COMMENTARY

Enhancing enhancers: new complexities in the retinoid regulation of gene expression

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Retinoic acid is a signalling molecule central to morphogenesis and musculoskeletal development. It can exist in several isomeric forms, of which all-*trans*- and 9-*cis*-retinoic acid are thought to be the most relevant as signalling molecules. Retinoic acid regulates gene expression via RARs (retinoic acid receptors) working as heterodimers with RXRs (retinoid X receptors). RXRs also heterodimerize with other nuclear receptors. In this issue of the *Biochemical Journal*, Harris et al. have shown that an enhancer responsible for chondrocyte-specific expression of the *coll1a2* gene is itself regulated by a retinoic-acid-dependent interaction with RXR β bound to a downstream response element. Thus,

RXRs bound to hormone-response elements can regulate gene expression indirectly via interactions with tissue-specific enhancers. This study raises interesting questions about the nature of the response element, the RXR β partner and the ligands able to influence *coll1a2* expression, and will provide a model system with which to understand tissue and ligand specificity of retinoid responses.

Key words: chondrocyte, hormone response element, retinoic acid receptor, retinoid X receptor, retinoid.

Retinoids, derivatives of vitamin A or retinol, have played an important role in attempts to understand morphogenetic processes in mammalian development for many years. The elegant demonstration of limb duplication in response to retinoic acid [1] is a powerful example of its potential role in morphogenesis. This signalling molecule regulates cell differentiation in diverse cellular contexts, underlying the importance of retinoids for clinical use and as research tools. Despite considerable advances in the molecular biology of retinoids since the nuclear receptors for retinoic acid (RARs; retinoic acid receptors) were first described in 1987, there is much still to be done to understand how retinoic acid as a natural signalling molecule regulates bone and joint development [2]. Therefore, studies that enlighten the roles of retinoids in musculoskeletal biology are particularly significant.

Retinoic acid can exist in several isomeric forms, of which the all-*trans* and 9-*cis* configurations are, on the basis of our current understanding, the most relevant to transcriptional regulation. Of these, the most predominant in biological contexts is all-*trans* retinoic acid, which is a ligand for specific nuclear RARs. According to the current paradigm of retinoid molecular biology, all-*trans*-retinoic acid regulates gene expression by binding to one or more of the three different types of RAR (α , β or γ). These work as heterodimers with similar receptors called RXRs (retinoid X receptors). In this context, RXRs are usually ligand-independent cofactors, and RXR–RAR heterodimers bind to REs (response elements) associated with the promoters of retinoid-responsive genes. Binding occurs with a specific polarity, and the RAR partner, occupying a downstream position, stimulates transcription by interacting in a ligand-dependent way with the transcription pre-initiation complex. This process is accompanied by the dissociation of co-repressors and recruitment of co-activators, facilitating access to the chromatin [3].

There are also three different types of RXR (α , β and γ), but, to make matters more complicated, RXRs are heterodimer partners for a diverse array of ligand-dependent and ligand-independent nuclear receptors or transcription factors, including the well-known thyroid hormone and vitamin D receptors (TRs and VDRs respectively), and the less well-known LXR (liver X receptor)

and NGFI-B (nerve-growth-factor-inducible gene B) families, amongst others. Let us not mention the fact that each RAR and RXR type can have variable N-terminal transcriptional activation domains as a result of differential promoter usage and alternative splicing: understanding the role of these N-terminal variants in differential gene expression remains a challenge for the future.

Although they are ligand-independent cofactors in the context of RARs, RXRs can be ligand-dependent in the context of LXRs and some other receptors. RXRs were so named because they are similar to RARs, but when originally identified the nature of their ligand was unknown. It was shown subsequently that RXRs bind 9-*cis*-retinoic acid with high affinity. 9-*cis*-Retinoic acid also binds to RARs. Although it is a minor isomer, reflecting its lower energetic stability, 9-*cis*-retinoic acid can be generated in cells by isomerization from the all-*trans* isomer [4]. Thus, as a result of isomerization, all-*trans*-retinoic acid applied experimentally has the potential to affect diverse nuclear receptor signalling pathways. The caveat ‘experimentally’ is important here: the ligand and stereoisomer specificities of cellular retinoic acid-binding proteins and the enzyme systems regulating retinoic acid levels in cells as part of developmental processes suggest that signalling by different retinoic acid isomers is carefully regulated, and all-*trans*-retinoic acid may be the main natural ligand in most contexts. The experimental addition to cells of all-*trans*, or other retinoic acid isomers for that matter, may bypass these normal regulatory pathways and generate abnormal concentrations of all retinoic acid isomers. 9-*cis*-Retinoic acid may not be the only ligand for RXRs, and recent studies show that polyunsaturated fatty acids such as arachidonic acid and DHA (docosahexaenoic acid) also bind to and activate RXRs [5].

The retinoic acid RE is just one member of a family of hormone or nuclear receptor response elements in which two repeats of a 6-bp consensus half-site (RGKTCA) are separated by up to 5 bases, with the spacing determining which RXR hetero- (or homo-) dimers bind with high affinity. Thus RXR–RAR heterodimers bind to DR5 REs, RXR–TR heterodimers bind to DR4 REs, RXR–VDR heterodimers bind to DR3 REs, and so on [6]. Other REs not conforming to this rule have been described, and the

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half-site repeats themselves can be rather variable, depending on the context and the heterodimer. Furthermore, bases outside the consensus sequence can also be important in determining high-affinity interactions between DNA and the receptor dimer.

The paper by Harris et al. in this issue of the *Biochemical Journal* [7] reports the characterization of a rather different mechanism of action of retinoic acid in chondrocytes, and, like all the best science, raises interesting questions. The story goes like this: chondrocyte-specific expression of the *coll1a2* gene is driven by two enhancer elements upstream of the promoter, but the more 3' of these (the D/E element) interacts with a retinoid-receptor RE (5A) further downstream, in response to culture with all-*trans*-retinoic acid. Their evidence suggests that a retinoic-acid-dependent interaction between the D/E element and the 5A retinoid-receptor RE increases transcription from the *coll1a2* promoter, and that this is mediated, in part at least, by RXR β . A particularly exciting feature of this study is that it is a novel example of an indirect regulation of transcription by retinoic acid in which the receptor–DNA complex does not interact directly with the transcription pre-initiation complex, but enhances the activity of a more-upstream enhancer. The retinoic acid dependency of *coll1a2* gene expression will thus depend on the presence of factors bound to the D/E enhancer. This, in turn, may depend on the differentiation status of the chondrocytes, or even their anatomical site of origin, and help to explain the diversity of chondrocyte responses to retinoic acid. In addition, since RXRs are heterodimers partners for other hormone- or ligand-dependent nuclear receptors, the availability of RXRs will be affected by the activity of these other receptors. Careful study of enhancer interactions in this and other experimental systems may bring other examples to light where retinoid-dependent transcriptional responses rely on interactions with transcriptional regulators bound at physically separate locations.

This study also raises interesting questions concerning the identity of the nuclear receptors bound to the retinoid response element and the way in which transcription is stimulated by interactions with proteins bound to the D/E element. Apart from the identity of proteins binding to the D/E enhancer, three key attributes to discover are the nature of the retinoic acid ligand, the nature of the response element, and the identity of the RXR β dimerization partner. Harris et al. [7] have excluded RARs as heterodimerization partners using supershift experiments. If this is correct, and the RXR β heterodimerization partner is not an as-yet-unidentified receptor for all-*trans*-retinoic acid, then the retinoic acid ligand doing the stimulation should be 9-*cis*-retinoic acid generated by isomerization during the experiments. This idea can be tested using an RXR-specific ligand, or rexinoid [8]. The converse experiment, using an RAR-restricted ligand such as TTNPB {4-[E-2-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl]1-propenyl benzoic acid} [9], would allow a test of the hypothesis that all-*trans*-retinoic acid is not involved. These would be interesting experiments to do. If it turns out to be true that the retinoic acid dependency of the D/E and 5A enhancer interaction is mediated exclusively through RXR β , this raises other interesting questions concerning the ability of other RXR ligands to achieve the same effect. If ligands such as arachidonic acid and DHA can enhance the activity of the D/E enhancer, then compounds other than retinoids may have an important role in regulating chondrocyte gene expression.

Identifying the RXR β dimerization partner and the nature of the response element are two important and closely linked questions. Therefore, although the RE described by Harris et al. [7] is an

exact direct repeat with a 4-bp spacing (DR4), it may be worth keeping an open mind on the orientation and spacing of the RE. As the authors imply, the 5' $\rightarrow$ 3' sequence on the antisense strand is a close match to the RE half-site consensus sequence, and if we allow for some variation between half-sites, an inexact repeat with a 3 bp spacing (i.e. a DR3) element in the antisense orientation would also be possible. DR3 elements are response elements for VDR–RXR heterodimers, and since vitamin D is involved in bone and cartilage development, this is an obvious candidate for elimination. LXR β , which is expressed more widely than LXR α and would bind to a DR4 RE as a heterodimer with RXR β , is another possibility [10]. These and other ideas could be explored using ligands for potential RXR β partners, and complemented by two-hybrid screens using chondrocyte cDNA and RXR β as bait.

RXR heterodimers occupy hormone-response elements in a specific polarity, often with the RXR partner occupying the 5' half-site. In this position, the RXR partner facilitates activation by the downstream partner of the transcriptional pre-initiation complex, either by acting as a ligand-dependent (such as in the context of RXR–RAR heterodimers) or ligand-independent (as with RXR–LXR heterodimers) cofactor. Therefore, since the 5A enhancer is interacting with an upstream (more-5') element, a reversed polarity with respect to conventional hormone-response elements and the direction of transcription may be important. It would be interesting to discover whether or not the interaction between the 5A RE and the D/E enhancer is independent of orientation of the 5A RE.

Clearly, there are exciting experiments to follow on from this work. RXR β itself is a rather enigmatic receptor with patterns of alternative splicing and intron retention of uncertain functional significance [11]. Having a well-defined gene expression model in which RXR β is a major player will help resolve these issues. Harris et al. [7] have developed an experimental model which will not only increase our understanding of chondrocyte gene expression, but which has the potential to make fundamental contributions to our understanding of gene regulation by retinoids.

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