A transcriptional basis for physiology

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Hormone
A substance, usually a peptide or steroid, produced by one tissue and conveyed by the bloodstream to another to effect physiological activity, such as growth or metabolism.
–The American Heritage Dictionary

In 1914, Kendall isolated thyroid hormone, the first lipophilic hormone, from more than 3 tons of porcine thyroid glands. In 1915, Osborne and Mendel, and McCollum and Davis, purified the first dietary 'vital factor' (vitamin A) from cod liver oil, butter and egg yolk. A few years later, Butenandt and Doisy discovered the female sex hormone estrogen, and Kendall and Reichstein isolated cortisol as the first adrenal steroid. Seventy years later, these powerful but seemingly independent strands of biology would be united by the unexpected kinship of their cognate receptors, which in turn would become the founding members of the nuclear receptor superfamily.

My graduate studies began in 1970 at UCLA in the laboratory of Marcel Baluda, a former postdoc with Renato Dulbecco and a benchmate of Howard Temin. Although I was soon in the thick of the exciting discoveries on reverse transcriptase, we never caught up with Temin and Baltimore, who went on to win a Nobel Prize for their work. I learned early on the breathtaking pace at which a field could progress, and that you either follow your instincts and work hard, or you get out of the way.

I subsequently joined the Darnell lab at Rockefeller University, whose members were focusing on RNA synthesis and processing, topics that are at the core of gene control. Most of the researchers there were studying the generation of the 3′-polyadenylated transcripts. In contrast, I focused on the 5′ end of the gene, with the presumption that a transcription promoter was a physical entity that could be mapped and subjected to molecular dissection. Ed Ziff and I characterized the adenovirus major late promoter, the first polymerase II promoter to be functionally identified and sequenced[1,2]. Cloning of cellular mRNAs was on the horizon, but fears about public safety led to the technique being subjected to a yearlong moratorium. During this time, I resolved to completely drop all adenovirus work in favor of a cellular model. After months of angst, I settled on the rat growth hormone, the secretion of which is inducible by glucocorticoids and thyroid hormone. Evidence that hormone secretion was reflective of transcriptional control was sketchy and mostly relied on pioneering studies by Gustafsson, Jensen, Yamamoto and O'Malley. Michael Harpold joined me on this project, and one midnight late in March 1977 we made our first cDNA library. Of the 20 clones that emerged on that memorable night, only one was growth hormone, but one was all we needed[3].

A year later I accepted a faculty position at the Salk Institute and trundled off to continue my studies on hormonal regulation (**Fig. 1**). In establishing the lab, the first order of business was to isolate the entire growth hormone gene, identify the promoter and establish assays to characterize its regulatory sequences. To the extent that the character of a lab is a reflection of its people, I was very lucky. My first technician, Lita Ong, was tireless and to this day sets high standards of work, dedication and loyalty. My first student Marcia Barinaga accepted the challenge to map the growth hormone promoter with naive enthusiasm[4]. We then joined forces with Geoff Rosenfeld, initially to clone prolactin mRNA, but his sabbatical with me soon evolved into a highly productive collaboration and a lifelong friendship.

In the 1981 renewal of my National Institutes of Health grant, I proposed to clone the glucocorticoid and thyroid hormone receptors. Because no known DNA-binding transcription factor had yet been cloned, this was a risky venture. A tireless graduate student, Stan Hollenberg, and a skittish but adventuresome postdoctoral fellow, Cary Weinberger, accepted this gauntlet. It was a terrific and lively group. We produced a complicated set of phage expression libraries, the lysates of which produced protein fragments that could be screened with receptor antibodies obtained from our collaborators, John Cidlowski and Brad Thompson. We screened, rescreened and rescreened again, but despite heroic efforts the strategy did not seem to be working. Then, in a screen of a new million-clone library from human lymphoid IM-9 cell RNA, one film showed a tiny spot that Cary insisted was the real deal. He was right, and that one-in-a-million clone yielded the glucocorticoid receptor (GR)[5]. We knew that other groups were attempting the isolation of the progesterone (O'Malley), estrogen (Chambon and Greene) and glucocorticoid (Yamamoto and Gustafsson) receptor cDNAs; the pressure on our small group was intense.

While the arduous task of sequencing our clone was underway, I went off to a Gordon Conference. At the meeting I received a call from Stan, who said that our collaborator, Russ Doolittle, had found homology between the GR and the v-erb-a oncogene. Astonished, I returned home immediately to share the excitement and begin writing the articles[6,7]. The discovery that the oncogene v-erb-a was a candidate transcription factor derived from a hormone receptor set off a chain reaction that is still being felt today. Clearly, the result implied the existence of a gene family. The feeling in the lab was like that of falling into the rabbit hole toward Wonderland. Magical excitement was in the air.

The cloning of the first nuclear receptor was important not so much for how it was achieved but for what it revealed. The first complete information on the protein sequence of a steroid hormone receptor subsequently allowed the characterization of the functional domains of these receptor

Figure 1 Ron Evans (1982).

proteins and the emergence of the shared features. At this time, however, virtually every step in the signal transduction process was clouded in either mystery or controversy. Furthermore, the technical approach to functional characterization was uncertain, because no transcription factor had previously been cloned. Would a single cloned polypeptide be sufficient to faithfully reconstitute glucocorticoid responsiveness in a recipient cell? After seemingly endless animated discussion with Vincent Giguere, a new postdoc in the lab, we hit upon the idea of transiently introducing a hormone-responsive reporter together with a receptor expression vector to see what would happen. It worked on the first try (I love it when that happens!). Thus, the cotransfection assay was born[8], and it would become the dominant assay for dissecting receptor function (**Fig. 2**). It was blisteringly fast, quantitative, cheap and adaptable to high-throughput formats. As such, it also became the standard way for pharmaceutical companies to screen for new ligands. Indeed, beyond nuclear receptors, the cotransfection assay would become the prototypical approach to characterizing any transcription factor.

We were in the rabbit hole but not yet through the door. During this time, Cary set out to clone and characterize c-erb-a, the cellular counterpart to v-erb-a. Pierre Chambon had just described the isolation of the estrogen receptor (ER), and it became clear that the ER and GR were more similar to each other than to v-erb-a. This suggested that, if a ligand existed, it might not be steroidal. At our weekly data club and in front of a skeptical crowd, Cary boldly proposed that the c-erb-a ligand was thyroid hormone. He was right, but it would take several months to devise the assays to prove it. In the meantime, Cary replaced me as a speaker at a European symposium on transcription. Bjorn Vennstrom, who originally isolated the v-erb-a gene, asked Cary about the c-erb-a identity, and Cary revealed our speculation. Vennstrom, who had apparently already proven this conjecture, was taken aback but nonetheless affirmed that our idea was correct. Vennstrom's brilliant insight and generosity in sharing an exciting result led to back-to-back publications identifying the chicken and human c-erb-a gene products as the thyroid hormone receptor[9,10]. We now stood before the door to Wonderland, but we still needed the key.

At this point our thinking changed. The recognition that steroid and thyroid hormone receptors share an ancient progenitor suggested to us that physiological homeostasis may be the manifestation of an interrelated genetic system sharing a common origin and therefore a common molecular logic. This concept had not been formally raised in the literature. As my colleague Geoff Wahl remarked to Cary, "In my lab all those faint hybridization bands would be background, but in your lab they probably represent a gene family." Indeed this became our assumption. Because this seemed to be a family of shared homology, we reasoned that it would be a family of shared physiology and function as well. What might be the nature of this family, and what secrets might it reveal about physiology or other life processes? As it turns out, this change in our thinking was the key. Looking at reality from a different perspective changes the reality. We were now in Wonderland.

During this transformation, I attended a symposium in Paris where Anne De Jean presented a soon-to-be-published observation that a piece of DNA from a liver cancer virus integrated into a chromosomal fragment with homology to the newly described steroid receptors. I called Vincent with the news, thinking that this might be a new receptor. A magician when it came to molecular biology, Vinnie already had under his hat what would turn out to be the first orphan receptors[11], and he now went fishing for this new one. Functional mapping studies from our lab and Chambon's indicated that the DNA- and ligand-binding domains could function independently[12,13], a concept that was berated publicly by a respected receptor biochemist who emphatically stated, "This simply is not how proteins work!" Nonetheless, the DNA-binding domain of the GR could seamlessly be substituted into other receptors, allowing us to create a powerful new assay to test ligand responsiveness of orphan receptors. The first orphan rabbit pulled out of Vinnie's hat turned out to be RAR, the receptor for retinoic acid, the active metabolite of vitamin A. From butter, egg yolk and fish oil in 1915, the first 'vital factor' had now found a home among the growing family of nuclear hormone receptors. Vinnie was so excited that we had to peel him off the ceiling. Unbeknownst to us, champagne corks were popping in Strasbourg as well, where Le Grand Fromage (Chambon) was

savoring the same discovery. Within a few weeks both articles would be published in consecutive issues of *Nature*[14,15]. Thus, within 3 short years, the pillars of a nuclear receptor superfamily were in place.

Curiouser and curiouser

There were many more rabbits yet to be pulled from the hat. As the eye of the Hubble telescope peers into seeming darkness to reach back in time, David Mangelsdorf, who had just arrived, focused on the faintest hybridization signals, and like the Hubble, brought a new galaxy of receptors into view. Receptors isolated in absence of a known ligand were termed 'orphans'. Classic endocrinology, such as that pioneered by Jensen, used labeled ligands to find the receptor. The discovery of orphan receptors raised the strong possibility that orphan ligands could also exist, and led to a strategy to use the receptor to guide the purification of the cryptic ligand. We dubbed this strategy 'reverse endocrinology'. David (Davo Mango as we called him) was an enthusiast with a zeal for discovery. His discovery of the retinoid X receptor (RXR)[16] and liver X receptor (LXR) would result in two paradigm shifts with far-reaching implications. The RXR project gained force when a lipid extract from out-of-date human blood showed weak activity. This was followed by a challenge using a series of ligand 'cocktails', one of which (containing retinoids) was weakly positive. After a year-long hunt, Davo and his benchmate Rich Heyman (and collaborators Gregor Eichele and Christina Thaller) identified 'retinoid X' as 9-*cis*-retinoic acid[17], the first new hormonal lipid discovered since aldosterone by Reichstein in 1952. Since then, 9-*cis*-retinoic acid has become an FDA-approved drug (Panretin) for cancer treatment. This result proved the orphan ligand hypothesis and, perhaps more importantly, provided the entrée to a new era in metabolic regulation.

A quietly ignored conceptual problem with orphan receptors was their divergent DNA-binding domains, which, annoyingly, had to be swapped and, more importantly, precluded target gene identification. The prevailing view was that each orphan must recognize a unique binding site. This idea proved to be wrong, and it was Kaz Umesono, a brilliant and creative force, who would soon crack the DNA-binding problem while Mango and others concocted elixirs of ligands. Kaz came to realize that a signature motif in virtually all nonsteroid receptors directed DNA recognition to the same nucleotide sequence, AGGTCA[18]. Kaz understood well before the rest of us that his work created a paradox, because receptors for thyroid hormone, retinoic acid, vitamin D and the numerous orphans could not possibly activate the same genes. From where does the specificity derive? The answer was in the nearby dimerization motif, which permitted each receptor to bind to the same sequence as a tandem repeat separated by a specific number of nucleotides[19]. This remarkable idea led to the spacing paradigm for hormone response elements initially termed the '3-4-5 rule'. Suddenly it was clear: a direct repeat of AGGTCA spaced by three nucleotides (a DR-3) was a vitamin D response element, a DR-4 was for thyroid hormone and a DR-5 was for retinoic acid[20]. Soon, every position on the spacing ladder would be assigned, and target genes began to percolate out of the genome. The last piece of the puzzle was identifying the competence factor that Kaz (along with Chris Glass and others) had shown was required for receptors of the nonsteroid class to bind DNA. This surprise was elegantly resolved by Steve Kliewer in my lab and by others in the laboratories of Chambon, Rosenfeld, Ozato and Pfahl, all of whom found that the associated factor was RXR[21–25]. Graduate students Tony Oro and Pang Yao went on to clone the *Drosophila* RXR homolog and show that heterodimerization through RXR was evolutionarily conserved from fly to human[26,27]. Thus, the concept of the nuclear receptor heterodimer, two molecules with one shared function, was born and would have profound impact on hormone signaling.

Like Alice after taking a bite of shortbread cake, the field underwent explosive development. A new generation of molecular endocrinologist was being born. Crystal structures of the first receptor DNA- and ligand-binding domains were being solved, receptor knockouts and transgenic mouse lines were being constructed, and the last of the orphan receptors were being teased out of the genome. While most laboratories wisely focused on one or a few receptors, our strategy of 'unity from diversity' was in peril.

Two developments would help unify the field and set new directions. The first was the recognition that receptors activate or repress transcription through the recruitment of common cofactors, which mostly function as chromatin-modifying enzymes. That is, the receptors do in fact operate through a common mechanism. The second was the realization that each RXR partner embodied a new physiological pathway (**Fig. 3**). That LXR was the long-sought oxysterol receptor regulating cholesterol metabolism was demonstrated by Mangelsdorf[28]. My postdoc Barry Forman, along with Peter Tontonoz and Bruce Spiegelman, and independently Jurgen Lehmann and Steve Kliewer[29–31], showed that the TZD class of antidiabetic drugs activated peroxisome proliferator-activated

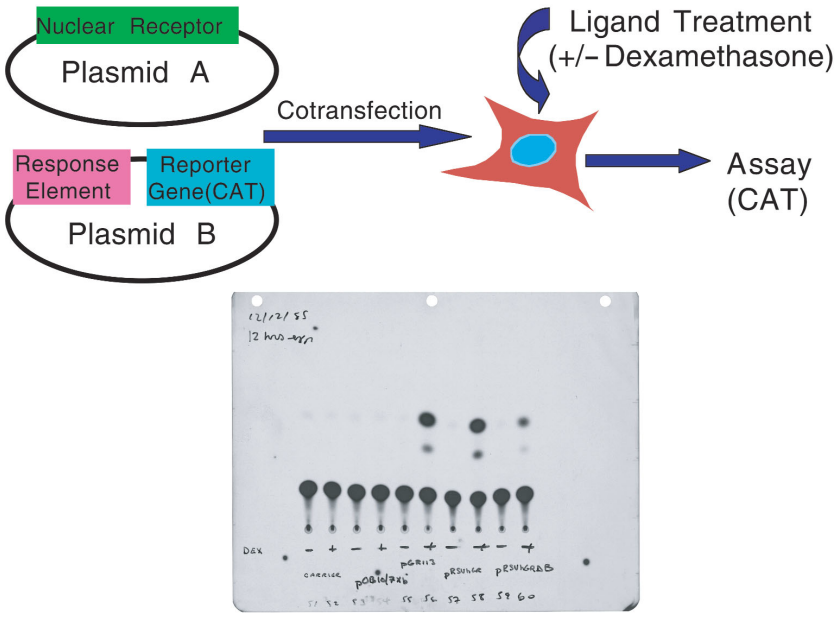

Figure 2 Schematic of the first cotransfection assay and results of the initial experiment. The autoradiogram depicts the activation of the CAT reporter gene in the presence of dexamethasone and expression vectors (pGR113, pRSVhGR) for the human GR[8]. Lanes 5 and 6 show the hormone-mediated induction.

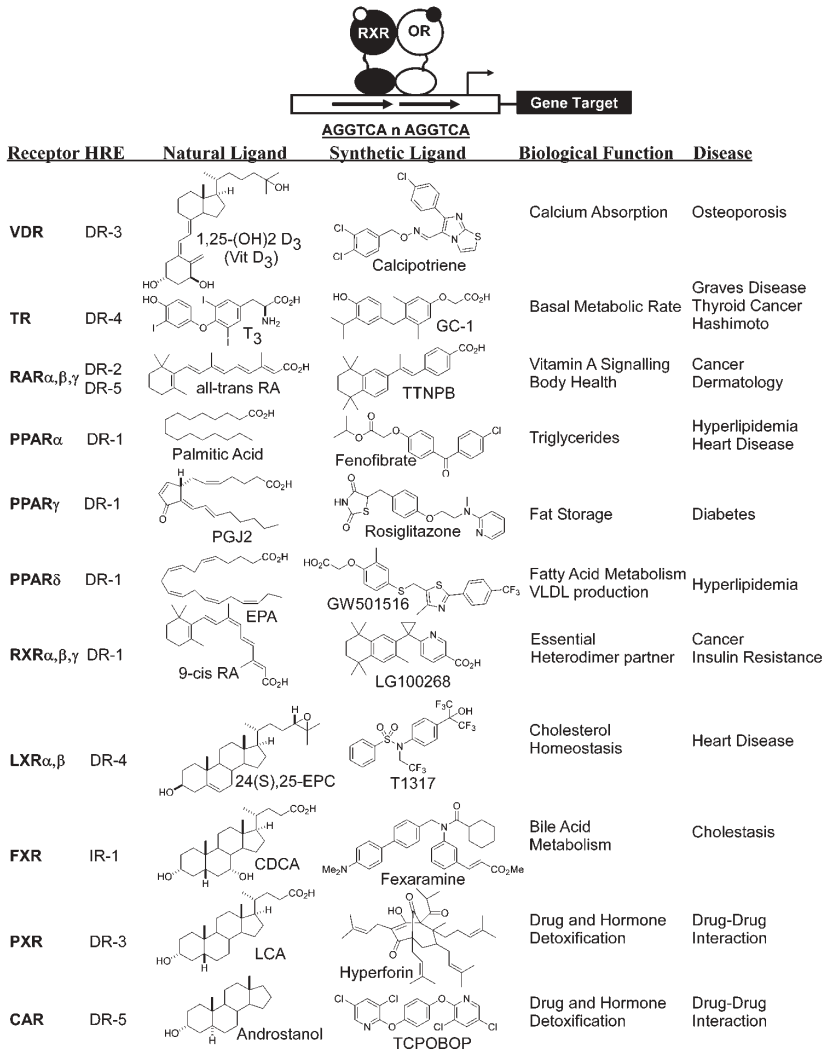

Figure 3 RXR heterodimeric partners and ligands. HRE, hormone response elements; OR, orphan receptor; DR, direct repeat; IR, inverted repeat; T3, 3,5,3′-triiodo-L-thyronine; PGJ2, prostaglandin J2; EPA, eicosapentanoic acid; 24(*S*),25-EPC, 24(*S*),25-epoxycholesterol; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; TTNPB ([(*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthylenyl)-1-propenyl]benzoic acid]; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene.

receptor γ (PPARγ). FXR was identified as the bile acid receptor by Mangelsdorf, Kliewer and Forman[32,33]. Finally, Moore, Kliewer and Bruce Blumberg in our lab characterized the roles of the xenobiotic receptors, PXR and CAR[34–36], thereby completing the RXR family. The common thread is that each of these receptors functions as a sensor for dietary lipids or for xenobiotics or drugs and, in so doing, acts as a potent regulator to control the storage, trafficking and metabolism of cholesterol and fats, and mediate the clearance of their metabolic side products.

Conclusions and perspectives

The discovery of the first hormones 100 years ago presaged the development of the burgeoning fields of endocrinology and physiological homeostasis as disciplines to decipher the most important aspects of reproduction, nutrition, metabolism and disease. However, because of the inherent limitation of technology, whole branches of endocrine and lipid signaling were missed. Through the discovery of orphan receptors, a 'hidden' wonderland of physiology has been unveiled. On a separate canvas, elegant pictures were being painted of development and physiological processes, including human disease, as the manifestations of genetic traits, the elaboration of which is dependent on gene control. To a great extent the discovery of the nuclear receptor superfamily showed that physiology and transcription are part of the same canvas. After nearly a century, these two parallel strands of biology have merged to empower transcription as an underlying regulator of health and disease and to help us rethink physiology as the most exquisite readout and window into the dynamic activity of the genome.

As mentioned earlier, studies over the past 20 years have not only identified a superfamily but unequivocally shown that each branch operates through a shared molecular logic. At another level, coordination among cholesterol, bile acid and fatty acid metabolism suggests a higher level of physiological control. The broader links to carbohydrate metabolism, inflammation, reproduction and nutritional status hint at the possibility that the entire superfamily is entwined by a megaregulatory principle that has yet to be described, which manifests itself as physiological homeostasis.

It is probable that the ramification of our work and that of our colleagues will continue to pull together medicine, pharmacology and cell biology into yet another synthesis. Prudence precludes speculating how deep into our core biology this might lead, but the adventure in Wonderland will clearly continue.

Ode to Wonderland

Where went the hormone estrogen
that Elwood sought to trace?
The nucleus was where it hid,
a most unlikely place.

And mightn't it act upon the genes?
a molecular Director
The curiouser of curious paths;
t'was a nuclear receptor.

In tandem with Le Grand Fromage
in search of mother lodes…
we riddled out the means by which
the DNA decodes.

The time has come, the lipids screamed
to cease your mad conniption
a superfamily was revealed
that modulates transcription.

The rabbit hole through which we fell
put us through our paces
to puzzle out the secret of
physiologic homeostasis.

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