Antibodies specific for nucleic acid modifications

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ABSTRACT

Nucleotide modifications constitute marks in RNA and DNA that contribute to gene regulation, development and other cellular processes. The understanding of their intricate molecular roles has been hampered by the high number of different modifications, the lack of effective methods and tools for their detection and quantification as well as by their complex structure-function relationship. The recent development of RNA and DNA immunoprecipitation followed by high-throughput sequencing (RIP- and DIP-seq) initiated detailed transcriptome- and genome-wide studies. Both techniques depend on highly specific and sensitive antibodies to specifically enrich the targeted modified nucleotides without background or potential biases. Here, we review the challenges and developments when generating and validating antibodies targeting modified nucleotides. We discuss antibody-antigen interactions, different strategies of antigen generation and compare different binder formats suitable for state-of-the-art high resolution mapping and imaging technologies.

Introduction

A wide range of chemically modified nucleic acids is present in DNA and RNA. It is generally accepted that the modification and isomerization of nucleotides serve as a regulatory layer to fine-tune vital cellular processes. More than 150 different modifications have been identified in different RNA families so far, approximately half of them in eukaryotes, but the functions of many of these modifications are still unclear. In the past, most studies focused on tRNA, rRNA and, to a lower extent, mRNA, mainly because rRNA and tRNA are the most abundant RNA families and also exhibit the largest diversity in modified nucleotides. In recent years, modified nucleotides have also been discovered in snRNA and miRNA precursor molecules. In mRNA, beside various types of N7-methylguanosine (m7G) as 5′-capping nucleotides, only very few types of modified nucleotides have been identified in coding RNA sequences, e.g. 5-methylcytidine (5mC) and its oxidized form 5-hydroxymethylcytidine (5hmC), N6-methyladenosine (m6A), N1-methyladenosine (m1A), pseudouridine (Ψ) and inosine (I). A more detailed review on modified RNA nucleotides and their role in gene regulation has been published recently.

In vertebrate genomes, 5-methyldeoxycytidine (5mCdC) was already discovered in 1948. 5mCdC has a relatively high abundance of about 4% in the human genome and is the major heritable modification in DNA. Once the 5mCdC pattern is established, it has to be maintained in dividing cells to ensure the lineage specific gene expression pattern. Therefore, 5mCcdC was believed to be a stable modification except during early embryogenesis. The fact that the 5mC mark is actively removed in the paternal genome of the zygote was reported earlier, but the discovery of TET-proteins in 2009 initiated a wealth of studies that shed light into the dynamic regulatory network which includes several oxidized 5mCdC variants. 5mCdC clusters in so called CpG islands in transcriptional regulatory regions. The balanced establishment and maintenance of the CpG methylation pattern is vital for development and normal cellular processes. After fusion of sperm and oocyte, an epigenetic reprogramming occurs including a massive reduction of CpG methylation. The development of the totipotent zygote into pluripotent stem cells and further cell fate decisions correlate with a cell-type specific re-establishment of CpG-methylation patterns (reviews). CpG methylation is challenged by active and passive processes which can lead to DNA demethylation, e.g., by reduced DNA methyltransferase activity or DNA repair pathways. It is becoming increasingly clear that lifestyle and environmental stress leads to altered methylation patterns, affecting aging and disease development including cancer progression. 5-methylcytidine is also found in tRNA, rRNA, and mRNA, with 5mC stimulating tRNA, regulating translational fidelity in rRNA, in mRNA it is overrepresented in UTRs and in the neighborhood Ago binding sites but the function of 5mCdC in mRNA is not understood. With the advent of more sensitive high-throughput profiling techniques, it was demonstrated that adenosine methylation in RNA and DNA provides an additional regulatory layer to many cellular processes including transcription, translation and epigenetic inheritance. For example, early studies proposed...
the existence of 6-methyldeoxyadenosine (m6dA) in eukaryotes, but direct evidence was only reported recently.30-33 The abundance of m6dA differs in the genomes of various eukaryotic species, but is less frequent than 5mC.30-33 These studies also revealed that m6dA is functionally involved in transcriptional regulation, albeit using different mechanisms in distinct species. In mammalian mRNA, m6A constitutes 0.1–0.4% of all adenosine nucleotides.34-36 m6A has also been identified in snRNA42 and miRNA.4 In mRNA, m6A is enriched around stop codons and levels increase during development.37,38 Functionally, m6A affects alternative splicing patterns,37 regulates translation by destabilizing mRNA38-41 and some steps of the translation process itself.32-44 A related modification, N6-2’O-dimethylenodesine (m6Am), was recently reported as a cap-associated modified nucleotide stabilizing mRNA.6,45 Currently, it is conceivable that some functions that have been originally attributed to m6A are in fact caused by m6Am, as the specificity of the used antibodies was unclear (ibid).

Transcriptome-wide mapping of m1A revealed an enrichment of this modification around the start codon. m1A dynamically acts as a positive regulator of translation upon stress conditions.46,47 Also pseudouridine, a modified nucleobase that has been mainly analyzed in tRNA, is discussed as a modification with a regulatory function in mRNA.48-50 These recent publications show that epitranscriptomics and epigenomics have become two of the most dynamic areas of research in cell biology. RIP- and DIP-seq techniques and also high-resolution imaging approaches to generate landscapes of appearance and dynamics of modified nucleic acids in eukaryotic cells depend on very specific and sensitive antibodies. However, many antibodies are often used without determining potential secondary effects in the experimental setting. In this review, we want to discuss the challenges in generating monoclonal antibodies against modified nucleotides. We will discuss aspects of antibody-antigen interactions, antigen generation, immunization and antibody validation.

**Antibody-antigen interactions**

The fundamental properties of specificity and affinity of each antibody molecule are determined by the variable domains of the heavy (VH) and light chains (VL), (Fig. 1). The affinity of the primary B cell repertoire is low and increases if immunoglobulin genes of activated B cells undergo somatic hypermutation upon repeated encounter with antigens.51 These mutations accumulate preferentially in the complementarity-determining regions (CDRs) of both the heavy and the light variable immunoglobulin genes (Fig. 1).52 Each VH and VL domain contains 3 hypervariable CDRs (CDRH1 to 3 and CDRL1 to 3). CDRs are surface-exposed loops that form the antigen-binding site, and hypermutation of the CDRs results in structural changes in the antigen binding sites.53 The CDRH3 loop is highly variable in length, sequence, and structure.54 In humans and mice, the other 5 CDRs are less variable and assume only a limited number of canonical backbone conformations (reviews55,56). This hypervariability, particularly in CDRH3, determines antigen specificity and affinity.56 In rabbits, however, due to a higher junctional diversity during VJ recombination, the CDRL3 loops are also heterogeneous in sequence and length and further contribute to high-affinity antigen binding.57,58

Crystallographic X-ray analyses of antibody-antigen complexes have revealed a closer insight into the residues of CDRs interacting with the antigen.59,60 The number and positions of the residues interacting with the antigen largely depend on the size of the antigen and determine the overall shape of the antigen-binding site.51,62 Comparative studies of more than hundred immune complexes showed that large antigens such as proteins are bound in planar interaction sites, peptides within grooves, whereas small antigens, so-called haptens, are burried in deep cavities (review63). Haptens are defined as small molecules of < 1kDa in size.64 Modified nucleotides, which have an average molecular weight of 0.35 kDa, are therefore haptens.

On the molecular level, the interactions between antibody and antigen are non-covalent and reversible. They are based on a combination of hydrogen bonds, hydrophobic interactions, electrostatic and van der Waals forces.65 To the best of our knowledge, no crystal structures of antibody complexes with modified nucleotides have been published yet. So far, analyses of several autoimmune anti-DNA antibody complexes revealed that high-affinity binding to DNA is mainly mediated through electrostatic forces between arginine residues in the CDR3H region and the bases or phosphate groups of the nucleic acid.66 Small changes in the antigen alter the electrostatic and hydrophobic interactions and have a profound effect on the strength of the antibody-antigen interaction. For example, the addition of a methyl group to guanosine completely abolished binding of an anti-ssDNA antibody.67 Similarly, substitution of a single arginine residue in the CDR3H loop by glycine abrogated antibody binding to ssDNA, while introduction of additional arginine residues into CDRH2 improved the binding affinity.68 A study comparing 6 monoclonal antibodies raised against various modified nucleotides (5mC, m7G, Ψ, m1In, m1A, 4AcG) revealed high specificity of each antibody toward the respective antigen and no cross-reactivity against the other nucleotides.69 These data demonstrate that modified positions of a nucleobase are part of the antibody interaction site and any change significantly alters the specificity and affinity of the interaction. Systematic structural analyses of antibodies bound to different modified nucleotides are required to gain insights of how specificity is achieved for nucleic acids that differ only in one position.

**Modified nucleosides as antigens for antibody generation**

Early attempts to generate antibodies against DNA were largely unsuccessful until it was discovered that carrier molecules improve the immune response (review70). It is thought that antibodies against the most common B-DNA-form are generally difficult to generate in experimental settings because responsive B-lymphocytes are eliminated as self from natural repertoires.71,72 Only certain ssDNA and less common DNA forms (e.g., Z-DNA), triplexes or DNA-RNA hybrids are targets of natural antibody responses (summarized73). Humans and animals therefore exhibit an immunotolerance to B-form DNA and autoantibodies against dsDNA are only found in patients diagnosed with e.g., systemic lupus erythematosus...
(SLE) or lupus nephritis as a result of progressive hypermutation in the CDR3. The first antibodies against unusual and modified nucleotides were generated in the 1970s and early 1980s when it was discovered that tRNA contains several differently modified nucleotides and that 5mdC is important in gene regulation and cell development (reviews).

RNA or DNA nucleotides are too small to elicit an immune response but it is possible to generate antibodies targeting single nucleosides by coupling the latter to an immunogenic carrier protein. Studies in the early 1960s identified BSA, a basic protein with many free –NH2 groups, as an appropriate carrier. A stable covalent coupling of ribonucleosides was achieved by oxidation of the ribose ring with sodium periodate followed by a reductive condensation of the resulting aldehyde groups to the NH2-group of the lysine side chain. The oxidation step opens the ribose ring between the 2’0 and 3’0 position and covalently couples the nucleobase via the opened ribose to the carrier protein (Fig. 2). As a consequence, antibodies generated against such antigens cannot discriminate between molecules differing at the ribose moiety, e.g., between RNA and DNA or nucleic acids with modified or non-modified 2’- and 3’-ribose OH-groups. All antibodies directed against modified nucleosides generated so far are based on this coupling method. They target single nucleotides in DNA or RNA chains and neighboring nucleotides appear not to influence antibody binding. Consequently, these antibodies allow an unbiased determination of sequences flanking modified nucleotides and their potential consensus motifs. For example, antibodies generated against m6A led to the identification of the DRACH motif flanking m6A. Other examples of antibodies currently in the spotlight are those specific for 5mC, m1A and m7G. Table 1 summarizes available antibodies against modified nucleosides and their applications.

Presently, a modified nucleobase-coupling protocol allows a more efficient binding of the hapten and also the coupling of nucleobases sensitive to oxidation or reduction such as m1A (pers. communication R. Hett / G. Meister). The use of cationized BSA, in which the carboxy groups of acidic amino acids are converted to aminoethylamide groups, enhances the resulting T-cell immune response and increases the immunogenicity of poorly immunogenic molecules. In addition, it increases the number of -NH2 groups available for coupling with the nucleoside. The higher coupling efficiency increases the likelihood that 2 or more modified nucleobases are in close vicinity to each other on the carrier so that antibodies that specifically recognize pairs of adjacent modified nucleobases might be raised. Such antibodies, if undesired, have to be eliminated during the validation process or by affinity purification. Modern Click Chemistry provides an alternative strategy to couple DNA or RNA nucleosides to the carrier protein. Here, the 5’OH group of the ribose is activated with azide allowing the copper(I)-catalyzed addition of the alkyne-linker molecule. This coupling method has the advantage that the ribose is kept intact, thus potentially allowing the generation of antibodies that discriminate between DNA and RNA nucleosides or recognize modified ribose OH groups. This is of special importance for the generation of antibodies that allow the discrimination between highly related modified nucleosides, for example nucleosides having the same chemical modification at one
position in the nucleobase but differ in an additional modification of the ribose such as a methylated OH-group (e.g., m7G versus m7Gm). We are currently trying to establish monoclonal antibodies using DNA nucleosides coupled by Click chemistry to the carrier protein as antigens (in collaboration with T. Carell). One possible drawback of this method is the generation of antibodies that might bind to the modified nucleoside only in conjunction with the 1,2,3-triazole resulting from the Click reaction. These antibodies need to be excluded during the antibody validation procedure.

**Comparison of different antibody formats**

The validity of high-throughput approaches in epitranscriptomics and epigenomics directly depends on the specificity and affinity of the used antibody. Currently, polyclonal and monoclonal antibodies are used to target modified nucleic acids (Table 1). Both have their advantages and disadvantages. A systematic comparison of monoclonal and polyclonal antibodies targeting modified nucleotides has not been performed so far. However, a detailed comparison of polyclonal and monoclonal antibodies targeting different post-translational histone modifications in ChIP-seq experiments showed that monoclonal antibodies exhibit the same sensitivity as polyclonal antibodies, yet offer higher reproducibility. Polyclonal antibodies, which are mainly produced in rabbits, comprise a heterogeneous mixture of antibodies that target different epitopes and bind the antigen with different affinities. They often perform well in multiple applications, as they can bind their targets under conditions of different pH and salt concentrations. A major drawback of polyclonal antibodies is batch-to-batch inconsistencies. Affinity column purification, e.g., on modified and unmodified nucleotides is required to reduce carrier-specific and other unspecific antibodies from the immune serum. However, due to their heterogeneous nature, validation for cross-reactivity, e.g., against similar nucleotide modifications or binding of 2 adjacent modified nucleotides, is difficult. In general, polyclonal antibodies exhibit a broader but less well characterized interaction pattern, which is the disadvantage of the larger antibody pool. In contrast, monoclonal antibodies, which are secreted from a single B cell clone, recognize a defined epitope with a given affinity and specificity. Their generation is more expensive and time-consuming as compared with polyclonal antibodies. However, once a monoclonal antibody is established it can be repeatedly produced in large amounts and with consistent quality between different batches. In addition, cross-reactive or unspecific clones can be eliminated already during primary antibody screening (see next chapter).

The development of a rabbit myeloma cell line allowing the efficient fusion of rabbit B cells from immunised animals was a major improvement on the antibody market. Because rabbit monoclonal antibodies often display high affinities they could be a valuable alternative to those from mouse and rat for antibody DIP-and RIP-seq studies. At present, we are not aware of rabbit monoclonals generated against modified nucleic acids. Unfortunately, the rabbit myeloma fusion cell line is not freely available for the scientific community. The recombinant cloning of smaller antibody formats, such as Fab or single-chain variable fragments (scFv) from established hybridoma cell lines or of nanobodies derived from camelid heavy chain antibodies can be useful especially for high-resolution imaging. Recombinant antibody technologies provide an alternative to the hybridoma technology. Different display technologies are now available to isolate the best binders from either immune or naïve antibody libraries (summarized). However, as far as we know, these technologies have not been applied for modified nucleotides so far.

**Antibody validation**

To serve as valuable and reliable research tools, antibodies need to be thoroughly validated regarding their affinity, specificity and reproducibility in the context of their intended use. The primary screening process during monoclonal antibody generation already identifies hybridoma candidate clones that produce the strongest and most specific binders. Supernatants from several hundred hybridomas can easily be tested in a high-throughput manner in solid-phase enzyme-linked immunosorbent (ELISA) assays. Detection ELISA assays are ideal to identify antibodies that recognize the desired antigen, e.g., by testing the binding of modified nucleobases coupled to a

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**Figure 2.** Conjugation of nucleosides to carrier proteins. In a first step the 2’ and 3’ hydroxyl groups of the ribose of the nucleic acid are oxidized with IO₄⁻ at pH 9.5. This allows the coupling to primary amino groups of carrier proteins, e.g., ε-NH₂-group of lysine residues. The resulting unstable acid is subsequently stabilized by reduction with NaBH₄.
Table 1. Overview of the most commonly studied nucleic acid modifications and their analyses using antibody-based approaches.

| Modified nucleoside (R=OH or H) | Occurrence^ (eukarya) | pAb application^ | mAb application | mAb sources^ |
|----------------------------------|------------------------|------------------|-----------------|--------------|
| 5-methyldeoxycytidine           | DNA                    | PAR-CLIP-RIP      | Immunoblot      | ms: 5-MeC^69 |
| 5-methylcytidine                | tRNA, rRNA, mRNA, viral ncRNA (reviewed in: 73) |                  | FMC-9^97        | ms: FMC-9^97 (m5dC) |
|                                 |                        |                  | IF              | scFv^97       |
|                                 |                        |                  | 33D3^104,105    |              |
| 5-hydroxymethyldeoxycytidine    | DNA                    | IF               | IF              | ms: Ab3/63.3^* |
| 5-hydroxymethylcytidine         | rRNA                   | hMeDIP^104       | AB63/3^106      | ms: 4D9^*     |
|                                 |                        | MeDIP            | 4D9^107         | rat: 633HMC^* |
|                                 |                        |                  | 633HMC^2/108    |              |
| 5-formyldeoxycytidine           | DNA                    |                  | RIP             | rabbit: DSD4K^* |
| 5-formylcytidine                | tRNA (mt)              |                  | AMA-2^69,99     |              |
|                                 |                        |                  | IHC             |              |
|                                 |                        |                  | AMA-2^99,109    |              |
|                                 |                        |                  | Immunoblot      |              |
| 6-methyladenosine               | tRNA, rRNA             | RIP^37-39,43     | DIP             | rabbit: EpiMark^8 |
| 6-methyldeoxyadenosine          | tRNA, rRNA, mRNA, snRNA, DNA | PAR-CLIP^19       | 212811^12       | N6-Methyladenosine Enrichment Kit |
|                                 |                        | Immunoblot^95    | Rip              | ms: clone 17^10,111 |
|                                 |                        |                  | ms: 212811^*    |              |
| 7-methylguanosine               | mRNA, tRNA, rRNA       | RIP              | ms: 7-MeGuo^69  |              |
| 2,7,2’-methylguanosine          |                        | H-20^12          | ms: H-20^112    |              |

(continued on next page)
different carrier protein or on small modified oligonucleotides to ensure the identification of hapten-specific antibodies and to exclude carrier-specific ones.69,83,92,98 Capture ELISA identifies antibodies that immunoprecipitate antigen with high affinity, a prerequisite for antibody-based mapping studies of modified nucleic acids. Competitive ELISA can further determine antibody specificity and cross-reactivity. Here, antibodies are pre-incubated with increasing amounts of the target antigen or potentially cross-reactive antigens as competitors before binding to the target antigen is assessed. The combination of these ELISA-based techniques allows identification of antibodies binding the desired antigens with high affinity and to eliminate those showing cross-reactivity.69,83,92,98

Further validation is essential to verify the antibodies’ performance in particular applications. Antibodies targeting modified nucleic acids were validated e.g., by DNA or RNA immunoprecipitation,32,46,99 immuno-Northern blotting99 or high-resolution nucleic acid mapping technologies.6,32,37,100 Potential off-target binding and biases can be detected by using methylation-deficient control cells, as recently demonstrated for anti-m6A antibodies in mapping studies with N6-adenine-methylases-negative bacteria or yeast cells.32,100 A study by Linder et al. revealed, that antibodies described as 6mA-specific, which were generated by coupling 6mA to the carrier protein by the reductive coupling method described above79 (Fig. 2) do not discriminate between m6A and m6Am.6 This is not surprising, as the antigen used for immunization does not contain the intact 2′- and 3′-moieties of the ribose. These studies highlight the importance of antibody validation, especially if highly related targets exist in the same molecule, as it is the case in RNA and DNA. Antibodies targeting nucleic acids or modified nucleic acids are generated by coupling single (modified) nucleosides to the BSA carrier protein.79 However, the resulting antibodies are used to detect the respective target in the context of complex structures, e.g., chromatin, dsDNA, protein-bound RNA or structured regions of RNA. These structures may impede antibody-antigen interactions. Most parts of RNA are single-stranded, but in DNA and in structured regions in RNA, nucleic acids pair with each other to make double-strands, and may therefore not be readily accessible for the antibody. For example, we observed that some antibody clones detecting a single modified nucleoside coupled to BSA in ELISA, did not bind to the same modified nucleotide in the context of RNA or DNA (own unpublished). In mammalian cells, the generation of CRISPR/Cas9-targeted methyltransferase knockout cell lines will be instrumental to identify antibodies without or low off-target activity.

Interestingly, apart from specific antigen binding, antibodies can be used to generate signatures on modified RNA after UV-induced crosslinking and mutations introduced during reverse transcription, allowing a precise mapping of modified nucleobases.6 However, although directed against the same modification, some antibodies were shown to induce an inconsistent mutation pattern.6

Summary and perspectives

Modifications of nucleic acids are widespread, and the function of many of these modifications is far from understood. Antibodies specific for modified nucleotides are essential tools to address fundamental questions on the molecular regulation of life in this new era of epigenomics and epitranscriptomics. At present, antibodies against a limited number of DNA and RNA modifications are available. The generation of new specific antibodies will be instrumental to decipher the function of those modifications that have been neglected so far. Comparative structural studies of antibody complexes with modified nucleobases are required for an improved understanding of specific target recognition.

Antibodies directed against modified nucleobases are traditionally generated by immunization with nucleosides coupled to a carrier protein using the ribose part as a linker. Modern Click Chemistry now allows coupling by maintaining the sugar intact, thus permitting the generation of antibodies that may, in addition to recognizing specific modified nucleobases, be able to distinguish between different forms of the ribose moiety, such as ribose, deoxyribose, and O-methylated variants. Careful antibody validation is essential to reduce background through off-target activity and also to verify the applicability in a particular technique. Whenever possible, cellular knockout controls should be part of the validation process. Polyclonal antibodies are often not sufficiently characterized, and their functionality may vary from batch to batch, which may hamper reproducibility and comparability of results. Monoclonal antibodies

Table 1. (Continued)

| Modified nucleoside (R=OH or H) | Occurrence (eukarya) | pAb application | mAb application | mAb sources |
|---------------------------------|---------------------|----------------|----------------|-------------|
| Pseudouridine                   | tRNA, rRNA, snoRNA, mRNA | Immunoblot     | ms: Ψ -Urd⁶⁹   |
|                                 |                     |                | ms: APU-6⁹⁸    |

Abbreviations: pAb: polyclonal antibody; mAb: monoclonal antibody; scFv: single-chain variable fragment; DIP: DNA immunoprecipitation; RIP: RNA immunoprecipitation; IHC: immunohistochemistry; IF: immunofluorescence; ms: mouse; mt: mitochondria.
from stable hybridoma cell lines have unlimited availability, allow for precise definition of specificity and affinity, and therefore more consistent experimentation. Several monoclonal antibody clones should be used to validate nucleotide mapping in RIP- and DIP-based experiments. The generation of high-affinity nucleotide-specific monoclonal antibodies in rabbits might further advance the epitranscriptomics display technologies, will be useful tools for high-resolution imaging.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed. Some antibodies described in this review are from commercial sources. We are not recommending a particular antibody for a specific experiment but are only citing publications where they have been used. We have no competing financial interest.

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