn^{2+}, thus dramatically changing the membrane potential and affecting the cell signaling pathways [103]. Changes in editing efficiency at the Q/R site of GluR-B were observed in forebrain ischemia [103] and were presumably due to a decrease in ADAR2 expression.

Five adenosine residues are subject to deamination in the pre-mRNA for G protein-coupled 5-HT2cR, and their deamination changes three amino acid residues. A combinatorial editing yields 24 different isoforms [104, 105]. Mice that express only the unedited INI isoform of the receptor are normal, while mice that express the fully edited VGV isoform have a substantially reduced fat mass in spite of hyperphagia [106]. Changes in 5-HT2cR editing level are associated with anxiety, depression, and suicidal behavior [107].

Adenosine residues in pre-mRNA introns are also subject to deamination to thereby affect splicing. Editing may generate a new 5'-GU splicing site and generate or eliminate a 3'-AG splicing site [82]. As an example of this editing, ADAR2 performs adenosine deamination in its own pre-mRNA, leading to a frameshift. This is an example of the regulation via negative feedback [82].

CONCLUSION

Modification of internal mRNA regions has been known for a long time, but its function remains unknown in the majority of cases. N6-methyladenosine is one of the most abundant and best studied of all modified nucleotides. Ample data are available for this modification, but the biological roles are still unclear for both modification itself and modification-related proteins. The inosine function is well established in certain cases. Inosine generation changes the amino acid sequence of the mRNA-encoded protein or regulates splicing. Less is known about the other modifications, and further studies are necessary to better understand their functions.

Considering RNA modifications, we focused mostly on mRNA because its modifications are the least understood and the most interesting in terms of new mechanisms that regulate eukaryotic gene expression. Modification of eukaryotic rRNA was intentionally left beyond the scope of this review as a separate problem studied in great detail.

It is clear from the above that data on mRNA modification are fragmentary and are difficult to summarize, especially with the purpose to focus on the roles of individual modified nucleotides. Studies of the relevant processes will certainly bring many unexpected interesting discoveries.

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