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REVIEWS

Letter

Knocking Out Enhancers to Enhance Epigenetic Research

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Advances in epigenetics have uncovered a myriad of transcription factor binding sites and chromatin modifications in presumed DNA control elements that regulate gene activation (enhancers) or repression (silencers). Identification of enhancers has relied extensively on the production of animals carrying enhancer reporter transgenes; however, recent studies reporting knockouts of such presumed enhancers have described many cases in which the enhancer is nonessential [1–5]. In one case, knockout of one presumed testis enhancer for *Sox9* had no effect on testis development, whereas knockout of another single testis enhancer reduced expression enough to result in reversal to an ovary fate [4]. In some cases, knockout of two presumed enhancers significantly reduced the expression of a nearby gene and resulted in a physiological defect, thus clearly demonstrating a required function and revealing enhancer redundancy [1–3]. However, two of these studies uncovered examples in which double enhancer knockouts had no effect on gene expression or development; specifically, knockout of two presumed enhancers for *Sox9* in limb bud [2] and two presumed enhancers for *Tbx5* in forelimb bud [5]. It remains to be determined whether the two presumed limb bud enhancers for either *Sox9* or *Tbx5*, or the nonessential testis enhancer for *Sox9*, are redundant with yet more DNA control elements or whether they are nonredundant (i.e., not able to control the nearby gene). Further enhancer knockouts may reveal that some presumed enhancers are indeed vestigial enhancers or pseudoenhancers that are not able to regulate the nearby gene.

Although enhancer reporter transgene technology has been thought to be a good method to identify enhancers that function in specific tissues *in vivo*, the recent knockout studies show that transgene analysis cannot on its own identify enhancers essential for gene regulation. The disconnect is likely to be due to the fact that an enhancer reporter transgene is generated by linking a potential enhancer to a heterologous basal promoter upstream of a marker gene, which is then randomly inserted into the genome of an animal. By doing this, the enhancer is removed from its endogenous location in the genome and placed in a foreign location close to a promoter, instead of being located in a position that is normally far from the promoter of the gene it is proposed to control. These recent studies demonstrate that knockout of the endogenous proposed enhancer in its normal location in the genome is required to validate function.

Genomic studies can identify global changes in transcription factor binding sites and epigenetic marks across the entire genome that are often reported to be responsible for changes in gene expression during development and in the adult. However, now that we know many presumed enhancers are nonessential, it is likely that many transcription factor binding sites and epigenetic marks do not provide true insight into gene regulation as they occur in nonessential DNA control elements. By identifying enhancers that are required to regulate a gene *in vivo*, efforts in epigenetics can be focused on examining the epigenetic changes that occur in these true enhancers. This increase in the signal-to-noise ratio will revolutionize epigenetics.

Recent advances in knockout methodology such as CRISPR/Cas9 make it realistic for journals and funding agencies to require demonstration of functions for presumed enhancers or silencers by *in vivo* genetic loss-of-function studies, thus validating such studies and providing a solid foundation for future studies. Although it is possible to obtain some insight by generating knockouts in cell lines, function *in vivo* is also needed to show how the cell line studies relate to biology *in vivo*. In this way, researchers will be able to make valid conclusions on gene regulation that can be reproduced by others.

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Spotlight

Post-Translational Modification, Phase Separation, and Robust Gene Transcription

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A few recent reports reveal fundamental new insights into the intricate regulatory mechanisms that govern RNA polymerase II (Pol II)-mediated gene transcription. Whereas a histidine-rich domain