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# Protein Methyltransferase Activities in Commercial *In vitro* Translation Systems

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**Protein arginine methylation is a well-known post-translational modification that has been shown to occur in rabbit reticulocyte *in vitro* translation lysates (RRL); however, it is not known whether this is a general feature of *in vitro*-produced proteins from other eukaryotic cell-free translation systems, particularly insect-derived lysates (ICL). Because methylation can affect protein localization, RNA binding and protein–protein interactions this may be of great importance as *in vitro*-produced proteins are often used in assays of protein function. Here, I report the presence of base-stable and base-labile methyltransferase activities in RRL, ICL and wheat germ *in vitro* extracts (WGE). Indeed, the presence of CARM1 in RRL and ICL and a class II protein arginine methyltransferase activity (PRMT5/7) is documented in all three systems. Additionally, the lysine methyltransferase that modifies eukaryotic elongation factor 1A (eEF-1A) was detected in ICL and WGE. Importantly, using a defined set of substrates under identical conditions I show that all three *in vitro* systems contain different complements of the various methyltransferases. These data suggest that three systems can be used in a complementary fashion to investigate the effect(s) of post-translational modification on protein function.**

**Key words: fragile X mental retardation protein, histone, myelin basic protein, protein arginine methyltransferase, protein lysine methyltransferase.**

Abbreviations: CARM1/PRMT4, Coactivator of arginine methylation 1; eEF-1A, eucaryotic elongation factor 1A; ICL, insect-derived lysates; MT, methyltransferase; PKMT, protein lysine methyltransferase; PRMT, protein arginine methyltransferase; RRL, rabbit reticulocyte *in vitro* translation lysates; SAH, *S*-adenosyl-homocysteine; SAM, *S*-adenosyl-methionine; WGE, wheat germ *in vitro* extract.

Post-translational modifications expand the repertoire of the proteome. They also provide novel mechanisms for regulating protein function. Cell-free protein translation systems have been widely used to study open reading frame expression and enzymatic activity (*1*), protein–protein interactions (*2*), protein–RNA interactions (*3*, *4*), protein–DNA interactions (*5*), translational arrest (*6*) and protein folding (*7*). While it is known that rabbit reticulocyte lysates (RRL) and wheat germ extract (WGE) harbour post-translational modifying activities for phosphorylation (*8*), isoprenylation, myristoylation (*9*) and ubiquitination (*10*, *11*), their protein methylating activities have not been exhaustively studied. However, certain RNA binding proteins [fragile X mental retardation protein (FMRP), iPABP, hnRNP A1 hnRNP K, hnRNP U, hnRNP D], splicesomal Sm proteins and splicing factors are methylated by RRL *in vitro* (*12–15*) as is histone H3 (*2*). Similarly, the post-translational modification repertoires produced in commercially available wheat germ and *Spodoptera frugiperda* cell lysates (ICL) have not been examined. To better characterize the protein methylating activities present in commercially available *in vitro* translation systems, the ability of these extracts to promote methylation of endogenous and defined protein substrates was examined. This manuscript reports the results of comparative studies focusing primarily on the protein *N*-methyltransferases, protein arginine methyltransferase (PRMT) and protein lysine methyltransferase (PKMT).

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## MATERIALS AND METHODS

*Antibodies*—Anti-FMRP mAb was purchased from Chemicon. Anti-H3 $(R17)^{DMA}$ pAb, Anti-H3$(K4/K9)^{Me2}$ pAb, anti-H4$(R3)^{Me2}$ pAb, anti-ASYM24 pAb, anti-SYM10 pAb, anti-CARM1 pAb and anti-EF-1A mAb were purchased from Millipore. Anti-H4$(R3)^{sMe2}$ pAb was purchased from Abcam. Anti-V5 mAb was obtained from Invitrogen.

*Methylation Substrates and Inhibitors*—Histone H2a, H2b, H3 and H4 were purchased from Roche Applied Biosciences. Myelin basic protein (MBP) (mouse) was obtained from Sigma. Recombinant GST was a generous gift of Dr Lataetia Davidovic, CNRS UMR6543, Faculté de Médecine, Université de Nice Sophia-Antipolis, NICE-France. FMRP and eucaryotic elongation factor 1A (eEF-1A) were made *in vitro* as previously described (*4*); *FMR1* exon 15–17 truncation mutants Ex15a, Ex15a-$R_{544}$K and Ex15a-$R_{546}$K were prepared as previously described (*16*). *S*-adenosyl-homocysteine (SAH) was purchased from Sigma.

 

In Vitro *Translation Systems*—TNT RRL and WGE *in vitro* translation kits were purchased from Promega. The insect cell *in vitro* translation kit (ICL) was purchased from Qiagen.

In Vitro *Methylation Assays*—*In vitro* methylation by endogenous methyltransferases (MTs) was performed with various substrates by incubation in HMTase-Buffer (50 mM Tris-HCl pH 9, 0.5 mM DTT) supplemented with 1 μCi of $^{3}$H- *S*-adenosyl-methionine (SAM) in a total volume of 20 μl. For the inhibitor studies a master mix of the particular lysate (5 μl/20 μl reaction), substrate (1–2 μg/20 μl reaction) and $^{3}$H-SAM (1μCi/20 μl reaction) were prepared in HMTase-Buffer. Equal aliquots of the master mix were then incubated with varying amounts of the inhibitor, as indicated. Incubations were allowed to proceed for 2 h at 30°C. Following the incubation, an equal volume of 2× Laemelli sample buffer was added and the samples were boiled for 5 min. The proteins were resolved in duplicate on SDS-polyacrylamide gels. The gels were fixed, in 30% methanol, 10% acetic acid overnight. After removing the fixing solution the gels were either soaked in En$^{3}$Hance (Perkin Elmer) for 1 h and then water for 30 min. and then dried and subjected to fluorography as described, or stained with Coomassie Brilliant Blue (*2*). The length of exposure was 5 days, unless otherwise indicated.

*Western Blotting*—Western blotting using anti-H3(R17)$^{DMA}$ pAb, anti-H3(K4/K9)$^{Me2}$ pAb, anti-H4(R3)$^{Me2}$ pAb, anti-H4(R3)$^{sMe2}$ pAb, anti-ASYM24 pAb, anti-SYM10 pAb and anti-CARM1 pAb was performed according to the manufacturer's instructions. Western blotting using the anti-FMRP mAb or anti-eEF-1A mAb was performed as previously described (*17*).

In some instances (as noted in the Figure legends) substrate proteins (20 μl) were partially purified following *in vitro* methylation by binding to 20 μl of carboxymethyl-Sepharose that was pre-equilibrated in HMTase-Buffer. Contaminating proteins were removed by three washes with 100 μl of HMTase-Buffer and the resulting proteins were recovered by boiling in 30 μl of 1× Laemelli buffer. This enhanced detection of Histone H3 with the anti-H3(R17)$^{DMA}$ pAb and Ex15a and the other FMRP truncation mutants with anti-V5 mAb.

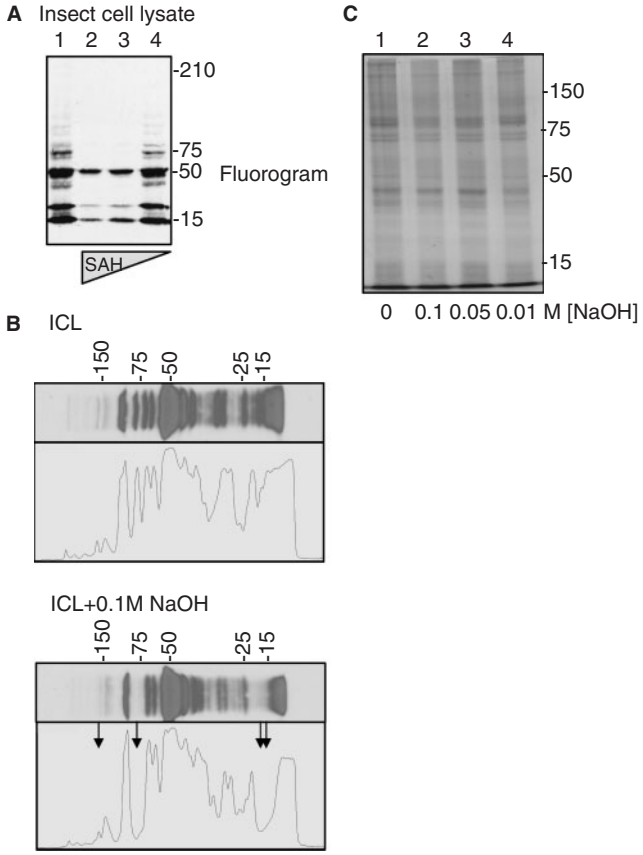


Fig. 1. **ICL *in vitro* translation lysates harbour MT activity.** (A) Fluorogram of ICL, following *in vitro* methylation in the absence and presence of various concentrations of SAH as indicated in the text. (B) Protein O-methyltransferase activity is present in ICL. *In vitro* methylation reactions of ICL were treated with NaOH (final concentration 0.1 M) at room temperature for 30 min. Aliquots of the sample and an untreated control were resolved by SDS–PAGE and subject to fluorography. Scanned images of the data are shown below each fluorogram. Proteins absent in the base treated extracts are marked by arrows. (C) ICL was subject to *in vitro* methylation as in (A). Subsequently, aliquots were treated decreasing concentrations of NaOH and resolved by SDS–PAGE. The resulting protein loads for each lane are shown.

## RESULTS

*Insect Cell Lysates Exhibit High Endogenous MT Activity*—To assess the endogenous MT activity of *in vitro* translation lysates methylation assays were carried out with equal aliquots of each lysate. As shown in Fig. 1A many endogenous proteins in ICL (panel 1, lane 1) were labelled in the presence of the methyl donor ($^{3}$H)-SAM. Thus, ICL appears to contain significant amounts of MTs. To confirm that the labelled protein resulted from methylation, reactions were carried out with decreasing concentrations of SAH, a direct product inhibitor of SAM-dependent MTs. In a previous study of the symmetrical dimethylation of spliceosomal proteins Sm B/B′ and Lsm4, Brahms *et al*. (*15*) demonstrated that there was a significant, but incomplete reduction of SAM-dependent class II PRMT activity in RRL using 400 μM SAH; therefore, we used three 10-fold dilutions of SAH starting at 1 mM. For ICL, a dose-dependent inhibition was observed, indicating that incorporation of the label into the protein resulted from *bona fide* MT reactions.

Protein MT activities can be classified as either base-stable or base-labile, depending whether nitrogen or oxygen atoms are modified. Base-labile protein *O*-methylation catalyzed by a variety of protein MTs occurs in prokaryotes and eucaryotes. Therefore, we next examined the stability of ICL methylation toward base. As seen in Fig. 1B the majority of $^{3}$H-incorporation was stable to alkaline conditions, indicating that most of the proteins modified by ICL are *N*-methylated. However, there were two notable exceptions. A protein that migrates between 75 and 100 kDa and another protein that migrates at 21 kDa were absent in the alkali treated samples. As these losses were not due to protein

degradation (Fig. 1C) the data are consistent with the presence of at least one *O*-methyltransferase in ICL.

In contrast to the results using ICL, very few endogenous proteins were labelled in either RRL or WGE (Fig. 2A, panels 1 and 2). This is not a function of the protein load, as roughly equal amounts of protein were present in each lysate (Fig. 2B). These data suggest that RRL and WGE contain very low levels of endogenous MT activity, or that the methylation reactions were not optimized, or that they lack the substrates for the activity (either by their absence or their prior methylation).

Since we and others have previously demonstrated that RRL harbours MT activity (*2*, *12*, *14*, *15*), the latter explanations appeared more tenable. However, to eliminate the possibility that the reactions were suboptimal, titrations with increasing concentrations of $^{3}$H-SAM at a fixed protein load and increasing protein concentrations at fixed $^{3}$H-SAM were conducted (Supplementary Figs S1 and S2, respectively). Both studies show that endogenous WGE and RRL proteins are poorly methylated under all conditions. In the case of the $^{3}$H-SAM titration, lack of methylation correlates with the acidification of the reaction.

To determine whether WGE contained methylated proteins aliquots of the extract were blotted and probed with rabbit polyclonal antibodies that detect asymmetric dimethylarginine (aDMA) or symmetric dimethylarginine (sDMA) residues (*18*). Extracts that were subject to *in vitro* methylation and ICL were used as controls. Several WGE proteins reacted with the antibodies (Fig. 2C, lane C). Furthermore, *in vitro* methylation of the extract did not increase the intensity of the immunoreactive proteins or result in dramatic changes in the distribution of the proteins that were recognized (Fig. 2C, lane 1). Consistent with these data was the finding that SAH had no effect on the level of endogenous aDMA in WGE (Fig. 2C, lane 2). Finally, the antibodies reacted much less strongly to proteins in ICL. This agrees with the robust SAM dependent methylation observed upon incubation with $^{3}$H-SAM (Fig. 1A) and indicates that the reactivity toward WGE is specific.

To begin characterizing the endogenous MT activity in RRL and WGE we subjected methylation reactions to alkali treatment. We found that some of the endogenous labelling in WGE and all of the endogenous labelling in RRL was base-labile (Supplementary Fig. S3). Thus, ICL,

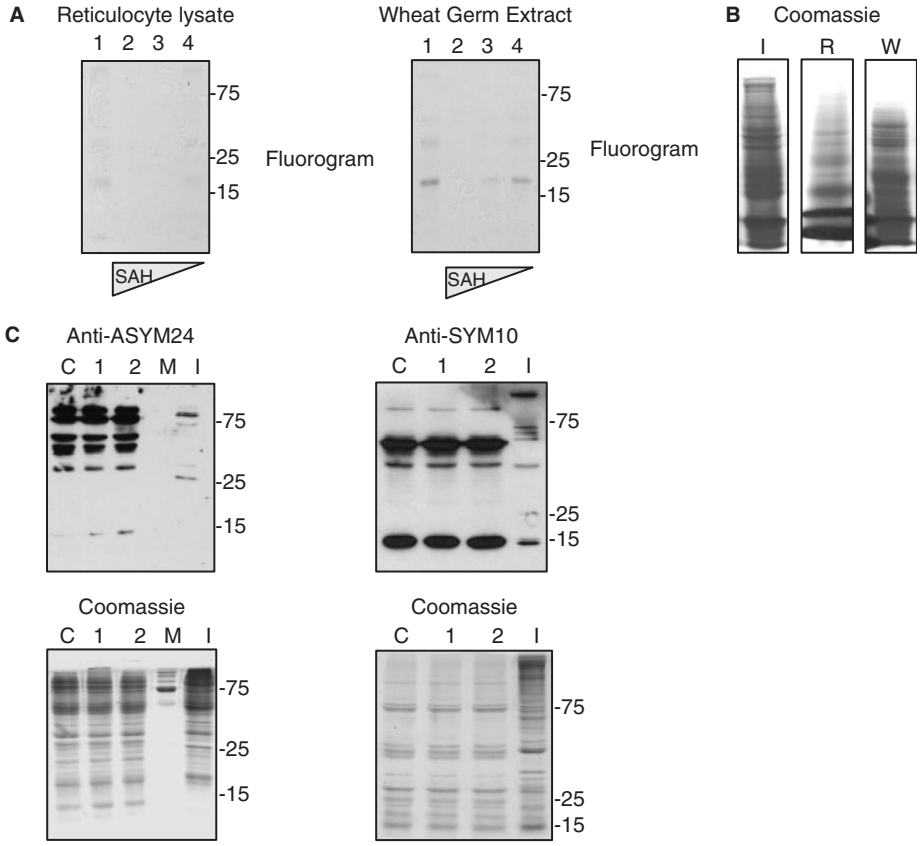


Fig. 2. **Endogenous protein methylation of WGE and RRL *in vitro* translation lysates.** (A) Fluorograms of RRL and WGE following *in vitro* methylation in the absence and presence of various concentrations of SAH as indicated in the text. (B) Protein loads of ICL (I), RRL (R) and WGE (W) from Fig. 1A and Fig. 2A. (C) WGE contains arginine methylated proteins. Western blot of WGE without prior methylation (lane C), WGE subject to *in vitro* methylation in the absence (lane 1) and presence (lane 2) of 1 mM SAH as in (A), molecular weight standards (lane M) and ICL without prior methylation (lane I) probed with anti-ASYM24 pAb or anti-SYM10 pAb as indicated. The protein loads for each lane are shown by the Coomassie-stained gel below.

RRL and WGE *in vitro* translation lysates harbour a variety of different MTs.

*Approach to Characterizing MT Activities Present in RRL, ICL and WGE*—MTs have been cloned from a variety of species (*19–26*) and in some cases antibodies have been developed to recognize specific MTs. However, most commercially available antibodies recognize mammalian proteins (human, mouse, rat). Although the PRMTs, for example, represent a well-conserved protein family sequences outside their core catalytic and cofactor binding domains diverge and it is these residues that are typically targeted for generating antibodies. The anti-PRMT4 pAb used in these studies represents a case in point. Based on the sequence differences of the non-mammalian PRMT4s in the epitope target region (Fig. 3A) one would predict that if a PRMT4 ortholog existed in ICL or WGE it would not be recognized by this antibody. Indeed, we previously assessed whether RRL contained a PRMT4 using this antibody and could not detect one (*16*). Figure 3B demonstrates that this antibody also does not recognize a corresponding protein in either ICL or WGE; it does recognize recombinant human PRMT4, albeit weakly. Similar sequence divergence can also be seen for other PRMTs (Supplementary Fig. S4). Thus, a strategy based on detecting the presence of particular PRMTs in *in vitro* translation lysates *via* western blotting using most commercial antibodies would be prone to error. However, MTs are characterized by their unique abilities to methylate particular proteins at specific sites. Thus, to further characterize the MTs present in RRL, ICL and WGE, methylation reactions were performed by incubating the extracts with known MT substrates.

*Methylation of MBP*—All three products of arginine methylation, monomethylarginine, aDMA and sDMA have been found in MBP isolated from mouse brain (*27*). Furthermore, the extent of these modifications in both mouse and humans differs according to age and the disease state of the brain (*28*). To determine whether RRL, ICL or WGE harboured PRMT activities that methylate MBP, *in vitro* methylation assays were carried out on purified mouse brain MBP. As shown in the fluorogram in Fig. 4 (lanes 1–4) RRL harbours SAM-dependent PRMT activity toward MBP. WGE also specifically methylated MBP; however, in this case the extent of methylation was much less than RRL (Fig. 4, lanes 5–8); ICL also methylated MBP (Fig. 4, lanes 9–12). Thus, all three extracts can methylate MBP to varying extents.

*Methylation of Histones*—Histones are subject to a wide range of post-translational modifications and these modifications affect their function. Histone H3 is a known substrate of coactivator of arginine methylation 1 (CARM1/PRMT4) (*29–31*) as well as lysine MTs (PKMTs) (*32*, *33*). We previously showed that RRL harboured a MT activity that modified histone H3 (*2*). To ascertain whether histone H3 could be modified by an endogenous SAM-dependent MT in ICL or WGE, *in vitro* methylation assays were carried out on histone H3 purified from calf thymus. As shown in the fluorogram in Fig. 5A (upper panel) an endogenous methylated protein co-migrated with histone H3 in ICL

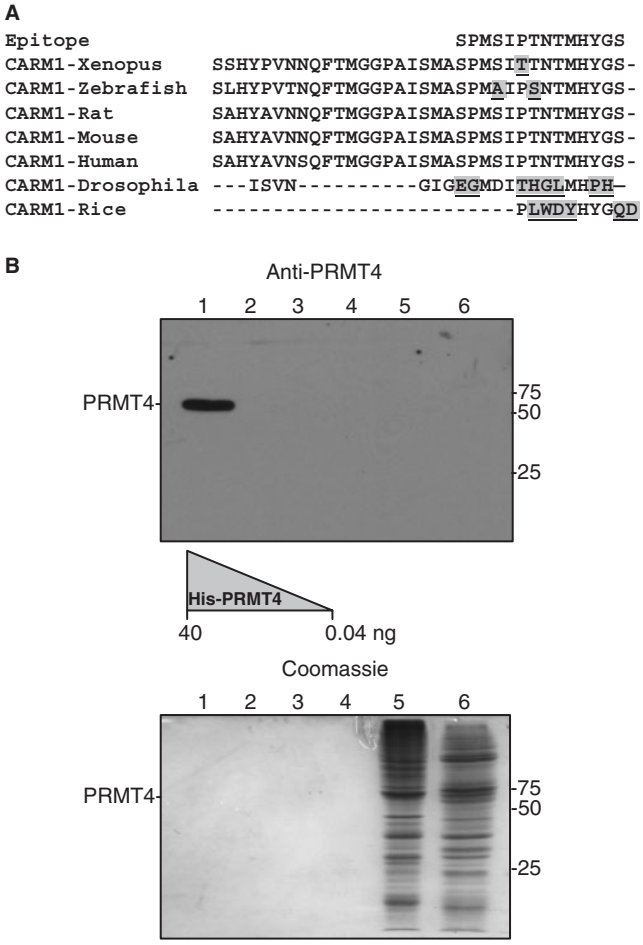


Fig. 3. **Anti-PRMT4 antibody does not recognize PRMT4 orthologs in ICL and WGE.** (A) Clustal sequence alignment of the C-terminal residues of PRMT4 from *Xenopus*, zebrafish, mouse, human, *Drosophila* and rice surrounding the peptide used to generate anti-PRMT4 antibody. Gray shaded residues indicate mismatches with the epitope-generating peptide. Note the divergence of the farthest members, *Drosophila* and rice, from the mammalian sequences. (B) Western blot showing a serial dilution of recombinant His-PRMT4 along with 5 µl of ICL and WGE performed according to the manufacturer's instructions. Under these conditions anti-PRMT4 detects 40 ng of recombinant PRMT4, but does not recognize ICL or WGE orthologs. The Coomassie-stained gel (below) shows the protein loads of the extracts.

(compare lane C and lane 1); however, when western blots of the same reactions were probed with a rabbit polyclonal antibody directed to dimethyl-arginine modified R17 in histone H3 a SAM-dependent band, which co-migrated with histone H3 appeared (middle panel lanes 1 and 4). To confirm that the antibody recognized CARM1/PRMT4 methylated histone H3, we performed *in vitro* methylation reactions with recombinant PRMT4 and found that the antibody specifically recognized histone H3, while MBP, which was also methylated by CARM1/PRMT4, was not recognized (Fig. 5A, right panels). Since R17 of histone H3 is an established substrate of CARM1/PRMT4 (*34*), it can be inferred that *S. frugiperda* ICL harbours a CARM1-like activity. This is not surprising as a CARM1 homolog (DART4) has been

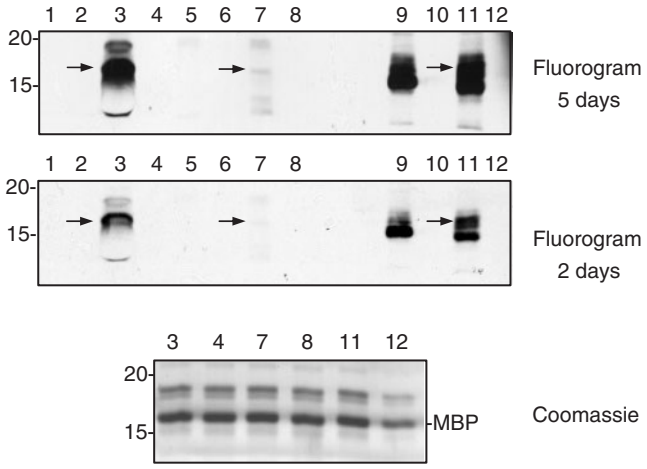


Fig. 4. **Methylation of MBP by ICL, WGE and RRL.** (Upper panels) Fluorograms of methylation reactions run in the presence of RRL (lanes 1–4), WGE (lanes 5–8) and ICL (lanes 9–12). Lanes 1, 2, 5, 6, 9 and 10 are run in the absence of MBP; lanes 3, 4, 7, 8, 11, 12 are run in the presence of MBP. Lanes 2, 4, 6, 8, 10 and 12 also contain 1 mM SAH. The 2-day exposure of the fluorogram clarifies the MBP methylation in ICL, which is partially obscured by endogenous protein methylation in the 5 day exposure. (Lower panel) shows Coomassie loads of MBP in the samples.

cloned from *Drosophila* (*21*). In contrast, while the fluorogram from WGEs showed the presence of a histone H3 MT activity, western blots with anti-H3(R17)$^{DMA}$ pAb were negative (Fig. 5B, left panels). This indicates that either the antibody is too weak to recognize the epitope, or that the WGE histone MT does not modify R17. To distinguish these possibilities, the reaction was repeated with twice the amount of WGE and twice the amount of histone H3. Western blots of the reaction were probed with anti-H3(R17)$^{DMA}$ pAb. Again, while the fluorogram showed SAM-dependent $^{3}$H-CH$_3$-incorporation into histone H3, the anti-H3(R17)$^{DMA}$ western blot was negative (Fig. 5B, right panels). Recombinant GST-CARM1/PRMT4 was previously shown to modify several arginine residues (R2, R17 and R26) of the N-terminal tail of purified calf thymus histone H3; however, the predominant site of modification was R17 (*29*). Although it is possible that a wheat germ homolog of CARM1/PRMT4 might modify histone H3 at a site different from R17 our data suggest that WGE likely does not contain such an activity.

To further investigate the methylation of histone H3 by WGE, its ability to be recognized by an antibody targeted to methylated K4 and K9 [anti-H3(K4/K9)$^{Me2}$] was assessed. Methylation of histone H3 at K4 is generally associated with transcriptional activation while at K9 it is associated with transcriptional silencing (*35*); however, the methylation of histone H3 by WGE did not correlate with this antibody either, Fig. 5C. Thus, it is unlikely that WGE harbours the lysine MT homologs that modify K4 and K9, respectively (*35*). Finally, as previously observed, RRL contains a SAM-dependent histone MT activity (Fig. 5D left panels). Furthermore, a western blot probed with anti-H3(R17)$^{DMA}$ pAb demonstrated the presence of a CARM1/PRMT4 activity (Fig. 5D right panels).

Using a similar strategy we next determined whether any of the translation lysates methylated histones H2a, H2b or H4. The results of these experiments reveal the diversity of MT activity in each lysate, (Fig. 5E–G). ICL moderately methylated histones H2a and H4 to a similar extent; potential methylation of histone H2b was obscured by the methylation of an endogenous ICL protein. WGE-induced methylation was detected in histones H2a and H2b, albeit very weakly. In contrast, WGE robustly modified histone H4. RRL methylated histone H2a, very weakly recognized histone H2b and robustly methylated histone H4.

Histone H4 is methylated at R3 by both PRMT1 and PRMT5 (*24*, *36*). To determine whether R3 is either symmetrically or asymmetrically dimethylated by ICL, WGE or RRL we probed western blots of H4 methylation reactions with anti-H4(R3)$^{Me2}$ and anti-H4(R3)$^{sMe2}$, respectively. The results show that all three lysates symmetrically dimethylated the R3 residue of histone H4 (Fig. 6A). In contrast, although histone H4 could be asymmetrically dimethylated at R3 by recombinant PRMT1 (Fig. 6B), this modification was not detected in any of the extracts.

*Methylation Controls*—Although it is quite clear from the above data that RRL, ICL and WGE differentially methylated various proteins, there was a concern that some of the weaker methylation may have occurred *via* contamination of a small amount of co-purifying PRMTs because the substrates were purified from tissues. To address this question we performed control methylation experiments of several of these substrate proteins minus the *in vitro* translation lysates. Figure 7 shows that under these conditions a purified recombinant *Escherichia coli* protein GST, which in the presence of PRMT8 can be weakly methylated (*37*) is not methylated in the absence of *in vitro* translation lysate. More importantly, MBP, histone H3 and histone H4 were not methylated in the absence of an added PRMT. Thus, all of the methylation observed for these substrates arises from the *in vitro* translation lysates.

*Methylation of eEF-1A*—eEF-1A is multiply methylated at $K_{36}$, $K_{55}$, $K_{79}$, $K_{165}$ and $K_{318}$ (*38*) and in some species it's C-terminus is carboxy-methylated (*39*). To determine whether any of the extracts contained MTs that could modify eEF-1A, *Xenopus* eEF-1A was prepared *in vitro* and then modified with WGE, ICL or RRL. Under the conditions used, WGE weakly modified eEF-1A, whereas ICL robustly modified eEF-1A (Fig. 8A and B). In each case, the methyl incorporation was found to be stable to base treatment (Fig. 8C). In contrast, RRL did not modify eEF-1A. These data are consistent with the hypothesis that both, ICL and WGE contain PKMT activity.

*Methylation of FMRP*—RNA binding proteins harboring RG-rich regions (RGG boxes) are known targets of PRMTs. We previously showed that one such protein, the FMRP, is methylated by RRL *in vitro* (*12*). Further studies determined that the modification occurred in C-terminal arginine residues located within an RG-rich region and that these residues were modified by a class I PRMT both in RRL and *in vivo* (*16*, *40*). To determine whether ICL or WGE harboured a class I PRMT activity

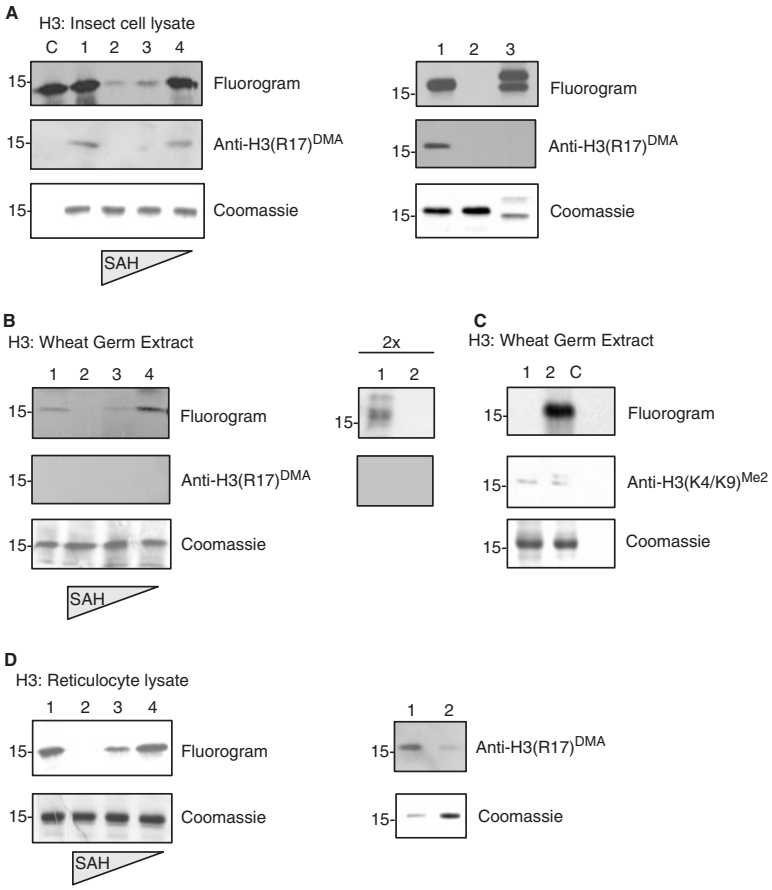


Fig. 5. **Methylation of histones by ICL, WGE and RRL.** (A) Histone H3 methylation reactions in the presence of ICL and in the absence (lane 1) or presence (lanes 2–4) of decreasing concentrations of SAH (left panel). Lane C shows ICL without added histone H3. Top, fluorogram; middle western blot with anti-H3(R17)$^{DMA}$ pAb; lower Coomassie staining showing protein load, as indicated. Methylation of histone H3 (lanes 1 and 2) or MBP (lane 3) by recombinant CARM1/PRMT4 (0.05U) (right set of panels). Lane 2 shows the effect of 1 mM SAH on histone H3 methylation. Note the methylation of MBP by CARM1/PRMT4 is in agreement with Boulanger *et al*. (*21*). (B) Histone H3 methylation reactions in the presence of WGE and in the absence (lane 1) or presence (lanes 2–4) of decreasing concentrations of SAH (left panel). Right set of panels shows a fluorogram and western blot of histone H3 methylation reactions in the absence (lane 1) or presence (lane 2) of 1 mM SAH using twice the amount of histone H3 and twice the amount of WGE. (C) Histone H3 methylation reactions in the presence of WGE, and in the absence (lane 1) or presence (lane 2) of 1 mM SAH. Lane C shows WGE without added histone H3. Note that anti-H3(K4/K9)$^{Me2}$ weakly recognizes calf thymus histone preparations independent of SAH indicating the protein has some basal methylation. (D) Histone H3 methylation reactions in the presence of RRL and in the absence (lane 1) or presence (lanes 2–4) of decreasing concentrations of SAH (left panels). Histone H3 methylation reactions in the presence of RRL and in the absence (lane 1) or presence (lane 2) of 1 mM SAH (lower panels). Note for this particular experiment H3 histone was isolated on carboxymethyl-Sepharose following *in vitro* methylation. Methylation of histones H2a, H2b (left panels) and H4 (right panels) by ICL (E), WGE (F) and RRL (G). Lanes 1 and 2 show results for histone H2a, lanes 3 and 4 show results for histone H2b and lanes 5 and 6 show results for histone H4. Lane C shows the lysate without added histone. Asterisks mark weakly methylated protein substrates.

that could modify FMRP, *in vitro* methylation assays were carried out with the full–length protein. As expected, FMRP was subject to SAM-dependent methylation in RRL (Fig. 9A). WGE did not methylate FMRP (Fig. 9B), which is consistent with its overall low methylation of endogenous substrates and its relatively weak activity toward histones MBP, histone H3, histone H2a and histone H2b, (Figs 4, 5B and F). However, ICL robustly methylated FMRP (Fig. 9C).

The RG-rich region of FMRP (Fig. 9D) is multiply methylated (*40*). Using RRL, we previously determined that conserved residues, $R_{544}$ and $R_{546}$, were both methylated and that $R_{544}$ methylation accounted for nearly 80% of the *in vitro* methylation activity (*16*); however, this ratio may be both context and PRMT-source dependent (*40*, *41*). To determine which, if any, FMRP arginine residues ICL favoured, we examined the *in vitro* methylation of FMRP truncation mutants Ex15a-$R_{544}$K and Ex15a-$R_{546}$K. As a control wild type Ex15a was simultaneously assessed. The results, shown in (Fig. 9E), demonstrate that ICL favours the $R_{544}$ site in the RG-rich region of FMRP like RRL.

A summary of all of the data is presented in Table 1.

## DISCUSSION

Protein *N*-methylation, particularly that catalyzed by the PRMT and the PKMTs, has emerged as a crucial

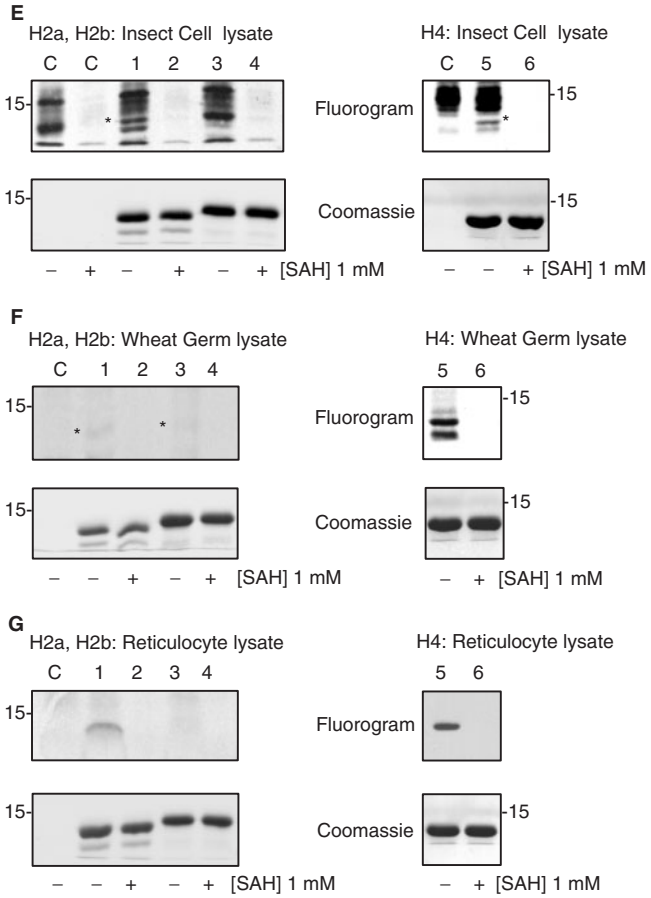


Fig. 5. **Continued.**

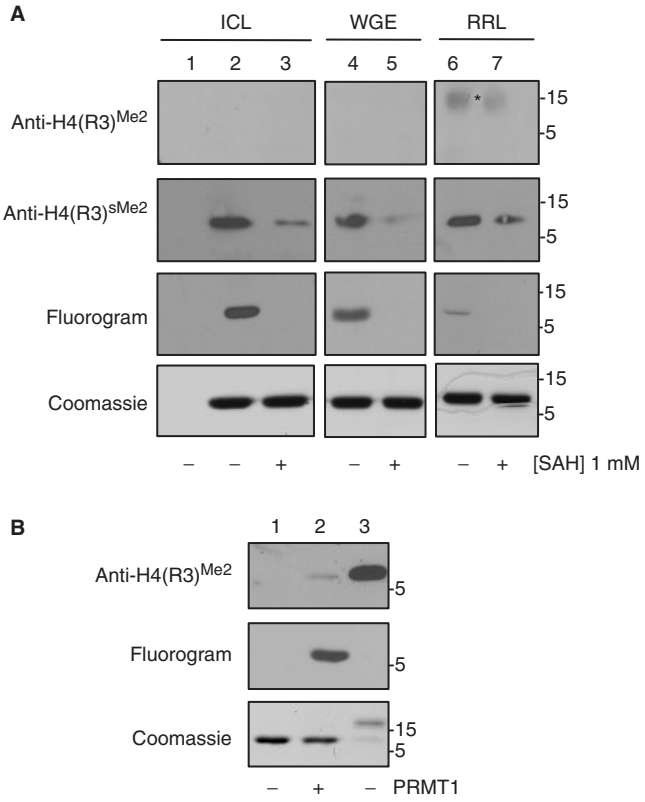


Fig. 6. **Histone H4 is symmetrically dimethylated by ICL, WGE and RRL.** (A) Recombinant histone H4 was methylated by ICL (lanes 1–3), WGE (lanes 4, 5) and RRL (lanes 6, 7) as described in Fig. 4 and subject to western blotting with anti-H4(R3)$^{me2}$ or anti-H4(R3)$^{sme2}$. The asterisk in lanes 6 and 7 of the anti-H4(R3)$^{me2}$ western blot represent non-specific recognition of the hemin in RRL. (B) Recombinant PRMT1 asymmetrically dimethylates histone H4 at R3. Recombinant histone H4 was incubated in the absence (lane 1) or presence (lane 2) of 0.3 μg of recombinant PRMT1. The resulting reactions were subject to western blotting with anti-H4(R3)$^{me2}$ and fluorography. The protein loads are shown by Coomassie staining. Lane 3 contains 10 μg of mouse histones as a positive control.

regulator of many cellular processes. Indeed, the lysine methylation mark on select histone residues can result in transcriptional activation or repression of certain genes (*35*, *42*, *43*). Protein arginine methylation has been shown to play a role in DNA damage repair (*44*), apoptosis (*45*), oncogenesis (*46*) and even the flowering time in *Arabidopsis* (*24*). Protein–protein interactions are the means by which the cellular changes due to PRMT- and PKMT-mediated methylation arise. In the case of histones chromo, PHD and Tudor domain-containing proteins are used to discriminate between methylated and non-methylated residues (*47*, *48*). PRMT-mediated methylation of RG-rich domains within RNA binding proteins also affects their protein–protein interactions. For example, methylation of the FMRP and the fragile X related protein 1 (FXR1P) enhances their interaction with each other, while inhibiting their methylation negatively impacts their association with stress granules (*2*). Similarly, methylation of the RG-motifs within the cold-inducible RNA binding protein (CIRP) is required for its nuclear export and incorporation into cytoplasmic stress granules (*49*).

In addition to affecting protein–protein interactions, methylation of RNA binding proteins can also affect their interaction with nucleic acids. The binding of poly U to Sam68 was recently shown to be negatively regulated by methylation, while methylation of FMRP inhibited the binding of G-quartet RNA to its RG-rich domain (*50*). Thus, it would appear to be crucial when conducting protein–protein interaction studies and protein-RNA interaction studies with recombinant proteins that their methylation state be established.

In that vein, Cheng *et al.* (*13*) recently developed a small-pool screening approach to identify methylated proteins using RRL. They corroborated previous reports of the presence of PRMT1 (*16*) and PRMT5 (*15*) in RRL and established the presence of CARM1/PRMT4, which is corroborated in this study (Fig. 5). They then used this approach to identify several novel proteins including CA150 and SAP-49 that are methylated by CARM1. In contrast, Hsieh *et al.* (*51*) took a different tack. They engineered the yeast HMT1 arginine MT, thus enabling the expression of asymmetrically dimethylated proteins in *E. coli*.

In this study, a comparative approach was taken to assess the methylating potential of *in vitro* translation lysates. Special attention was given to ICL from *S. frugiperda*, which is a new addition to the *in vitro*

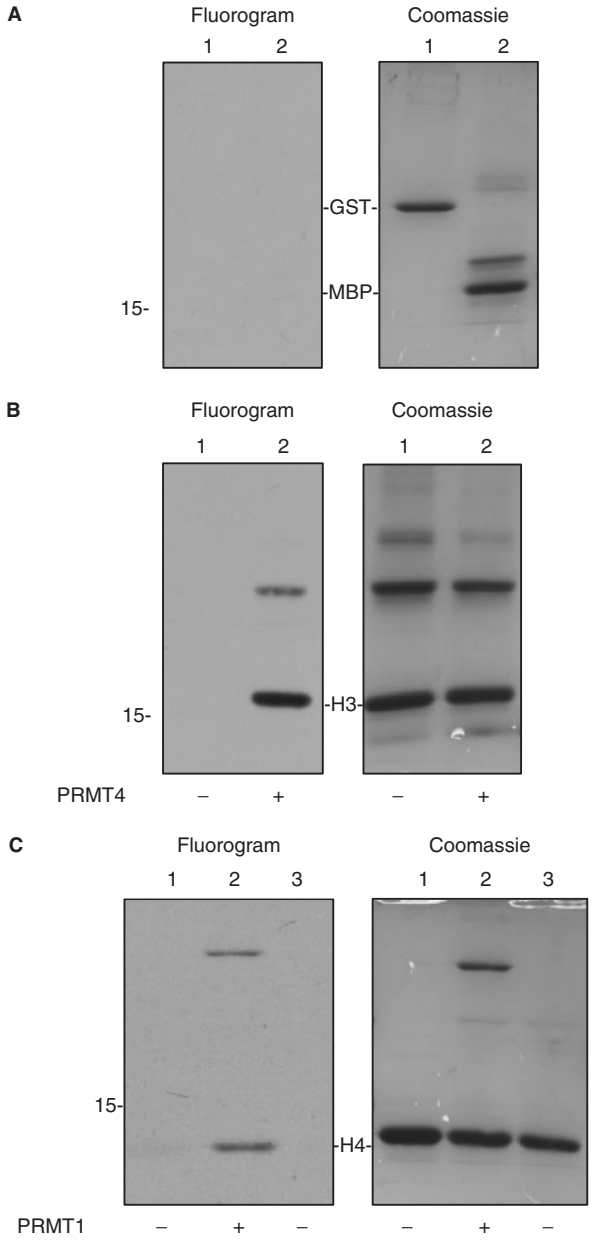


Fig. 7. ***In vitro* methylation results from addition of a MT source.** (A) *Escherichia coli*-produced recombinant GST (lane 1) and mouse brain purified MBP (lane 2) were subjected to *in vitro* methylation as described except that no source of exogenous MTs were added. (B) Calf thymus purified histone H3 was subjected to *in vitro* methylation in the absence (lane 1) or presence (lane 2) of recombinant PRMT4. (C) Calf thymus purified histone H4 was subjected to *in vitro* methylation in the absence (lane 1) or presence (lane 2) of recombinant PRMT1. Lane 3 shows an *in vitro* methylation reaction of *E. coli*-produced recombinant histone H4 in the absence of a MT source. The Coomassie-stained gels in the right-hand panels of (A–C) show the protein loads.

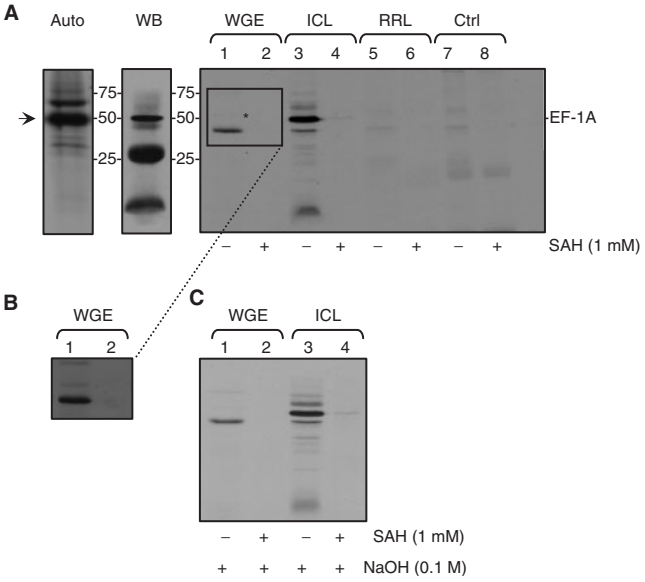


Fig. 8. **Methylation of eEF-1A by ICL, WGE and RRL.** (A) eEF-1A was translated in the presence or absence of $^{35}$S-methionine *in vitro* using RRL. Aliquots of the reactions were resolved by electrophoresis and subjected to either autoradiography left panel or western blotting with anti-EF-1A mAb middle panel. Nonradiolabeled eEF-1A was denatured for 10 min at 42°C and then subjected to post-translational methylation with WGE, ICL and RRL in the absence or presence of 1 mM SAH (right panel, lanes 1–6). A control showing that the denatured protein does not have residual eEF-1A methylating activity is shown in lanes 7 and 8. An asterisk marks the methylation of eEF-1A by WGE. (B) A longer exposure of the fluorogram in (A) shows the SAM-dependent methylation of eEF-1A by WGE. (C) *In vitro* methylation reactions of lanes 1–4 in (A) were treated with NaOH (final concentration 0.1 M) at room temperature for 30 min. The samples were resolved by SDS–PAGE and subjected to fluorography.

translation repertoire, but which is widely used to express recombinant protein *via* baculovirus. Analyses using a defined set of protein substrates revealed a rich diversity of MT activity in each type of lysate. Not surprisingly, each system harboured unique activities toward specific substrates. For example, both ICL and WGE contained the PKMT that methylates eEF-1A, whereas this activity is not present in RRL as was previously shown (*13*). Conversely, both ICL and RRL had detectable CARM1 activity toward histone H3(R17), which was absent in WGE.

Interestingly, the results of histone H4 *in vitro* methylation reveal a unique interplay between the PRMTs within these lysates for particular substrates. Previous studies had established that PRMT1 methylates H4(R3) and, as mentioned above, PRMT1 has been confirmed in RRL. Thus, it was unexpected that all of the detectable methylation of H4(R3) in RRL was due to a class II PRMT. Apparently, the endogenous PRMT5/7 outcompetes PRMT1 for this substrate, and this is a consistent feature of all of the extracts.

Finally, differences due to substrate specificity of particular MTs were also observed here. For example, WGE class I PRMT activity was either extremely weak or absent for most substrates *i.e.* H3(R17), FMRP. This is consistent with the finding that the robust methylation of H4(R3) is due to either PRMT5 or PRMT7. However, MBP is also an *in vitro* substrate of PRMT5 and PRMT7 (*52*, *53*); nevertheless, it is much more weakly methylated by WGE than histone H4.

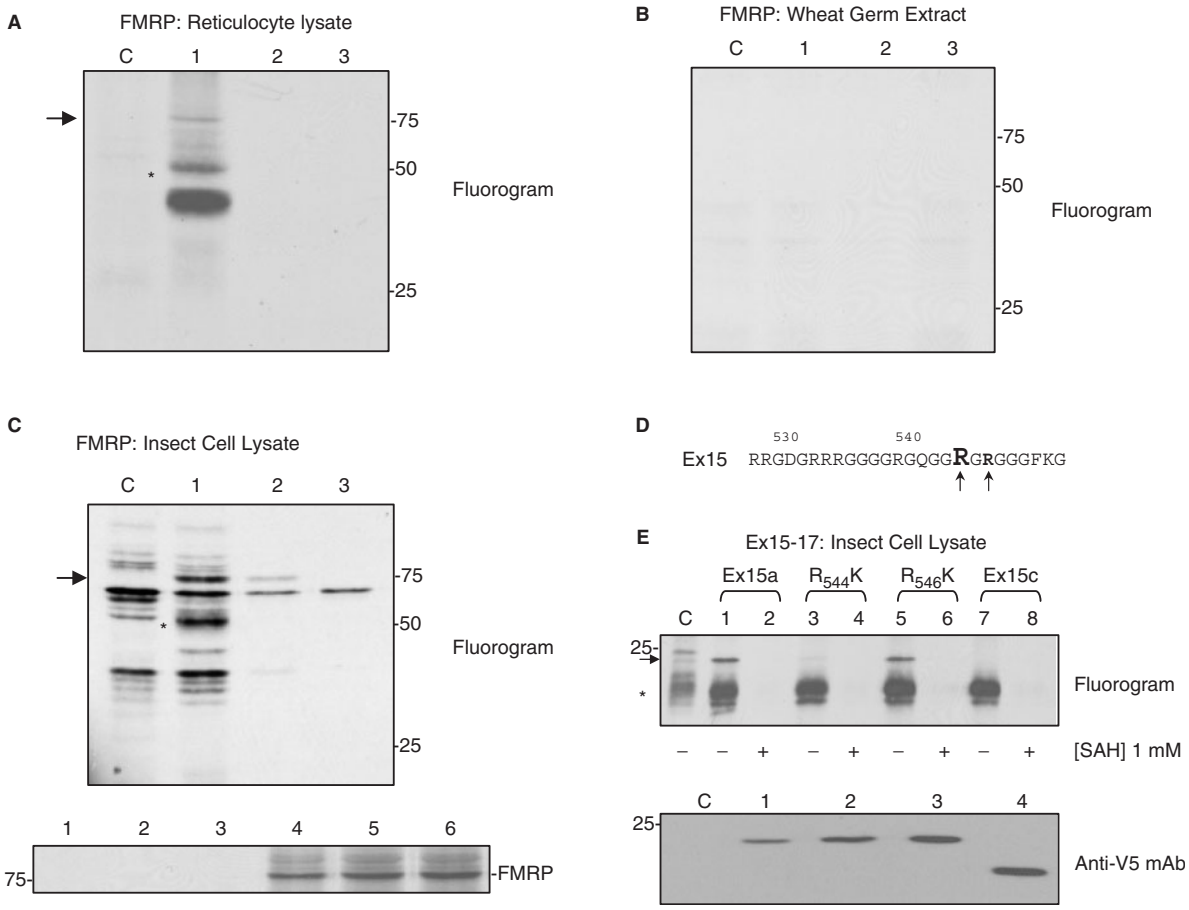


Fig. 9. **Methylation of FMRP by ICL, WGE and RRL.** (A) (Upper panel) Methylation reactions in the presence of RRL and in the absence (lane C and 3) or presence (lanes 1, 2) of FMRP. Lanes 2 and 3 included 1 mM SAH. Arrow shows the position of full-length FMRP, asterisk shows the main proteolytic cleavage product (*4*, *17*). (B) As in (A) except assayed with WGE. (C) As in (A) except assayed with ICL. (Lower panel) lanes 1–3 show the C lanes for each lysate and lanes 4–6 show the lane 1 reaction of each lysate blotted and probed for FMRP expression. (D) The RG-rich region of human FMRP showing methylated residues $R_{544}$ and $R_{546}$. (E) ICL recapitulates the methylation of FMRP RG-rich region residues. Wild type, Ex15a, and Ex15a mutants, $R_{544}K$ and $R_{546}K$ (lanes 1–6) were methylated in the absence or presence of SAH. Lanes 7 and 8 contain the FMRP splice variant Ex15c, which is not methylated by RRL (*16*); its methylation in ICL, if it occurs, is obscured by an endogenous ICL protein. Lane C shows the lysate without the added FMRP variant. The lower panel shows the loads for the proteins in the fluorogram above. Note for this particular experiment Ex15a, Ex15a-$R_{544}K$ and Ex15a-$R_{546}K$ was isolated on carboxymethyl-Sepharose following *in vitro* methylation.

Table 1. **MT in *in vitro* translation lysates.**

| Substrate | Target[a] | Enzyme[b] | ICL[c] | WGE[c] | RRL[c] |
|---|---|---|---|---|---|
| Histone H3 | General | PRMT/PKMT | + | + | +++ |
| Histone H3 | $R_{17}$ | CARM1/PRMT4 | + | – | +++ |
| Histone H3 | $K_4/K_9$ | SET7/9/Suv39h | + | – | ND |
| Histone H4 | General | PRMT/PKMT | ++ | – | +++ |
| Histone H4 | $R_3^{Me2}$ | PRMT1 | – | – | – |
| Histone H4 | $R_3^{sMe2}$ | PRMT5/PRMT7 | +++ | +++ | ++ |
| Histone H2A | General | PRMT5/PKMT | ++ | + | +++ |
| Histone H2B | General | PKMT | ? | + | – |
| MBP | General | PRMT | +++ | + | +++ |
| eEF-1A | General | PKMT | +++ | + | – |
| FMRP | General | PRMT | +++ | – | +++ |
| FMRP | $R_{544}$ | PRMT | – | NA | – |
| FMRP | $R_{546}$ | PRMT | ++ | NA | ++ |

[a]Target: the specific residue of the substrate when known, or the entire protein (general). [b]Enzyme: activity attributed to a PRMT, a PKMT or a specific MT, as indicated. [c]Methylation by ICL, WGE and RRL was scored as absent (–), weak (+), moderate (++) or strong (+++) from the figures; ?, methylation of the substrate was obscured by endogenous protein methylation.
ND, not determined; NA, not applicable.

All together, the data demonstrate that the proteins generated by these three *in vitro* systems will have different methylation patterns and different extents of methylation at particular sites. The functional consequences of this will vary from protein to protein, and it would be wise to keep this in mind when protein–protein and protein–RNA interaction data are interpreted.

Supplementary data are available at *JB* Online.

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