5.08J Biological Chemistry II (2016)

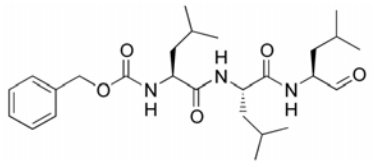
ANS to Problem Set 8 Chemistry 5.08

This PS is focused on HMGR regulation and revisits the PCSK-9 paper.

1. HMG-CoA reductase (HMGR) is a 97 kDa integral membrane glycoprotein localized to the ER. The N-terminal membrane domain has eight transmembrane helices and is sufficient to regulate the enzyme's stability. At the time of Experiment 1 described below, the signaling pathway or pathways for the degradation of HMGR in response to metabolic cues were not understood. Experiment 2 describes experiments to further address this issue that were carried out in 2014. Review the model for controlling HMGR activity presented in your class notes.

Experiment 1 was carried out to determine the fate of HMGR in the presence and absence of sterols and in the presence or absence of a proteasome inhibitor, MG-132. The results of these experiments are shown in Figures 1 and 2 and are described in the figure legends associated with each of the experiments.

Digression:



MG-132 is a cell permeable proteasome inhibitor with a K_d of 4 nM.

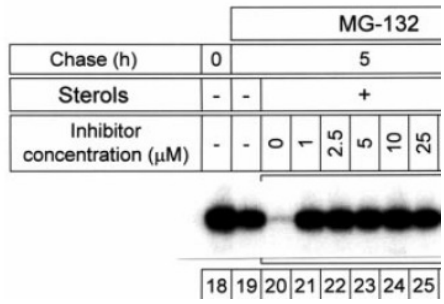


Figure 1: Effect of MG-132 proteasome inhibitor on the amounts of HMGR in a pulse-chase experiment. LP-90 cells were pulse-labeled for 30 min with 150 mCi of [^{35}S]-Met and either lysed immediately (lane 18) or chased for 5 h in the absence of sterols (lane 19) or in the presence of sterols (lanes 20-25) and the indicated concentration of the proteasome inhibitor. Cells were lysed in lysis buffer in the presence of the detergent deoxycholate and the lysates were centrifuged for 30 min at 16,000 x g to remove cell debris. HMGR was immunoprecipitated from the supernatant fraction with an antibody (Ab) targeting the HMGR membrane domain and analyzed by SDS PAGE and fluorography(Phosphorimager technology where the phosphor detects radioactivity, see recitation 2/3 notes).

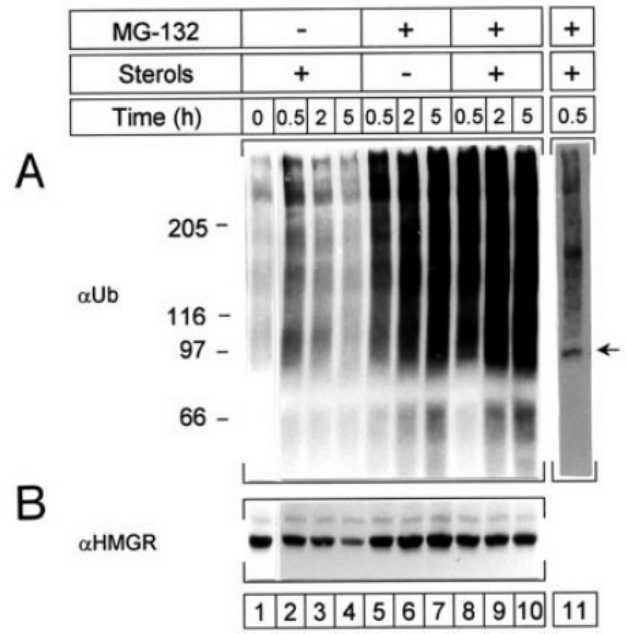
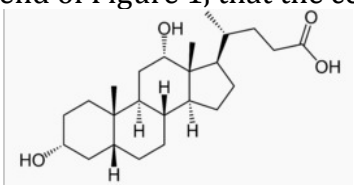


Figure 2: Effect of Sterols on modification of HMGR in chinese hamster ovary (CHO) cells. CHO cells were treated with sterols (25-hydroxycholesterol and cholesterol), MG-132, or both for the indicated time periods. The cells were lysed in lysis buffer, the lysate was centrifuged at 16000 x g to remove cell debris, and HMGR was immunoprecipitated from the supernatant with antibodies against the HMGR membrane domain. Immune complexes were resolved by SDS-PAGE (5 to 15% gradient gel) and blotted onto a nitrocellulose membrane. Ubiquitin conjugates were detected with anti-Ubiquitin monoclonal antibodies (αUb) from mice as the primary antibody and HRP-conjugated anti-mouse-IgG antibodies from goats as the secondary antibody and color developed using HRP. In lane 11 of part A and in all lanes of B, the membrane was stripped and reprobed with A9 monoclonal Ab to HMGR (arrow indicates HMGR band).

Questions:

1. You are given in the figure legend of Figure 1, that the cell lysis buffer contained deoxycholate. Its structure is: 

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Why was deoxycholate included in the lysis buffer?

Deoxycholate is a bile acid and an ionic detergent used to assist cell lysis and to solublize membrane proteins. In this case, it solubilizes membrane-bound HMGR. The remainder of the cell membranes are then separated in the subsequent centrifugation and the detergent-solubilized HMGR can be used for further analysis. Note: some of the HMGR could remain unsolublized.

2. Describe the conclusion(s) that can be drawn from the data in Figure 1.

Figure 1 is obtained from a pulse-chase experiment, that is, only proteins that were synthesized during the pulse are visualized by detecting the radioactivity incorporated in this time period. Recall that ^{35}S is a β-emitter. The data indicate that, in the absence of

sterols, HMGR is stable, as its levels decrease only marginally during the 5 hour chase. In the presence of sterols, almost all of the HMGR synthesized during the pulse is degraded after 5 hours, indicating that its half-life is significantly shorter. The presence of the MG-132, a proteasome inhibitor, prevents the degradation of HMGR at concentrations as low as 1 μM, indicating that HMGR is usually degraded by the proteasome in the presence of sterols.

3. In Figure 2 lanes 1-4 (no MG-132) provide an explanation for why there are so many high molecular weight species observed?

HMGR is a membrane protein that is glycosylated and gets ubiquitinated in the presence of sterols to target it for degradation. All three of these factors could contribute to the heterogeneity in the gel: Membrane proteins can often exhibit significant heterogeneity in gel electrophoresis because of variable loading with SDS. Different extents of glycosylation can alter the total molecular weight of a protein and also affect its loading with SDS. Finally, ubiquitination changes the total molecular weight of the protein, depending on the number of ubiquitin molecules that are attached. Which of these factors contributes most will depend on the experimental conditions, e.g. how the samples were prepared.

One way to examine the basis for the heterogeneity, for example modification with sugars, is to treat the sample with a deglycosylase that removes all the sugars attached to an asparagine in a defined motif amino acid motif on the cell surface. To perhaps remove heterogeneity due to incomplete unfolding, one could boil the sample longer in SDS. To remove any heterogeneity associated with ubiquitination, you could treat the sample with deubiquitinases. Note that the primary antibody used in the Western Blot was an anti-ubiquitin antibody, so only ubiquitinated HMGR is detected.

4. Describe conclusions that can be drawn from this experiment.

In this experiment, HMGR was immunoprecipitated and analyzed by Western Blots after cell growth with and without sterols and with and without MG-132. Without sterols and without MG-132 (lane 1), there are a few high molecular weight bands that react with the ubiquitin antibody. Upon addition of sterols (lanes 2-4), higher molecular weight bands for ubiquitin appear that then lose intensity over time. The highest levels of ubiquitination appear to be at the 30 min time point. As can be seen in part B, HMGR gets modified and/or degraded over this time period, as the levels for the 97 kDa version decrease. Likely, HMGR is getting rapidly ubiquitinated upon addition of sterols and is then degraded by the proteasome. In the presence of MG-132, but in the absence of sterols (lanes 5-7), there is a strong accumulation of high molecular weight bands that react with the ubiquitin antibody. At the same time, levels of HMGR increase (part B, lanes 5-7). When sterols are added in the presence of MG-132 (lanes 8-10), the intensity of the high molecular weight bands increases even further. Under these conditions, however, there is no increase in levels of HMGR (part B, lanes 8-10).

There are a number of conclusions to be drawn from this experiment. First, addition of sterols leads to polyubiquitination of HMGR and degradation by the proteasome. Inhibiting the proteasome leads to significant accumulation of ubiquitinated HMGR

(compare lanes 2-4 and 8-10). Second, it seems that HMGR also gets polyubiquitinated in the absence of sterols (lanes 5-7), but these species only accumulate when the proteasome is blocked. Because the half-life of HMGR in the absence of sterols is long (see Figure 1), maybe there is a proteasome-dependent deubiquitination activity that prevents HMGR degradation in the absence of sterols. Inhibiting the proteasome leads to accumulation of the ubiquitinated species.

Third and much more subtly, HMGR is transcriptionally regulated as well: in the absence of sterols, its levels increase when the proteosome is blocked (part B, lanes 5-7), but its levels do not increase in the presence of sterols when the proteasome is blocked (part B, lanes 8-10). Note the images in Figure 2 cannot be quantified by the "eyeball" method. It is thus very difficult to tell about small changes without analyzing the intensity of the bands more quantitatively.

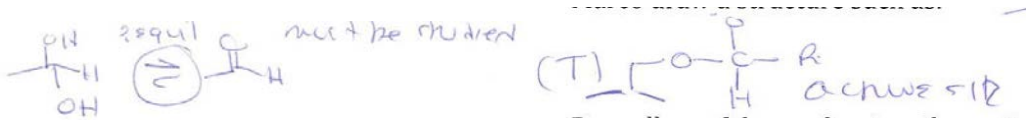
5. Describe how the αUb-HRP-anti-mouse antibody sandwich works to allow visualization of the proteins of interest, in this case ubiquitin.

The primary antibody, αUb, binds to the ubiquitin on the nitrocellulose membrane. αUb was raised in mice. The secondary antibody binds mouse IgG and is conjugated to HRP. HRP can oxidize precursor substrates into colored dyes that deposit where they are generated and allow for visualization of ubiquitin.

6. Given what you have learned about the mechanism of the proteasome, propose a mechanism by which MG-132 inhibition might occur. What may be the issues with using an aldehyde as an inhibitor in general in humans and with MG-132 specifically?

MG-132 is a competitive reversible, covalent inhibitor of the proteasome. The aldehyde gets attacked by the active site hydroxyl of the threonine nucleophile with the help of its α-amino group that functions as a general base catalyst. Recall that the active site threonine is at the N-terminus in the human proteasome. The reaction may also be time-dependent, if the hemiacetal formed by the attack by threonine is stabilized. The peptide part of the inhibitor provides the specificity.

Aldehydes can be problematic as inhibitors for several reasons. They are often hydrated and since the active form of the inhibitor must be the aldehyde, the reactive concentration is lower than the "weighed" amount of aldehyde depending on the level of hydration. Inside the cell aldehydes are in general rapidly metabolized to acids or alcohols that will be much poorer, non-covalent inhibitors.



7. Are the data presented in Figures 1 and 2 consistent with the model for HMGR regulation that you learned about in class and your reading?

The data presented here are in principle consistent with the model described in class and presented on the PP slides. Note, however, that there is no information on the other

proteins involved in this process. The data only suggest that HMGR is indeed ubiquitinated and degraded in the presence of sterols.

2. From the data described above there are NO molecular details, that is, information about the proteins E1, E2 and E3 that must be involved in this process. In fact, the model discussed in class should include gp78 and Ubc7 which are E3 and E2 proteins involved in the ERAD (endoplasmic reticulum associated degradation process).

Experiment 2: Studies in the last decade have shown that there are three E3 protein complexes involved in ERAD, one of which includes gp78. Song et al reported that gp78, which they showed was associated with Insig1, is responsible for degradation of HMGR in response to sterols. They also reported that another ERAD E3, TRC8, interacts with Insig1 and also plays a role in HMGR degradation.

Recently Tsai et al studied mouse embryonic fibroblasts (MEFs) from a liver-specific gp78 knockout mouse. Initially they carried out the experiment shown in Figure 3.

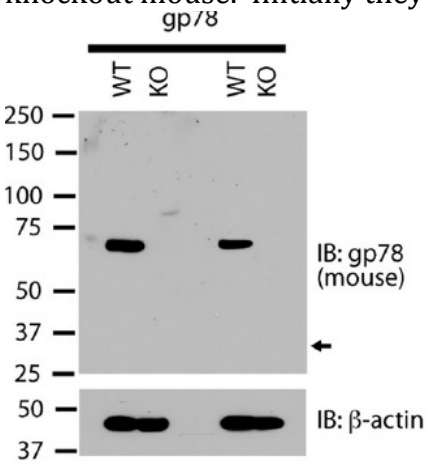


Figure 3: Levels and length of gp78 in two different sets of primary MEFs (from two separate pregnancies of mice) were examined because of the complexity of the targeting strategy for the knockout of gp78 in order to determine if the knockout experiment was successful. Ignore the black arrow in the Figure. The β-actin serves as a loading control (actin should be, and is, present at the same levels in WT and KO cells).

They then used ^{35}S pulse-chase metabolic labeling and immunoprecipitation to quantitatively assess the degradation of the endogenous HMGR in these MEFs. The cells were grown under complex conditions to maintain their viability (details have been omitted) and then pulse labeled for 30 min with [^{35}S]-Met and Cys. This labeling was followed by a chase in "cold" medium in the absence or presence of sterols. At indicated times (Figures 4 A and B), the endogenous HMGR was immunoprecipitated with antibodies to the transmembrane domain of HMGR (as described above).

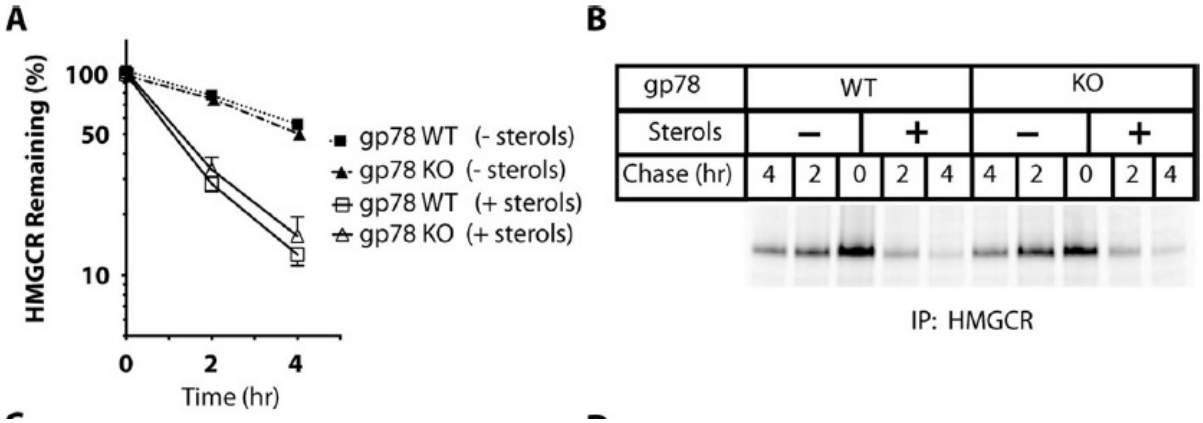


Figure 4 A and B. Results of pulse-chase experiments described above.

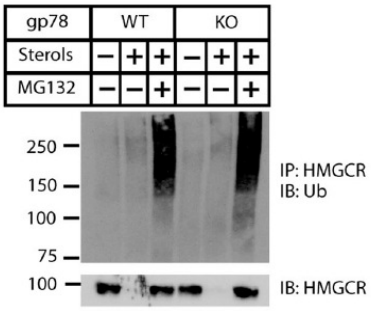


Figure 4C. (IP = Immunoprecipitation; IB = Immunoblot/Western blot)

In the experiment whose results are shown in Figure 4C, the cells were allowed to accumulate HMGR by media manipulation, followed by addition of sterols (where indicated) in the presence or absence of the proteasome inhibitor MG-132. After 60 min, the cells were lysed and HMGR was immunoprecipitated with HMGR antibodies raised in rabbit. The immunoprecipitates were sequentially immunoblotted with mouse monoclonal antibodies to ubiquitin (top part) and HMGR (bottom part).

Questions:

1. Describe what the data in Figure 3 tell you about the gp78 knockout mouse.

Figure 3 indicates that gp78 is indeed knocked out in the mouse. There is no band for gp78 in the Western Blot, while the β-Actin control lane shows no change.

2. Describe what the results in Figure 4 A-C tell you about the studies with embryonic fibroblasts relative to the model for HMGR regulation.

The data in Figure 4 A and 4 B indicate that the levels of HMGR in the gp78 knockout are **the same** as in the wild-type in the presence or the absence of sterols. The results in Figure 4 C further indicate that HMGR in the gp78 knockout is still getting ubiquitinated in the presence of sterols. Thus, the gp78 knockout behaves like WT under all conditions tested, indicating either that gp78 is not involved in ubiquitinating HMGR or that there is an alternative pathway.

3. The results given above are different from the earlier reported studies of Song et al. How might the differences between this study and the earlier study be resolved? I am not asking for details, but for how you would go about determining the basis for the differences between the two studies, which would in turn, provide the impetus for future experiments.
4. The experimental data in this PS were taken in part from *Molecular Biology of the Cell* 23 4484 (2012). In this paper, which is very thoughtful, they go step by step through the differences in their experimental protocol in comparison with those reported by Song et al. However, they also offer explanations for how the earlier experiments could have been carried out more carefully. The complexity of the experiments and the model cells occur in many experiments done today. Thus this paper is important to think about, as many issues that must be considered in experimental design are articulated.
   Think about the details of how Song et al. determined that gp78 and TRC8 are involved in mediating HMGR degradation. Were there experiments done in vitro or in vivo? What were the types of cells used? Did they have good antibodies to the proteins or were the antibodies to the epitope tags (e.g. myc)? In vitro and after HMGR detergent solubilization, maybe gp78 can mediate ubiquitination of HMGR. Etc

3. In recitation 8, you read the paper in which Crispr-cas9 was used to remove the PCSK-9 protein from 3T3L1 cells and from mouse liver cells. This question is focused on the experimental details described in that paper. The gene for *pcsk-9* is shown in Figure 5.

Figure 5. *pcsk-9* gene where the black rectangles are the targeted exons within this gene: exon one and exon two are at the far left and far right of the gene.

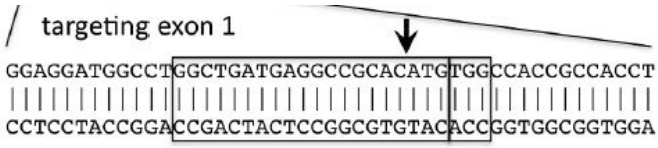


Figure 6. The target for the gRNA from exon 1

Both exon 1 and exon 2 of *pcks-9* (Figure 5) were targeted for guide RNA design with the choice for exon 1 targeting shown in Figure 6. Shown in Figure 7 are the results of the experiment to determine which exon (one vs two) would be best target.

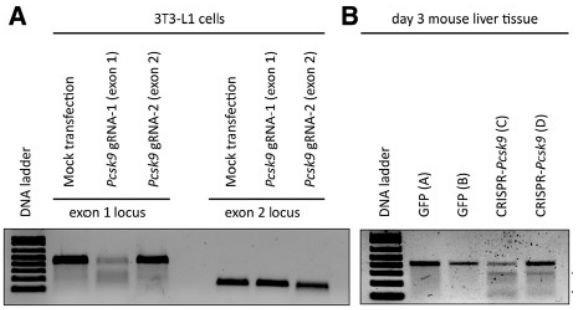


Figure 7 Target effects in mouse cells and livers subsequent to CRISPR-cas9 expression. A. Surveyor assays performed with genomic DNA from 3T3-L1 cells transfected with Cas9 and guide RNA targeting either exon 1 (gRNA-1) or exon 2 (gRNA-2). B. Surveyor assays performed with genomic DNA from liver samples taken from mice from mice 3 days after receiving a control adenovirus expressing GFP or adenovirus expressing Cas9 and gRNA-1. In panel B, there are two GFP controls (A and B) and two mouse liver tissue experiments (C and D).

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Finally, PCR amplification and Sanger sequencing of the target site of many samples of mouse liver genomic DNA subsequent to the CRISPR-cas9 studies gave results, some of which are shown in Figure 8.

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GGAGGATGGCCTGGCTGATGAGGCCGCAC-TGTGGCCACCGCCACCT    del_1 x 2
GGAGGATGGCCTGGCTGATGAGGCCGCA-ATGTGGCCACCGCCACCT    del_1
GGAGGATGGCCTGGCTGATGAGGCCGCACcATGTGGCCACCGCCACCT   ins_1 x 9
GGAGGATGGCCTGGCTGATGAGGCCGCA--TGTGGCCACCGCCACCT    del_2 x 6
GGAGGATGGCCTGGCTGATGAGGCCGCAC--GTGGCCACCGCCACCT    del_2 x 2
GGAGGATGGCCTGGCTGATGAGGCCGCgcCATGTGGCCACCGCCACCT   ins_2/del_1
GGAGGATGGCCTGGCTGATGAGGCCGCACAccaTGTGGCCACCGCCACCT ins_3
GGAGGATGGCCTGGCTGATGAGGCCGCAC-----GCCACCGCCACCT    del_5
GGAGGATGGCCTGGCTGATGAG------CATGTGGCCACCGCCACCT    del_6
GGAGGATGGCCTGGCTGATGAGGCCGCACcg-------CCGCCACCT    ins_2/del_9
GGAGGATGGCCTGGCTGATG--------CATGTGGCCACCGCCACCT    del_8
GGAGGATGGCCTGGCTGA-----------ATGTGGCCACCGCCACCT    del_11
GGAGGATGGCCTGGCTGATGAGGCC------------ACCGCCACCT    del_12
GGAGGATGGCCTGGCTG------------ATGTGGCCACCGCCACCT    del_12
GGAGGATGGCCTGGCTGATGAGGCCGCAC-----------CCACCT     del_12
GGAGGATGGCCTGG----------------TGTGGCCACCGCCACCT    del_16
GGAGGATGGCCTGGCTGATGAGGCCccgttgcctgg-----------    ins_11/del_27
GGAGGATGGCCTGGCTGATGAGGCCGCAC------------------    del_21 x 2
GGAGGATG---------------------ATGTGGCCACCGCCACCT    del_21
GG-------------------------CATGTGGCCACCGCCACCT    del_26
GG-----------------------ATGTGGCCACCGCCACCT        del_27
G------------------------ATGTGGCCACCGCCACCT        del_28
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Figure 8. Sequencing of liver genomic DNA based on the target from the gRNA sequence.

Questions:

1. Why were the exons and not the introns targeted by Ding et al ?

The exons end up in the mRNA and ultimately code for the protein. Altering the introns that are removed by splicing will likely still result in protein beng present.

2. The targeted region of exon 1 is shown in Figure 6. There are three features of this target DNA that are highlighted: the large rectangular box, the small box and the arrow. Given your understanding the Crispr-cas 9 technology describe why these three features are highlighted and why they are essential for the successful targeting *pcks-9*.

GGT (GGN) in the small rectangle is the required PAM sequence, that is the **p**rotospacer **a**djacent **m**otif that is required for Cas9 (with the two nuclease domains) to bind the target DNA. It must immediately follow the target sequence in the large rectangle on the 3' side in the non-target strand of the DNA. gRNA targeting sequence is in the large rectangle and experimentation in this system has shown that 20 nucleotides, in this case, preceding the PAM sequence in the genomic DNA was effective for obtaining the required double strand DNA cutting. The two strands of ds DNA targeted must be separated (see your recitation handout). Remarkably this system does not require a helicase or ATP to effect this strand separation.

3. The first experiments described in the paper determined whether exon 1 or exon 2 would be the target of choice. An agarose gel is shown in Figure 7 describing the results. Describe what you observe in Figure 7 A and B and the how these observations led to the next sets of experiments.

The experiments in A are carried out on the tissue culture cells (in vitro) and the experiments in B are carried out on the mice. The results may or may not be the same if the tissue culture cells are not good models for the animal. (Note, mice not be good models for humans). The results use PCR and agarose gels to determine if the sequence targeted remains intact or if it is degraded. In the tissue culture cells (3T3-L1) one can see in the control mock infection that the sequence remains intact. However in the adjacent two lanes in the experiment targeting exon 1 the sequence has been extensively degraded, while in the experiment targeting exon 2, the sequence remains intact. This type of experiment provide the impetus to focus on gRNA targeting exon 1. gRNA targeting exon 2 would have no effect on exon 1. Similar experiments were carried out with mice. The adenovirus targets the liver and DNA from the liver was examined. A control used GFP. The results from two separate experiments on mice are shown in B. Both samples are extensively degraded, but to different extents. Thus the Crispr-cas9 targeting appears to be working when exon 1 is targeted.

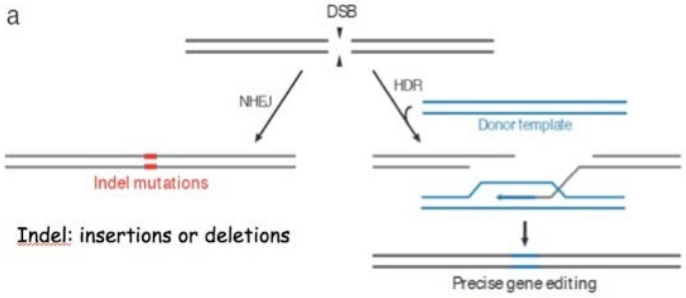
4. On target and off-target DS DNA cleavage is a major concern with the Crispr-cas 9 technology. What experiments using methods similar to those described in Figure 7 were carried out to determine off-target cleavage?

Off target cleavage is apparently still a problem with this technology. They thus sought the 10 sites within the genome most closely matched to the on-target site and most likely to give rise to off target cleavage. They examined as stated in the paper 6 sites with 3 mismatches and some with 4 mismatches. Using the Surveyor assay they found and the method described in Figure 7 they found no evidence of off target cleavage.

5. The Crispr cas9 technology leads to ds DNA cleavage that can be repaired in a number of ways. What are the two general mechanisms of repair? Explain how the data shown in Figure 8 was obtained and what it tells you about which mechanism of repair predominates in this particular set of experiments based on the data shown.

As shown below, taken from your recitation handout, their are two methods for repair of DS breaks. One is non template dependent, non homologous end (NHEJ) repair were the blunt ended cleavage is repaired via insertions or deletions of a few nts, or via frame shifts.

The second method HDR involves template dependent repair and results in insertion of a sequence of interest, among other outcomes. The PCR reactions used in Figure 7 was used to acquire the data in Figure 8. One can see that most of the examples shown reveal deletions of insertions of a few nts and thus the NHEJ repair appears to be occurring.



5.08J Biological Chemistry II

Spring 2016