Eukaryotic Gene Expression: Basic and Benefits

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Lecture No. # 22

Regulation of Gene Expression by Type II Nuclear Receptors

Welcome to this lecture series on eukaryotic gene expression: basics and benefits. And, we have been discussing so far about transcription regulation, how it takes place in eukaryotic cells, various general transcription factors and what kind of promote elements they recognize, how RNA polymerase in response along with general transcription factors, binds and interacts with number of upstream transcription factors and regulates transcription. And after looking at various events that actually takes place inside a nucleus, we started looking at how molecules by interacting either with cell surface receptors or with intra-nuclear receptors are going to influence gene expression. So, we had a series of lectures on discussing how molecules which interact with membrane receptors transtitute signals inside the nucleus ultimately leading to the activation of transcription.

And, in the last class, we discussed about intracellular molecules – molecules which diffused through a cell membrane bind to intracellular receptors and then this ligand receptor complex goes inside the nucleus and activates transcription of various genes. As an example, we started discussing about steroid hormone receptors. And, in the last class, we discussed about hormones like glucocorticoid hormone, mineralocorticoids, estrogens progesterone, androgens and so on; how these molecules diffused through the cell membrane bind to specific intracellular receptors, which are present in the cytoplasm. And, once the hormone binds to this cytoplasmic receptor, it undergoes a conformational change. And then, their hormone receptor complex goes inside the nucleus, binds to specific response elements known as hormone response elements and activates transcription of target genes. So, what we will discuss today is to discuss another group of receptors known as the type II receptors. And first, we will start discussing about how this type II receptors are different from the type I receptors (( )) hormone receptors and

how these receptors were discovered and what kind of response elements these receptors bind and so on and so forth.

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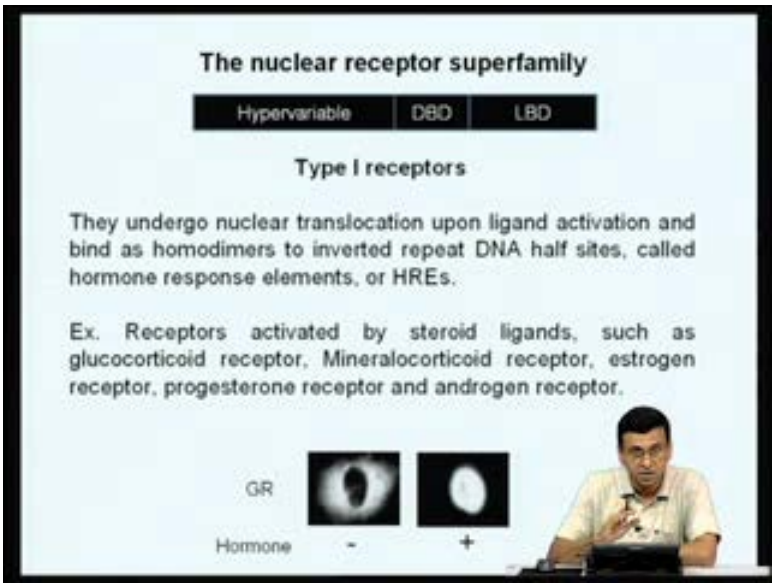

So, let us start discussing about the regulation of gene expression by type II nuclear receptors. As I said, in the last class, we discussed about one of the members of the nucleus receptor superfamily namely, the type I receptors. The type I receptors are characterized by the presence of a domain structure. There are three major functional domains: a hypervariable amino terminal domain, a DNA binding domain or the DBD, which consists of two zinc fingers, and the ligand binding domain, which actually binds a specific ligand. So, in the case of glucocorticoid receptor, the DNA binding domains specifically recognizes the glucocorticoid response elements in the promoters of various target genes; whereas, in the case of estrogen receptor, the DNA binding domain will recognize the estrogen response elements of various target genes.

In the same way, the ligand binding domain of glucocorticoid receptor will bind to glucocorticoid hormone; whereas, the ligand binding domain of an estrogen receptor bind to an estrogen hormone. So, by differential bind DNA binding as well as differential ligand binding, these groups of receptors are able to manifest their physiological reserves by binding to specific response elements and activating transcription response to specific hormones. So, the type I receptors – one of the major characteristic features of the type I receptors of the steroid hormone receptors are: they undergo nuclear translocation upon

ligand activation and bind as homodimers to inverted repeat DNA half-sites called hormone response elements or HREs. So, the major distinction between this type I receptors and the receptor that we are going to discuss now is that the steroid hormone receptors are primarily present in the cytoplasm; and, when the hormone like glucocorticoid hormone estrogen binds, then it undergoes a conformational change; and, the ligand bound receptor then translocates the nucleus and binds to specific hormone response elements, which primarily consist of inverted repeats. For example, in the case of glucocorticoid receptors, it is AGIACATGTTCT. So, it will read hope the same on opposite strands.

The examples that we have discussed under this category are receptors activated by steroid ligands, such as glucocorticoid receptor, mineralocorticoid receptor, estrogen receptor, progesterone receptor and androgen receptor. This just shows what is called as immunophorsons picture, where you try to localize where the receptor is present in the absence or presence of a hormone; and, which you can see here, if you use a antibody, which is fluorescently tagged to a secondary antibody, and then you look at the hormone receptor both in the absence or presence of a ligand; and, you can see in the case of glucocorticoid receptor, when you do such immunophorison studies, you can see the glucocorticoid receptor is primarily present in the cytoplasm when there is no hormone. The moment you add the hormone, the receptor translocates the nucleus and you can see, due to no longer seen the receptor in the cytoplasm, all the receptors mold in the nucleus. So, these are one of the hallmark characteristics of type 1 receptors; that means, they bind as homodimers to those palindromic sequences. They are normally present in the cytoplasm and then translocate a nucleus when ligand is present.

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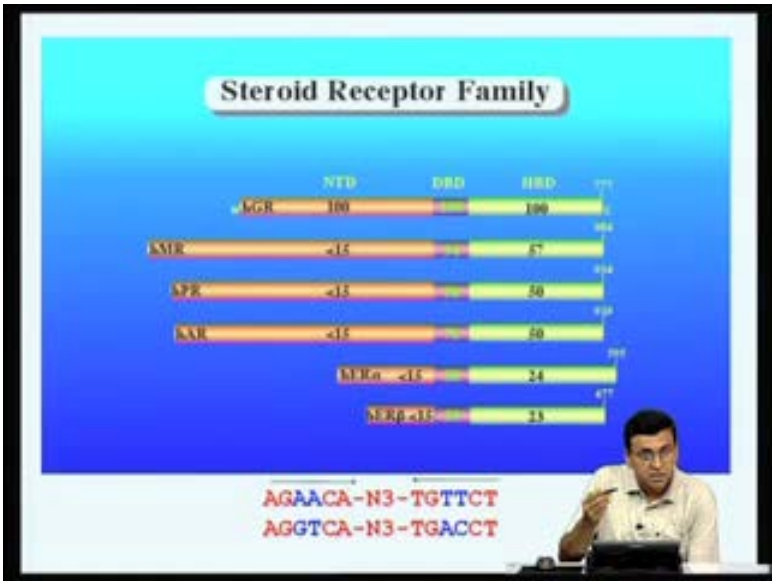

Some of the members of the steroid receptor family that we discussed about are the glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, androgen receptor and estrogen receptor. There are different isoforms of these receptors: alpha, beta and so on either because of differential spoising and so on and so forth. This auto also tells you that the DNA binding domain of all these receptors contain two zinc fingers and you can see there is very high degree of homology in the DNA binding domain of (( )) receptors; whereas, the ligand binding domain, the homologies not that much, because they bind different ligands. But, they all share certain common characteristic feature and these members that they constitute together, the steroid receptor superfamily or steroid receptor family, which again comes under the nuclear receptor superfamily.

And, I also discussed the glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, androgen receptor; they all recognize a sequence called AGAACA-three nucleoid spacing-TGTTCT. It will read the same way the other opposite strand AGAACA-three nucleoid spacing-TGTTCT. So, they are called as palindromes or inverted repeats. In the case of estrogen receptor, there will be two bases, which distinguishes an estrogen response element from glucocorticoid response element. And, in case of estrogen response element, instead of AGAACA, you have AGGTCA; that is the only difference.

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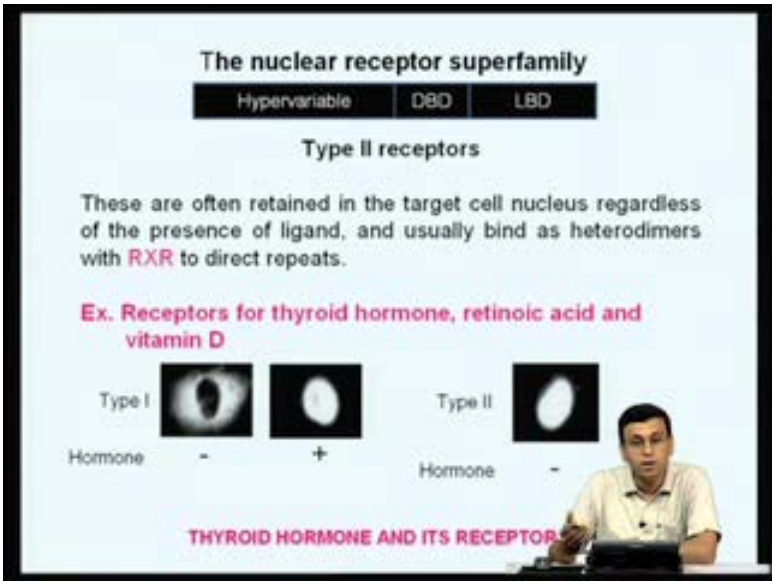

Today, let us not start discussing about what are these type II receptors. The type II receptors also have the same domain structure as the type I receptors by the fact that they also contain a hypervariable amino terminal domain; they also contain a DNA binding domain, consists of two zinc fingers; and, they also contain a ligand binding domain to which certain non-steroid hormones go and bind. Certain vitamins and non-steroid hormones bind to the ligand binding domain. But, the important characteristic features, which distinguish a type II receptors from type I receptors are: these receptors are often present in the target cell nucleus regardless of the presence of a ligand. So, the ligand is there or not, the receptor is already present in the nucleus. And usually, they bind as heterodimers with another important receptor called as retinoid X receptor or RXR and two sequences, which are direct repeats.

So, there are three important points, which distinguish the type II receptors from type I receptors or the steroid receptors. Number 1 – they are already present in the nucleus even if there is no hormone. Number 2 – the steroid receptors bind to DNA as homodimers, but these type II receptors usually bind as heterodimers; and, the heterodimeric partner for all these receptors is a receptor called as retinoid X receptor or RXR. We will discuss in detail what exactly is this receptor. And, the third important difference is that while the steroid hormones receptors bind to palindromic sequence or inverted repeats, these type II receptors bind to direct repeat motifs.

Some of the examples or classic examples of type II receptors are receptors for thyroid hormone, retinoic acid and vitamin D. So, if you now do an immunophoresis just as the way I showed in the last slide, (Refer Slide Time: 08:10) in the case of steroid hormones, in the absence of hormone, you do not say the receptor is in this hydrogen nucleus; they are all present in cytoplasm. When you add the hormone like glucocorticoid hormone, the glucocorticoidal receptor now transfer from the nucleus; whereas, in the case of type II receptors like retinoic acid receptor or thyroid hormone receptor or vitamin receptor, even in the absence of hormone, you can localize the receptor in the nucleus. So, these are the three major distinguishing features that discriminates the type II receptors from the type I receptors.

Now, let us begin by discussing the type II receptors by taking a thyroid hormone receptor as an example and see what let to the discovery of these type II receptors and how the knowledge of steroid hormone receptors actually help to understand some of the major functions of these type II receptors. So, the thyroid hormone receptor – what is thyroid hormone and what is its receptor and how was it discovered and what kind of response elements this thyroid hormone receptor goes and binds?

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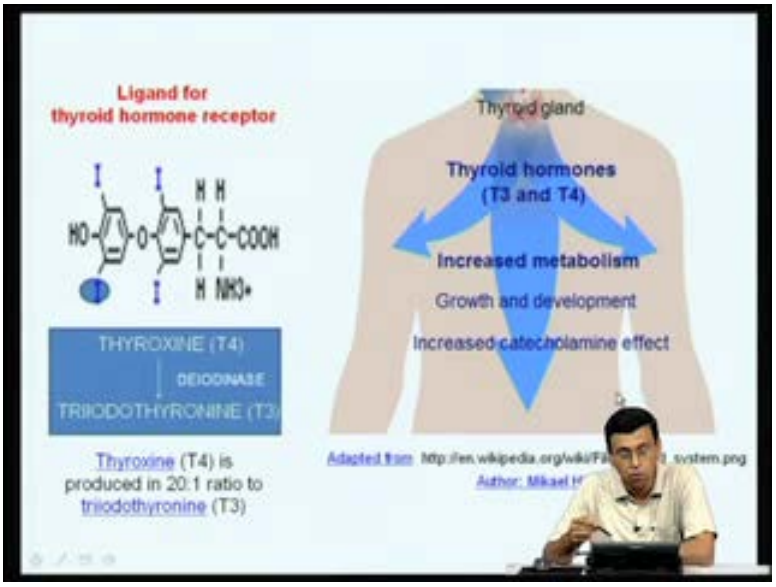

The thyroid hormone or thyroxin is actually synthesized from tyrosine. So, tyrosine is a precursor of a number of hormones like epinephrine or thyroxin and so on and so forth. Thyroxin basically contains is nothing but, is also known as T4 contains four iodine

molecule atoms. And, in the body, the thyroxin exists, say that as T4 or triiodothyronine. If you remove one atom of iodine from T4 by enzymes known as deiodinases, the T4 gets convert into T3. And usually, the T4 and T3 are present in the ratio of 20 is to 1 inside the cells. So, both coexist, but the T3 is at least three times more potent than the T4. So, today, when I am going to talk about what are the physiological effects of thyroid hormone, the thyroid hormones, which are synthesized by the thyroid gland and the hormone by the thyroid gland performs a number of important functions. Some of which are listed here. They play a very important role in metabolic regulation. They increase metabolic rate. They are involved in growth and development. They have increased catecholamine effects. There are number of other physiological effects thyroid hormones. But, these are some of the major effects. All these effects of thyroid hormones are probably mediated by the thyroid hormone receptors. And, let us now discuss and see what these thyroid hormone receptors are and how they were restored. So, the ligand for thyroid hormone receptor is thyroxin – either T3 or T4.

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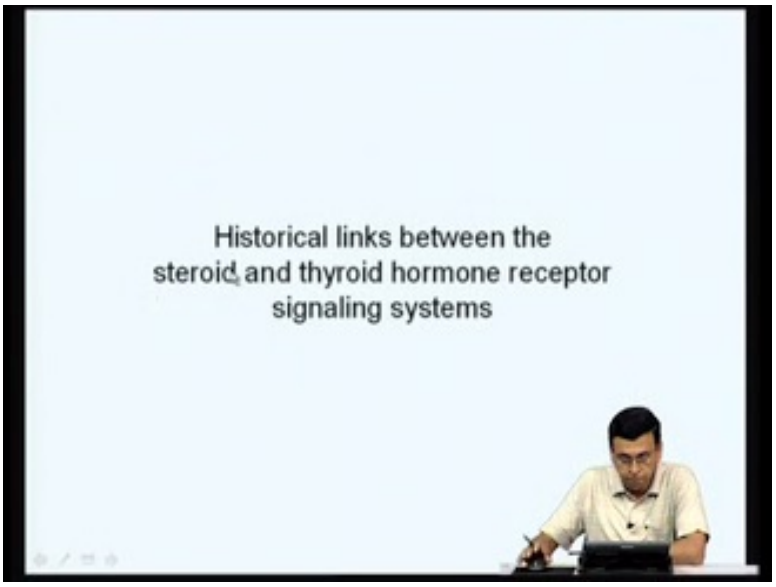

So, let us now start looking at what are the historical links between the type I receptors or the steroid hormone receptors and thyroid hormone receptors, because it is the knowledge that we gain from the steroid hormone receptor that led to the understanding of the thyroid hormone receptors of the type II receptors.

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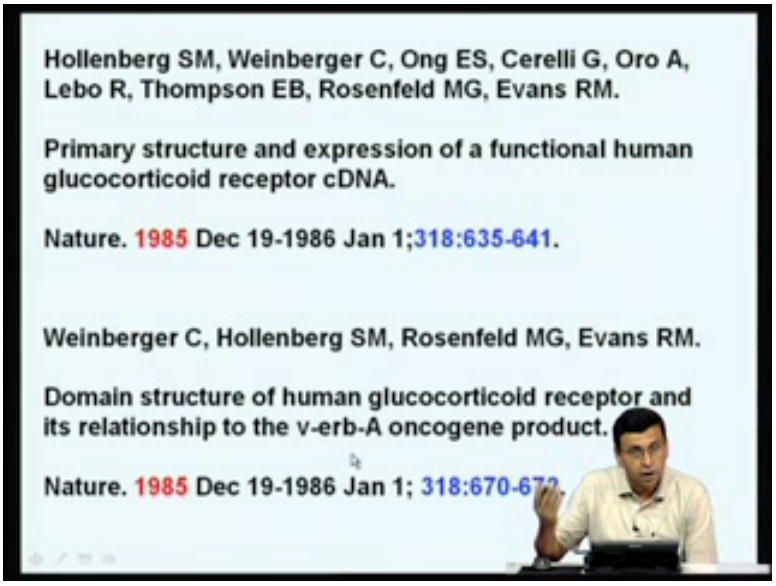

I had mentioned in the previous class, how with the advent of molecular biology in the early 80s the advent of cloning techniques, the advent of genomic libraries and cDNA libraries and ability to screen these libraries using antibody-specific of proteins or using oligonucleotides, which are designed based on amino acid sequences really helped to clone the genes coding for the various steroid hormone receptors. And, I also mentioned that one of the first hormone receptors, which were cloned is the glucocorticoid receptor, which was cloned in the Ron Evans' lab and was published in nature 1985. And, for today's topic, what is most important is that in the same year, and in fact, the same issue of nature, the same group also demonstrated that if you look at the domain structure of the glucocorticoid receptor, which contains a amino terminal domain, a DNA binding domain and the ligand binding domain, it was very similar to a oncogene product known as v-erb-A. For today's topic, this is going to be very relevant. Now, what is this v-erb-A and why this paper became very important? That is, the demonstration that the structure of glucocorticoid receptor is very similar to an oncogene product coded by an oncovirus like erythroblastosis virus.

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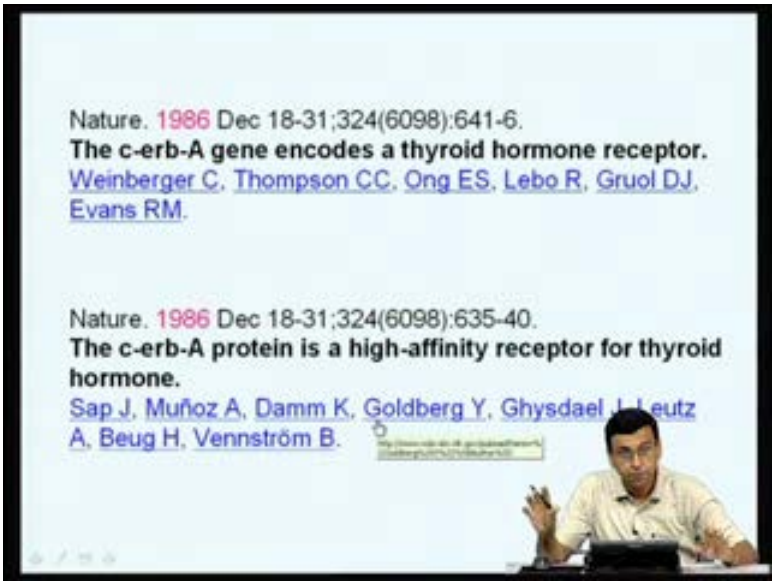

In the following year, again, two groups, from Ron Evans' lab and from Vennstrom's lab, actually demonstrated the cellular homolog or the proto oncogene of this c-erb-A, is nothing but thyroid hormone receptor. The c-erb-A gene encodes a thyroid hormone receptor; the c-erb-A protein is a high-affinity receptor for thyroid hormone. These two are the classic papers, which actually linked the thyroid hormone receptor on one hand to the steroid receptors on another hands to cancer.

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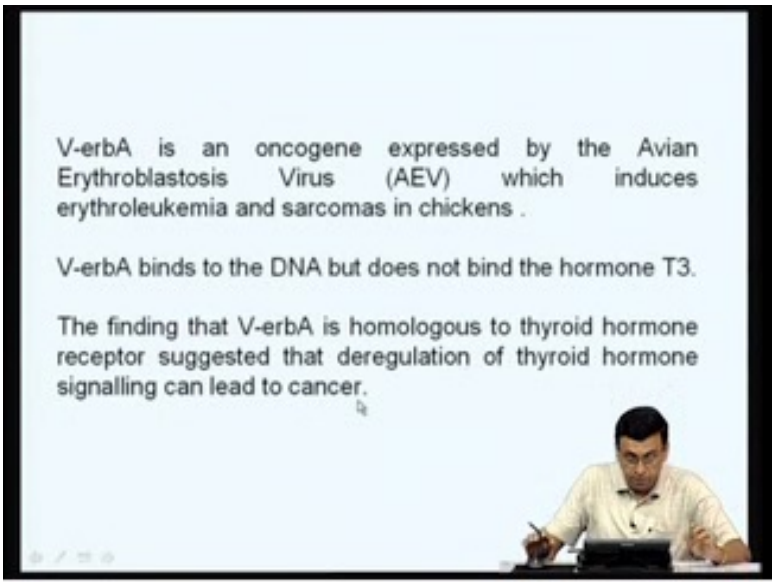

So, let us now examine little bit in more detail. What is V-erb-A. Now, V-erb-A is an oncogene expressed by Avian Erythroblastosis Virus or AEV, which induces erythroleukemia and sarcomas in chickens. So, it is a cancer-causing protein. When you introduce this V-erb-A into cells, the cells become cancerous. But, what became clear is that once it was discovered that the V-erb-A has very high degree of identity or homology to the thyroid hormone receptor, people started asking question, what is the difference between V-erb-A and a thyroid hormone receptor? And, soon they discovered that V-erb-A also binds to the same DNA sequence as the thyroid hormone receptor, but it does not bind to hormone. So, the viral oncoprotein V-erb-A is nothing but a Newton-form of thyroid hormone receptor, which cannot bind ligand, but it can bind of the same DNA sequence as a thyroid hormone receptor. This knowledge clearly indicated that V-erb-A is homologous to thyroid hormone receptor. And therefore, it gave a hint that deregulation of thyroid hormone signaling may lead to cancer.

So, you can see, the cloning of glucocorticoid receptor and their demonstration that the domain structure of glucocorticoid receptor is very similar to viral oncoprotein. And then, the knowledge that the viral-erb-A gene and if you look at the cellular-erb-A gene, there are some differences. And, one of the major differences between V-erb-A and the cellular V-erb-A – C-erb-A is nothing but thyroid hormone receptor is that when the thyroid hormone receptor can bind to thyroid hormone, the V-erb-A cannot bind thyroid hormone. So, on one hand, we had steroid hormone receptors, you have thyroid hormone receptor and you have cancer. So, they found a common link between three different areas of research. This is also a very important observation.

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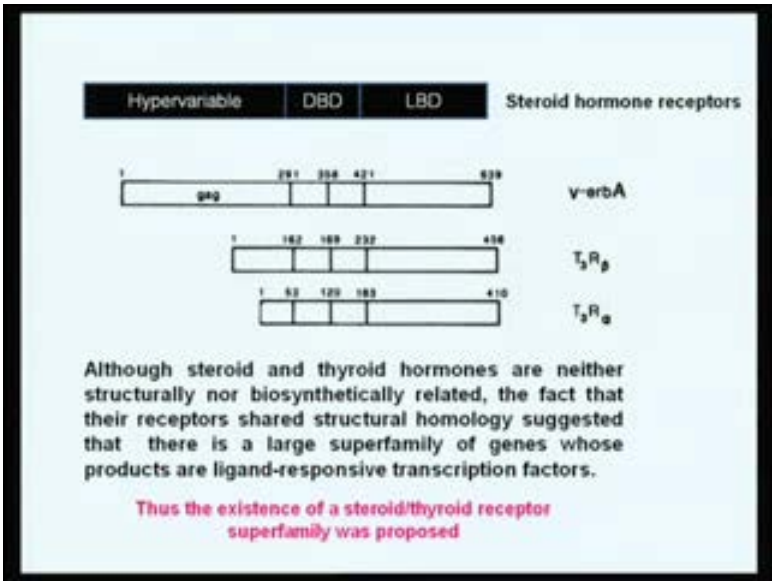

The steroid hormone receptors share the same kind of a domain structure either with v-erb-A or with thyroid hormone receptors. And, although steroid and thyroid hormones are neither structurally nor biosynthetically related, the fact that their receptors shared a structural homology suggested that there exists a large superfamily of genes whose products are ligand-responsive transcription factors. So, these in the early 1980s actually revolutionized the entire field of nuclear receptors, because till then, people thought, the mechanism by steroid hormones activate gene expression is different; the mechanism by thyroid hormones activate gene expression result is different; and, nobody thought thyroid hormone may be involved in cancer. But, with these two or three papers that were published, it became very clear that although steroid hormones and thyroid hormones are not structurally stimular, they all seem to binding to the same kind of receptor molecules. And therefore, the mechanism by which they are activating expression of target genes may be very similar or identical. And, this also suggested that not only thyroid hormone receptor and glucocorticoid receptor, there could be many other receptor molecules. And therefore, all these receptor molecules may actually constitute a superfamily of nuclear receptors. So, this was a conceptually a very important advancement in the area of transcription factors. So, it was proposed that there exist a steroid thyroid receptors superfamily of proteins.

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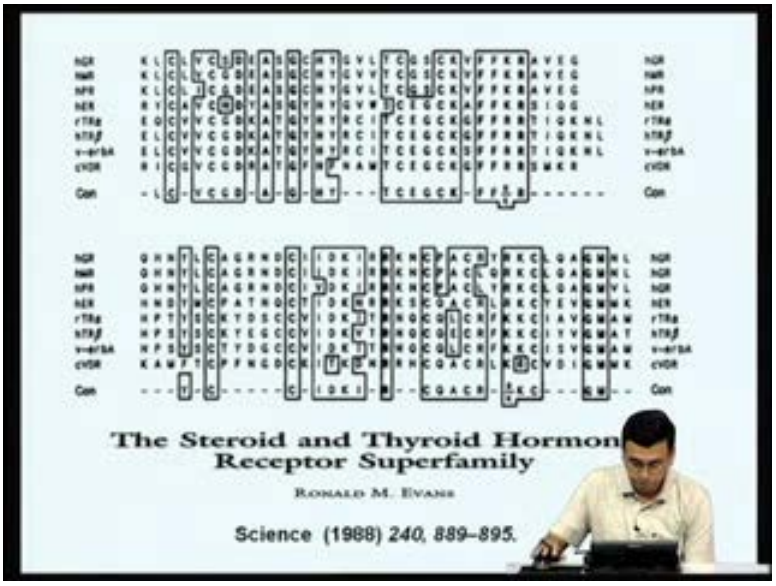

This was a classic review, which was published in science by Ron Evans, who really proposed that there exists a steroid thyroid hormone receptor superfamily. He actually compared the DNA binding domains of various steroid hormone receptors like G R M R P R E R as well as the thyroid hormone receptors V-erb-A as well as the vitamin-D receptors. And, he demonstrated that extensive homology exists in the DNA binding domains of all these receptors suggesting that all these probably belong to a superfamily of transcription factors known as the steroid-thyroid hormone receptor superfamily.

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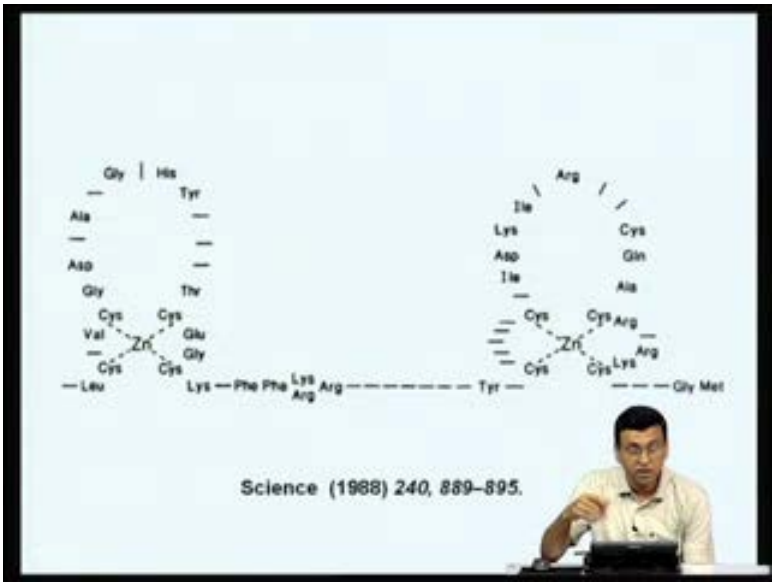

And, they also shared the same zinc finger DNA binding domain. At least hypothetically, they are all capable of coordinating the zinc finger. And therefore, these amino acids in the DNA binding domain can actually form two zinc finger domains. But, a question is what kind of DNA sequences they recognize and how do they bind to DNA? And, do they bind same sequences or do they bind to different sequences? What we now discussed is that despite having homology in the DNA binding domains, despite they all containing zinc finger domains, the sequences, which they recognize are quite different. That is what will be the crux of today's talk.

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Once people realized that before the advent of molecular biology, researchers were purifying receptors like glucocorticoid receptors, progesterone receptors. And, by using techniques like electrophoretic mobility shift assay, DNA zone foot printing, identification of DNA zone hypersensitive sites, etcetera. Within these techniques, they have been able to demonstrate that genes, which are responsible to these hormones contained binding sites for these receptors. For example, promoters like that of mouse mammary tumor virus promoter, human growth hormone, metallothionein, tyrosine hydroxyls, tyrosine aminotransferase. All these genes are responsible of glucocorticoid hormones. And, when they looked at the promoter regions of all these genes, they found that the purified glucocorticoid receptor can actually bind to promoter regions. And in fact, based on these sequences to which these glucocorticoid receptors bind, a concern sequence are being derived like for example, GGTACATGTTC or AGAACATGTTCT.

Now, this is an inverted repeat. So, by comparing the sequences to which these glucocorticoid receptors binds to various promoter elements, a concern sequence was derived for the binding of glucocorticoid receptor.

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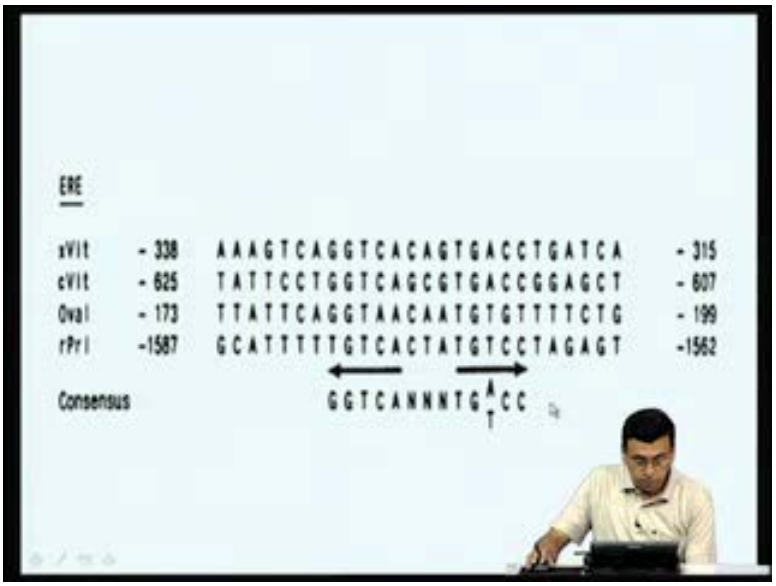

Similarly, in the case of estrogen responsible genes, by analyzing the promoter regions of the estrogen responsible genes and studying by using techniques like msal, DNA zone foot printing, etcetera, by identifying exact binding sites of either the purified receptor; or, once have a cloned receptor, you can over express the receptor in the equalize cells; or, you can use the receptors, which are translated in vitro using rapid heterocyclic sites. And, using these receptor preparations, people have been able to demonstrate, identify exact binding sites for estrogen receptor as well, which is GGTCA three nucleotide spacer TGACC.

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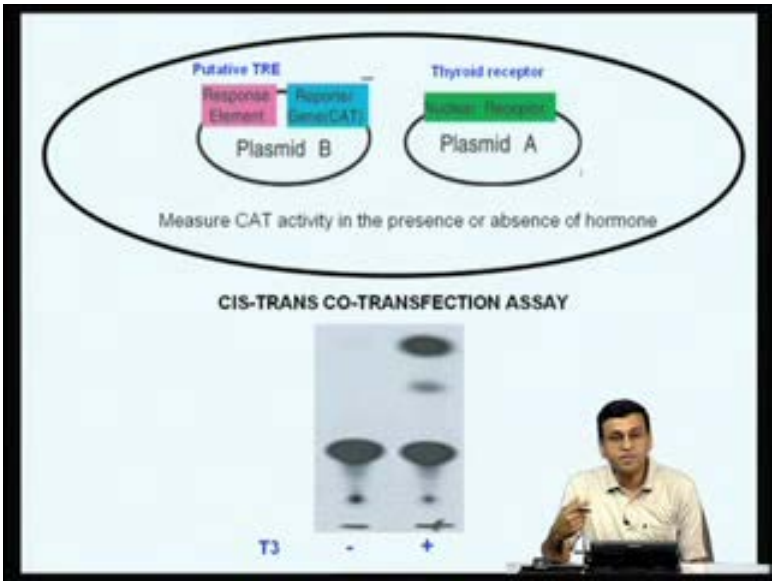

But, once you demonstrate that these receptors actually combine to specific response elements, then the question comes when they bind to the response elements, do they really activate transcription? For this, a very well-designed assay called as cis-trans co-transfection assay was developed. So, what you do in this assay is that you design two different kinds of plasmids; one of the plasmids, you suspect. Suppose you suspect that the promoter region between minus 338 and minus 315 in the (Refer Slide Time: 19:39) vitlogenic gene contains an estrogen response element. And, you want to really demonstrate that this region of the promoter when estrogen receptor binds, it activates transcription.

Now, how do you demonstrate that? What you do is that you take this response element (Refer Slide Time: 19:53) and clone it in front of a reporter gene, which can be either a chloramphenicol acetyltransferase, which converts chloramphenicol to acetyl chloramphenicol; or, it can be luciferase, which will convert luciferin, which can oxidize luciferin (( )) of light; or, it can even be a reporter genes like beta-galactosidase, which when the enzymes is made will give a color when you use a chromogenic substrate. So, by using by linking these response elements with a minimal promoter, which only consists of binding sites for RNA polymerase to a general transcription factors and linked them, putting them in front of a reporter genes.

And then, when you now express another expression plasmid, which I call plasmid A or plasmid B, in this case, the nuclear receptor CDNA is cloned in front of a ubiquitous promoter, it can be a promoter from a cytomegalovirus promoter or rossocromo virus promoter. All these promoters work very well in any eukaryotic cell lines; many of the mammalian cell lines. So, when you introduce this CDNA, what we call as a mammalian expression vector, these cell will start expressing the nuclear receptor. And now, the nuclear receptor will go and bind to the response element. And now, when you add the hormone, you will now start seeing the chloramphenicol assay transferase activity. So, I have just shown here (Refer Slide Time: 21:09).

If you want to demonstrate that a thyroid hormone response element actually present in the promote region of a certain gene, you take this, you design in oligonucleotide or take out this promote region, put in front of a reporter gene. And now, when you now express the thyroid-hormone receptor in the same cell, which have been transferred to this plasmid, you transfer both the cis-plasmid and the trans-plasmid. Cis-plasmid is the one in which the response element is linked to a reporter gene; trans-plasmid is the one which contains the receptor of your interest. The receptor now expresses from this receptor, from this plasmid, goes and binds to the response elements and expresses the reporter gene. And now, you can do what is called as a reporter gene assay. If you use CAT as a reporter gene, you can actually measure the CAT activity in the presence or absence of a hormone. And, this is how a typical result looks like. So, this is chloramphenicol; this is acetylated form of chloramphenicol (Refer Slide Time: 21:57).

And, you can see, when you make these cell extracts, in the absence of hormone, chloramphenicol is not converted into acetylated form. But, when you add hormone, now, chloramphenicol is getting converted into acetylated hormone. What it means is that the thyroid hormone receptor, which has been synthesized from this trans-plasmid bound to the thyroid hormone, gone and then bound to the response element and activate the expression of the CAT gene. And, this CAT is now able to convert chloramphenicol into acetylated chloramphenicol. So, by using this kind of cis-trans co-transfection assays, people have been able to demonstrate that binding of the receptor whether it is glucocorticoid receptor, thyroid hormone receptor or estrogen receptor when they bind, it can actually activate transcription of a downstream reporter gene when the cognate ligand is present. So, this cis-trans co-transfection became a very useful assay for

demonstrating that a binding site or a response element, the binding site for a nuclear receptor can actually function as a hormone response element in vivo. So, in these kinds of assay, people have identified the binding sites for thyroid hormone receptors, because the cDNA for thyroid hormone was present. So, you can now take the cDNA for thyroid hormone receptor; put it in a trans-plasmid and suspect that we know many genes, which are responsible thyroid hormones; and, take those promoter regions; put it in front of these reporter genes and do this cis-trans co-transfection assay and see what are the exact binding size to which thyroid hormone receptor binds and activates transcription.

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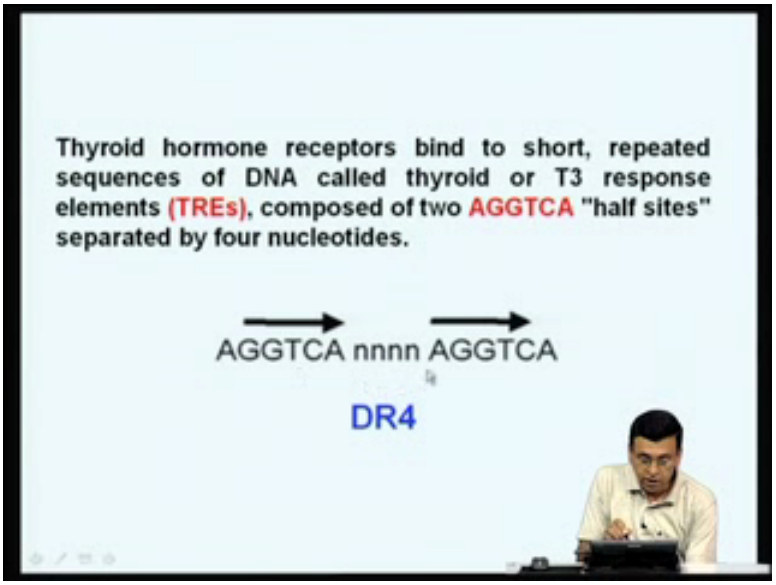

With such studies, people have demonstrated that the thyroid hormone receptor actually bind to short repeated sequences of a DNA called thyroid or T3 response elements or TREs. And, these thyroid hormone response elements actually consist of AGGTCA separated by four nucleotides. So, the response element for thyroid hormone or the TRE is nothing but AGGTCA four nucleotides spacer AGGTCA. And, this is called as DR4, because the two directed repeats are separated by a 4 base-pair spacer. So, a DR4 element is a thyroid hormone response element or a TRE. And, you can see very clearly, this response element is very different from the steroid hormone response element.

For example, if we take estrogen receptors, it is AGGTCA TGACCT. So, it is in palindrome. So, AGGTCA is actually present on the others strand. So, AGGTCA AGGTCA, the palindrome sequence and estrogen receptor binds on the two sides of the

double strand DNAs; whereas, in the case of the thyroid hormone response element, the half-site sequence is same as that of estrogen receptor, but in this case, the half-site is present as a direct repeat separated by a 4 base-cell spacers. So, the thyroid hormone receptor is a type II receptor, binds a direct repeat motif; whereas, the type I receptor like the estrogen receptor and glucocorticoid receptor bind to a inverted repeat or a palindromic sequence, where the two half-sites are present as palindromic sequence or inverted repeats. So, an important observation is that the thyroid hormone response element or TRE consists of two half-sites containing the sequence AGGTCA separated by a 4 base spacer. And, this is normally known as direct repeat 4 or DR4 element. So, if a promoter has a DR4 sequence, it is likely to be response of the thyroid hormone. Thyroid hormone receptor can bind to such sequence and activate transcription.

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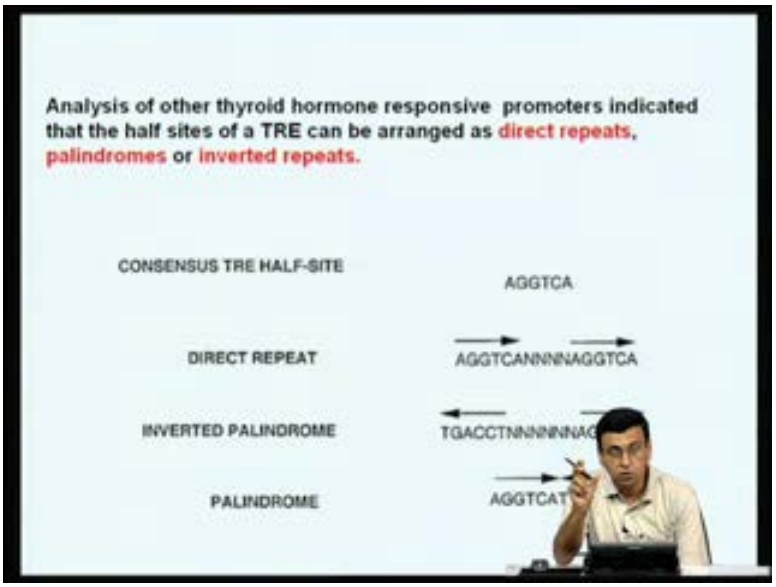

By analyzing a number of other thyroid hormone responsive promoters, people came to realize that not only the direct repeat can present as a direct repeat separated by 4 base-first space or a DR4. In some of the promoters, the same direct repeat can also be provided as inverted palindromes or palindromic sequences. And, such variance of these direct repeat elements can also function as thyroid hormone response elements. So, analysis of other thyroid hormone response elements promoters indicated that the half-sites of the TRE can be present either as direct repeats palindromes or inverted repeats. But, to avoid complication, just remember the most important observation that came is

that the thyroid hormone receptor actually binds to promoters in which the direct repeats, the AGGTCA motif is present and is separated by a 4 base-pair sequence.

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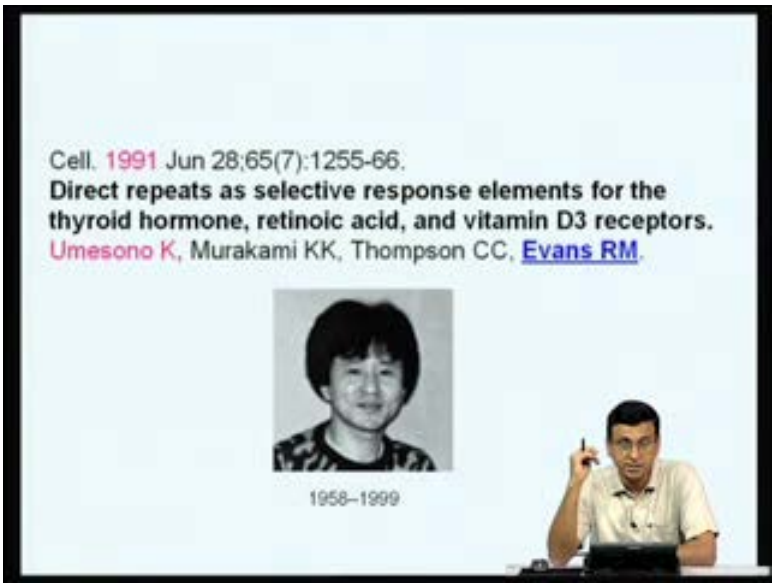

A very important discovery in the area of type II receptors of the nuclear receptors is the proposal of a rule known as 345 rule. This is a land mark paper, which is actually published by (( )) Umesono, a very brilliant post-doc in the laboratory of Ronald M Evans at Salk Institute, who unfortunately passed away at a very early age. And, he came up and published this paper, which is titled direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. These people in this field often consider as a very important paper or land mark discovery, wherein he actually proposed that a very simple rule exists at the molecular level for the binding of vitamin D receptor, thyroid hormone receptor, and retinoic acid receptor.

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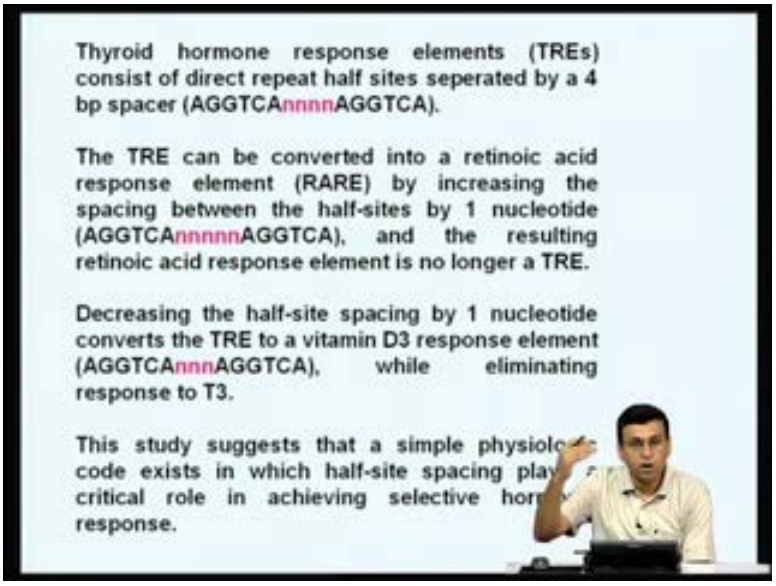

Now, what is this rule? What he actually proposed is that he started looking at the binding of thyroid hormone receptor to thyroid hormone response elements and he found the thyroid hormone response elements consist of direct repeat half-sites separated by a 4 base-pair spacer. This is what we discussed now. So, AGGTCA when you separate by 4 base-percep sequence, it becomes a thyroid hormone response element. Now, what he now did is that if you now increase the spacing by one more nucleotide; that means, instead of 4 base-pair spacer, if we make it a 4 base-pair spacer, thyroid hormone receptor does not bind it any longer. So, the TRE, that is, a DR4 element can be converted into retinoic acid response element by increasing the spacing between half-sites by 1 nucleotide sequence. So, the half-site sequence is the same. But instead of 4 nucleotide spacer, if you now increase the spacing to 5 nucleotide spacer, a TRE now becomes an RARE or a retinoic acid response element. So, when a DR4 becomes DR5, a thyroid hormone response element now becomes a retinoic acid response element. And, by just increasing 1 nucleotide spacing, you can convert a thyroid hormone response element into a retinoic acid response element. So, by increasing the spacing by 1 nucleotide, the resulting retinoic acid response element no longer functions as a thyroid hormone response element.

And, more interestingly, instead of adding 1 nucleotide, now if you remove 1 nucleotide; that means, if a 4 base-pair spacer now become 3 base-pair spacer, now, it becomes a vitamin D3 response element; or, vitamin D receptor can now go and bind to this.

Decreasing the half-site spacing by 1 nucleotide, converts a thyroid hormone response element to vitamin D3 response element; that means, here AGGTCA is separate by 3 base-pairs and this now no longer binds to thyroid hormone receptor. So, this study suggested that a simple physiologic code exists in which half-site spacing plays a critical role in achieving selective hormone response elements.

You can see now, at the molecular level, how a simple code can lead to different physiological responses. Now, I have vitamin D, which has totally different physiological effects; thyroid hormone entirely different physiological effects; retinoic acid, which is a morphogen, again the physiological receptor are still different. But, at the molecular level, the DNA sequences, which these receptors are recognizing and therefore, activating specific target genes, is just different by 1-1 base. So, if you have two half-site sequences separated by 4 base-pair nucleotide, it becomes a thyroid hormone response element. If you have two half-sites separated by 3 base-pair sequence, 3 base-pair spacer, it becomes a vitamin D response element. And, if two half-sites separate by 5 base-pair spacer, it becomes a retinoic acid response element. So, that is why, this came to be known as 3-4-5 rule; that means, DR3 is a vitamin D response element; DR4 is a thyroid hormone response element; DR5 is a retinoic acid response element.

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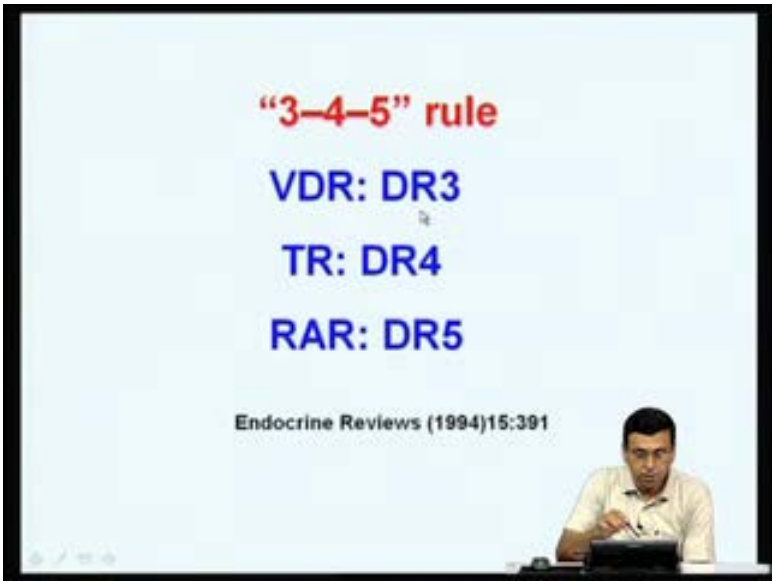

The following publishing of this paper – this entire observation became known as the "3-4-5" rule; where, a DR3 is a vitamin D receptor binding site; DR4 is a thyroid hormone receptor binging site; and, DR5 is a retinoic acid receptor binding site.

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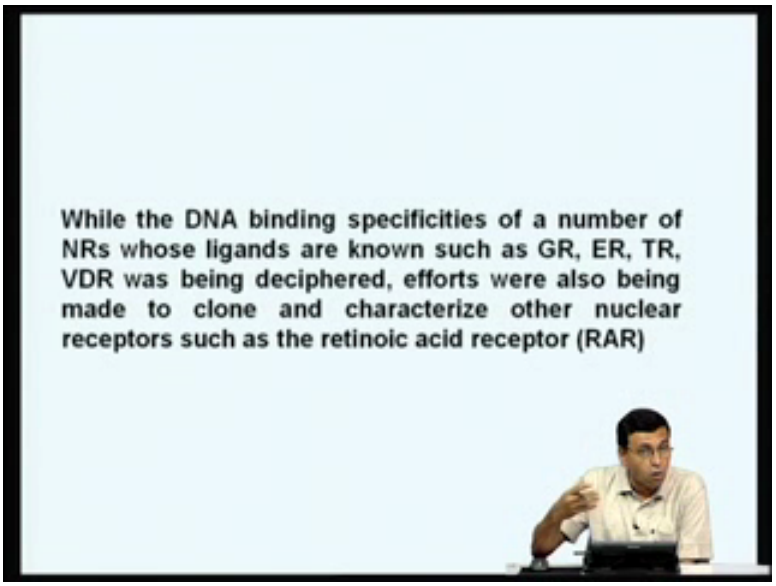

While the DNA binding specificities of a number of nuclear receptors whose ligands are known such as glucocorticoid receptor, estrogen receptor, thyroid hormone receptor, vitamin D receptor was being deciphered, efforts were also being made to clone and characterize other nuclear receptors such as the retinoic acid receptors. As I told you, Ron Evans proposed the existence of a steroid-thyroid hormone receptor superfamily and he proposed that in addition to the receptors, which bind to known ligands, there may be number of other receptors for which the ligands are not known.

In fact, using the DNA binding domains of the glucocorticoid receptor or estrogen receptor or thyroid hormone receptor as probes, people starts screening CDNA libraries sometimes under low tendency conditions to see or the other receptors like molecules with the same kind of zinc finger domain as that of glucocorticoid receptor or thyroid hormone receptors. So, when you do these hybridizations under low stringency, in fact, people pulled out a number of other CDNA clones, which also had the same kind of a domain structure as either glucocorticoid receptor or thyroid hormone receptor. But, they did not bind to glucocorticoid response element, they did not bind to a thyroid hormone response element, nor they bound to those ligands, indicating that the exact physiological

function of these receptors is not clear, because we do not know what kind of sequences the receptor binds and what the ligands for the receptors are. And, such receptors became to be known as orphan receptors.

Orphan receptors are nothing but these receptors share the same structural features as that of steroid or nuclear receptors. But, the sequences to which they bind or the ligands with which they interact is not known. So, the exact physiological functions of these orphan receptors were not clear. So, a major area of research during the late 80s and early 1990s is to understand what the function of these orphan receptors is, what kind of response elements they bind, what are the ligands for these orphan receptors. So, let us see how these discoveries were made.

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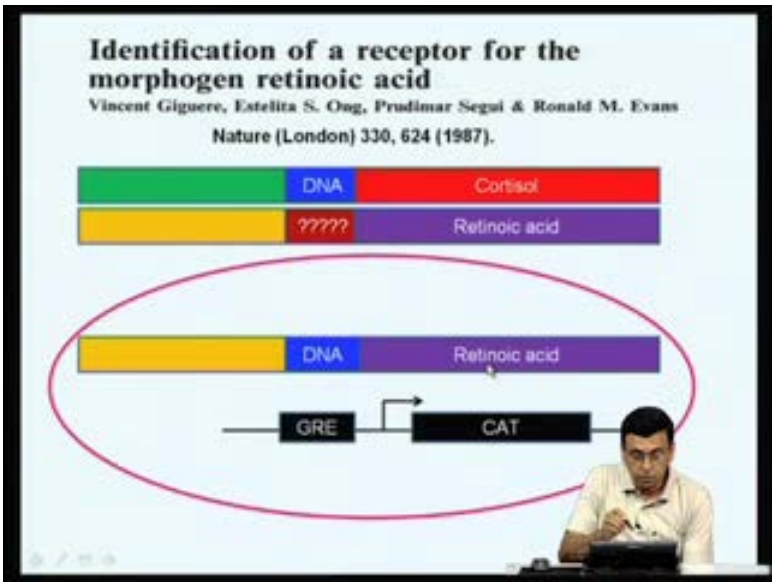

For example, a CDNA, which has very high homology to that of glucocorticoid receptor or thyroid hormone receptor, was identified; it had the same kind of domain structure. And, in fact, this receptor was found to bind to retinoic acid – label retinoic acid suggesting that this can actually be retinoic acid receptor. But, the response element to which this retinoic acid binds was not very clear. So, this is a very novel strategy was, used to identify response elements or the recognition sequences of this orphan receptors. So, what you do is you take advantage of the fact that these orphan receptors have the same kind of a domain structure as the known receptors, which have known ligands. For example, if we take glucocorticoid receptor, it has an amino (( )) domain, a DNA binding

domain and a ligand DNA domain, which binds to glucocorticoid hormones like cortisol. And, we know, the DNA binding domain of glucocorticoid receptor binds to glucocorticoid response elements.

Now, you have for example, retinoic acid receptor and we know the ligand binding domain of a retinoic acid bind retinoic acid, but we do not know what kind of sequences to which the retinoic acid binds. But, to actually demonstrate that this induces a retinoic acid response receptor and it can actually activate transcription retinoic acid bind, a very clever strategy was used, wherein you introduce restrictions sites in the DNA binding domains like a NotI site here, XhoI site here; NotI site here, XhoI site here. And now, you remove the DNA binding domain of glucocorticoid receptor as a NotI XhoI fragment and replace it with the NotI XhoI fragment of retinoic acid receptor. And, you can actually see, this is what is a (( )) receptor is made. So, you replace the DNA binding domain of retinoic acid receptor as a NotI XhoI fragment, pull it out; and then, put the NotI XhoI fragment of glucocorticoid receptor into the retinoic acid receptor. So, this is now called as RGR.

Now, this is GGG, this is RRR and this is RGR; that means, it has the amino (( )) domain of retinoic acid receptor, ligand bind domain of retinoic acid receptor, DNA binding domain of glucocorticoid receptor. Now, if you transfect this chimeric receptor into cells and if you now put a reporter plasmid and glucocorticoid response element, this RGR was now able to activate transcription in response to retinoic acid. So, by using this kind of domain swapping experiments, people have started demonstrating or screening ligands for this various orphan receptors. So, in this land mark paper, where identification of receptor for the morphogen retinoic acid, they conclusively demonstrate that when retinoic acid binds to this chimeric receptor, it activates transcription clearly indicating that the serene that I have isolated is actually retinoic acid receptor although the sequences to which this receptor binds was not clear at that time.

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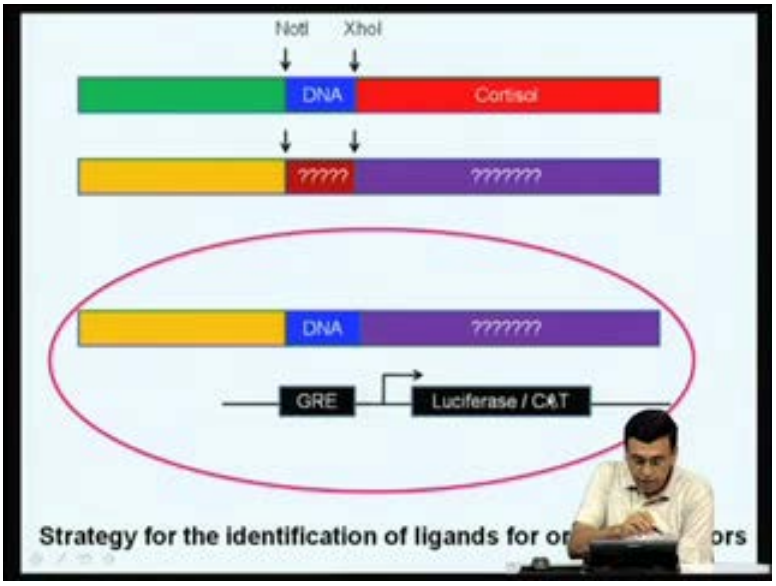

So, using these kinds of strategies, people have started screening a number of orphan receptors to see what kind of ligands when the receptors bind can activate transcription. So, basically, what you do is, you go add and make extracts of various liver tissue or brain tissue or different stages of development; and then, fractionate these extracts; and then, separate these various fractions through HPLCs and take these various fraction (( )), which of the fractions can actually activate an orphan receptor. So, the strategy…

Since we do not know what kind of sequences these orphan receptors bind, you use this chimeric approach. So, you take the DNA binding domain of a well-characterized receptor like glucocorticoid receptor and put it into the… and replace it with the DNA binding domain of the orphan receptor. And, this chimeric receptor now introduce to cell lines. And, now you add various fractions of tissue extracts and see which fraction will now contains a ligand for these orphan receptors. So, it became possible to screen or demonstrate or look for biological activity or a receptor activation ligands in various cell extracts although you have no knowledge of the sequences to which these orphan receptors bind. By using these kinds of studies, people started identifying ligands for various orphan receptors.

So, on one hand, people started identifying what kind of response elements to with these binds; on the another hand, since a large number of orphan receptors have been isolated using this low stringency hybridization techniques, people even without the knowledge

of what kind of sequences these receptors bind, by making these kind of chimeric receptors, people started screening for ligands from various cell extracts or synthetic libraries, what kind of molecules can actually bind to the ligand binding domains and activate transcription. So, this became the common strategy for the identification of ligands for the number of orphan receptors for which we do not know what the ligand is nor we know what kind of sequences these receptors bind.

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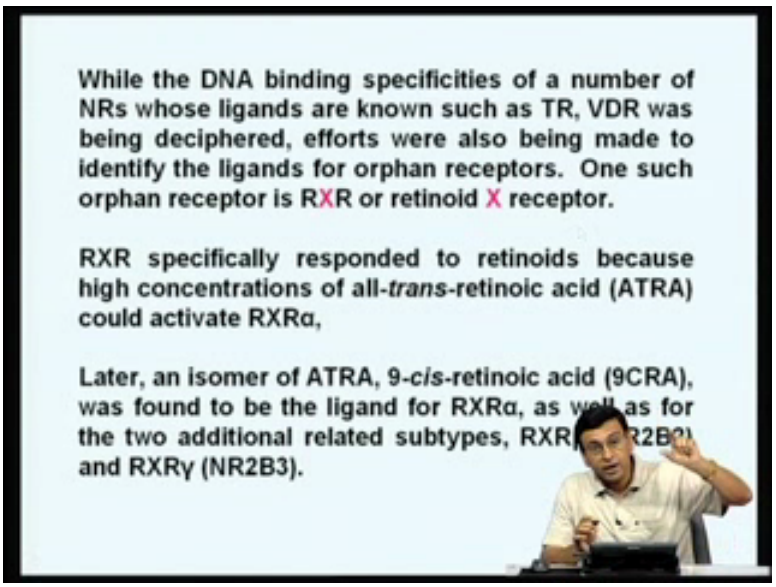

While the DNA binding specificities of a number of nuclear receptors whose ligands are known such as TR, VDR was being deciphered, efforts were also being made to identify ligands for orphan receptors. One such orphan receptor, which later was identified as retinoid X receptor is a very important discovery. So, of a number of orphan receptors (( )) were available, one of the receptor was soon identified as retinoid X receptor.

Now, what is this X? Because in the previous couple of (( )) I had actually shown the identification of retinoic acid receptor. The retinoic acid receptor activates transcription in response to all trans-retinoic acid. That is the ligand for retinoic acid receptor. But, there is another receptor, which was called as retinoid X receptor or RXR. Now, what is the difference between RAR and RXR? The only difference is that when you take the cDNA coding for RXR or cDNA coding for RAR and replace the DNA binding domains with that of glucocorticoid receptor, and now if you have alterized retinoic acid, even at very low concentrations, even at nano molar concentration, the RAR, the ligand binding

domain of retinoic acid receptor is able to activate transcription; whereas, in the case of retinoid X receptor, it requires very high concentrations of all-trans-retinoic acid. In fact, maybe micro molar concentrations. So, RXR specifically responds to retinoids, because high concentration of all-trans-retinoic acid could activate RXR alpha; whereas, the RAR alpha could be activated by even very low constant of all-trans-retinoic acid. So, people suspected that it is not the all-trans-retinoic acid, but when you add all-trans-retinoic acid, it is now getting converted to some other isomer; and, it is an isomer of all-trans-retinoic acid, which is acting as a ligand for this other variant of RAR. So, we do not know what that isomer of retinoid was called as X. Since this isomer is different from all-trans-retinoic acid, it is called as RXR or some derivative of retinoid, which is the ligand for this receptor.

And soon, this retinoid X was found to be an isomer of retinoic acid called 9-cis-retinoic acid. An isomer of all trans-retinoic acid called as 9-cis-retinoic acid was found to be a ligand for RXR alpha as well as two other related subtypes called RXR beta and RXR gamma. So, you can see how using this kind of chimeric approaches, people started identifying ligands for orphan receptors. So, you do not know what kind of response element this RXR binds. But, by replacing the DNA binding domains of RXR with that of another one and by screening various synthetic derivatives of all-trans-retinoic acid, people came to know that 9-cis-retinoic acid is actually the ligand for this particular receptor. And therefore, the retinoid X receptor is actually the receptor for 9-cis- retinoic acid. So, retinoic acid receptor activates transcription response to all-trans-retinoic acid retinoid X receptor activates transcription response to 9-cis-retinoic acid. So, this is just an example to show how ligands for orphan receptors were identified although even without the knowledge of the exact sequences to which these receptors actually bind.

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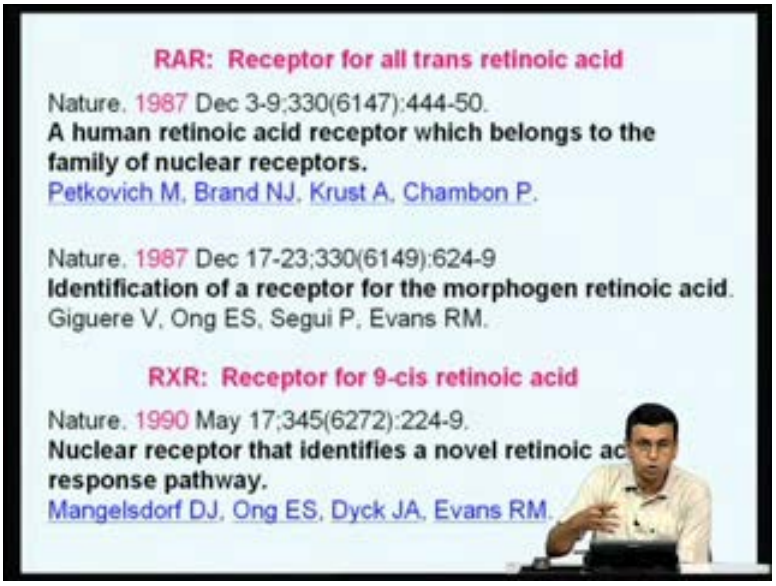

While in the 1987, two groups: one from Ron Evans' group and one from Chambon's group actually identified the demos of the cloning of the retinoic acid receptor; and, took about three years to actually clone the retinoid X receptor and actually demonstrate the retinoid X receptor is the receptor for 9-cis-retinoic acid; whereas, the retinoic acid receptor, which was cloned in 1987 is the receptor for all-trans-retinoic acid. So, two major isomers of retinoic acid – we have all-trans-retinoic acid, which binds to RAR and activates transcription, and another isomer of retinoic acid, 9-cis-retinoic acid binds to RXR and activates transcription. So, these are again two land mark papers.

Now, you also got the retinoic acid into the realm of nuclear receptor superfamily. And, say that in fact, there are three isoforms of RAR called RAR alpha, beta and gamma. They are expressed in different tissues ending that there could be variations in this receptor cell type. So, in the RXR also, they found out there are three different isoforms of RXR alpha, beta and gamma and so on and so forth. So, using these kinds of a chimeric receptor strategy, people started screening libraries of compounds and see either these compounds are made from various tissue extracts or cell extracts; or, it could be synthetic libraries made by organic chemist. And, by using this kind of chimeric approach, people started screening libraries of compounds to identify what is the receptor for all these orphan receptors.

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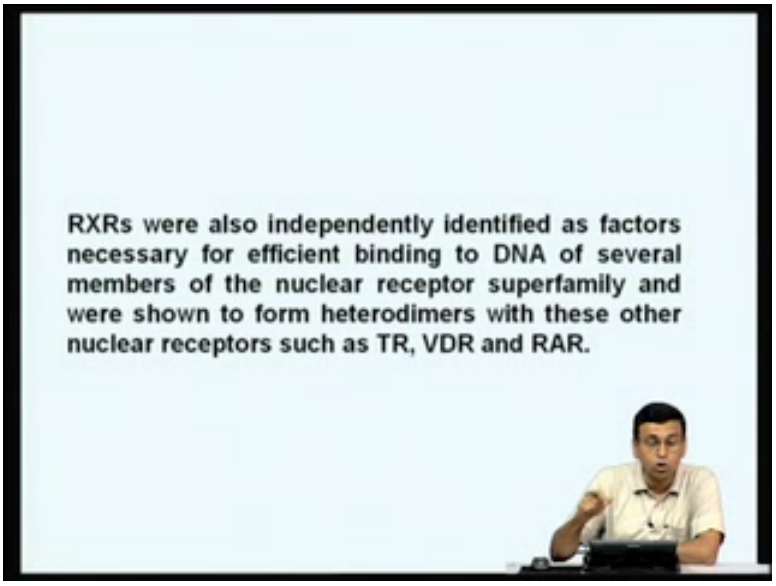

Another important land mark in the area of type II receptor research is that the RXR not only was the receptor for 9-cis-retinoic acid and activate transcription response 9-cis-retinoic acid, it also became clear, that is, RXR also is very important for the binding of other nuclear receptors like thyroid-hormone receptor, vitamin D receptor and retinoic acid receptor. Now, how was this discovered? Now, once the cDNA for thyroid hormone receptor, vitamin D receptor and retinoic acid receptor are cloned, when they take this cDNAs and do what is called as in vitro transcription and translation, you can now take the cDNA put it into an expression vector and put into a vector, which is now controlled by T7 RNA polymerase. Now, if you add T7 RNA polymerase, you can make large amounts of this RNA. So, you have RNA coding for either vitamin D receptor, thyroid hormone receptor or retinoic acid receptor. And then, if you now take this RNA and put it into rabbit reticulocyte lysate, you can make a protein. These are called as in vitro translated proteins.

Now, if you take such in vitro translated proteins and then do what is called as electrophoretive mobility shift assay with a suspected thyroid hormone response element or vitamin response element or retinoic acid response element, these in vitro translated proteins could not bind; that means, either we need to translate a thyroid hormone receptor or a receptor that was made in equalized cells. They did not actually bind to thyroid hormone response element. But, when you take a nuclear extract for example, see one (( )) eukaryotic cell lines and add little bit of this extract to this inverted

translated receptor or battle express receptor, it bound very well. So, these kinds of studies very clearly suggested that for the vitamin D receptor or thyroid hormone receptor or retinoic acid receptor bind to the response element, there is some other factor that is required. So, some factor, which is present in these cell extracts is actually essential for the efficient binding of thyroid hormone receptor or vitamin D receptor or retinoic acid receptor to the response elements.

So, people started hunting what is this factor that is present in the cell extracts that makes these receptors bind very efficiently with the response elements. And, soon this factor turned out to be retinoid X receptor. So, you can see, the retinoid X receptor not only activates transcription in response to 9-cis-retinoic acid, it also is very essential for the binding of thyroid hormone receptor, vitamin D receptor and retinoic acid receptor.

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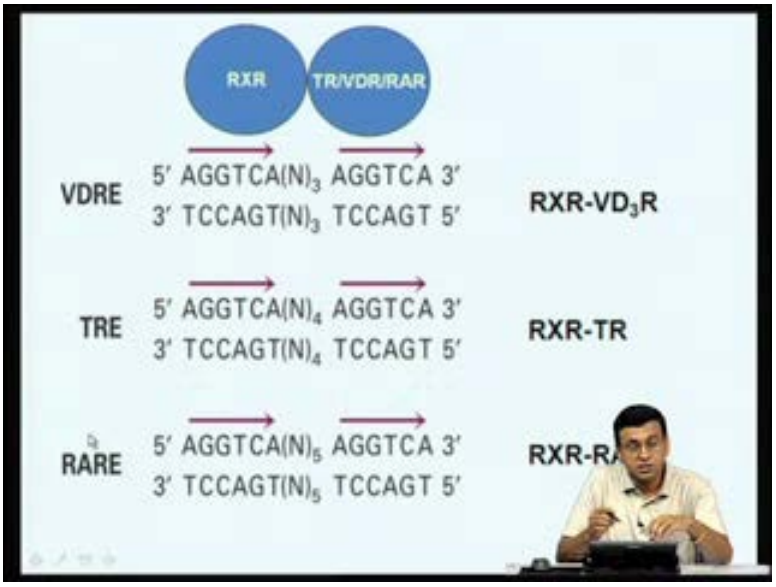

So, it became very clear in order for the vitamin D receptor to bind to a DR3 element or a vitamin D response element, you require RXR. And, by using a number of studies from a number of laboratories, it became very clear, of the two half-sites, which are present in the vitamin D response when separate by 3 base-pair spacer, the RXR bind to the 5 prime half-site and the vitamin D receptor binds to the 3 prime half-site. So, the RXR-VDR heterodimer is the one that can bind to this DR3 element; whereas, vitamin D receptor alone cannot bind. Similarly, the RXR in combination with thyroid hormone receptor heterodimer can now bind to a DR4 element or a thyroid hormone response element;

whereas, RXR and RAR heterodimer can bind to a RARE or a DR5 element. So, you can see, the RXR became the common heterodimeric partner for vitamin D receptor, thyroid hormone receptor and retinoic acid receptor. So, again, is a very important observation in the area of nuclear hormone receptor research.

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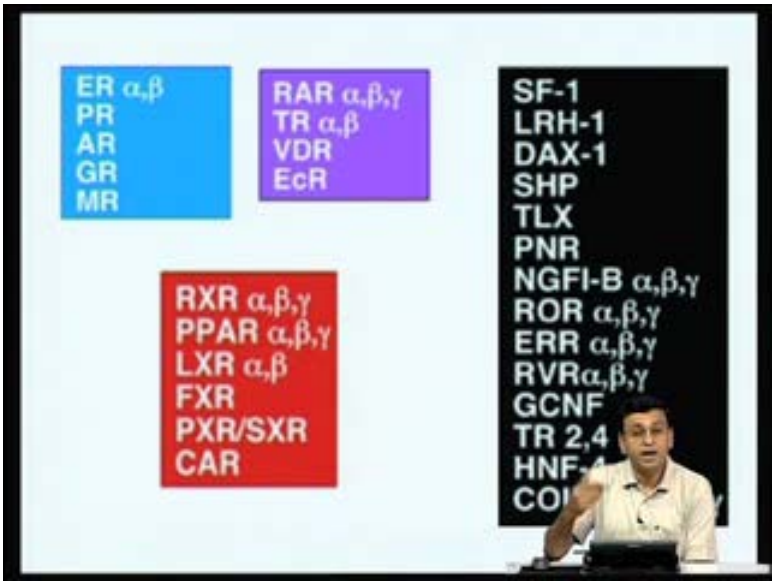

Soon people started looking at, as I said, number of orphan receptors were cleaned. So, like we began this discussion with steroid hormone receptors, then we have now discussed about retinoic acid receptor, thyroid hormone receptor, vitamin D receptor, the same time, people also started looking at steroid hormones like epti zone in the case of insect cells and they found that headdizm receptor also has the same structure as that of steroid hormone receptors. And, people started identifying (( )) response elements in the insect promoters and the (( )) receptor also became a member of the nuclear receptor superfamily. And, many other insect receptors discovered later like ultraspiracle and so on and so forth.

Today, actually we know that there are number of other orphan receptors like LXR, which is liver X receptor, farnesoid X receptor, pregnane X receptor and so on and so forth. All these which were actually known as orphan receptors in the late 1980s and early 1990s, now people have identified what are the ligands for all these receptors. So, receptors, which were classified as orphan receptors in the late 1980s and 90s became full-fledged members of nuclear receptors once the ligands for these receptors were

discovered and also the response elements to which this receptors were identified. So, they all have very important physiological functions. And today, one of the major areas of research is to see what the physiological functions of these receptors are and can we develop agonize or antagonize against the nuclear receptors. And, we will discuss some of these things and see how understanding the structural function of nuclear receptor can now lead to development of number of drug molecules, which can either be agonize or antagonize of these nucleus receptors and have very important biomedical applications.

I have listed here a number of receptors, were actually discovered: HNF-4, COUP-TF – chicken ovalbumin upstream promoter transcription factor, and (( )) growth factor, induced protein, NGFI-B and so on and so forth. But, what I am trying to tell you is that the story, which started with the cloning of steroid hormone receptors led to the discovery of number of other receptors, which had same structural features. And, a major activity was initiated in the area of nuclear receptors to see what kind of response elements to which these receptors bind and what the ligands for these various receptors are.

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And soon it became clear the "3-4-5" rule actually became 1 to 5 rule or "1-2-3-4-5" rule, because RXR was found to be a heterodimeric partner not only for binding to receptors for thyroid hormone receptor and the TR, VDR and RAR, it also was required for other receptors.

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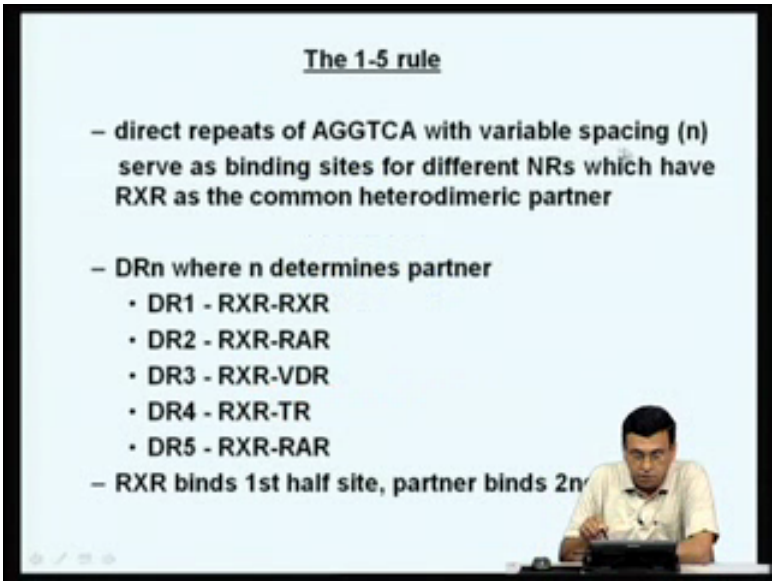

Now, what are these? The direct repeats of AGGTCA with variable spacing was now found to serve as binding sites for different nuclear receptors which have RXR as the common heterodimeric partner. And, so far, I told you that for DR3, DR4 and DR5, vitamin D receptor, thyroid hormone receptor, RAR to bind to these elements, we require RXR. But, soon it became clear that the RAR-RXR heterodimer can not only bind to DR5 element, but it can also bind to a DR2 element. That is, AGGTCA AGGTCA separated by 2 base-pair sequence. And soon, another receptor called peroxisome proliferator-activated receptor (PPAR) was discovered. And, the PPAR again was found to bind to DR1 kind of a sequence along with a heterodimer of RXR.

And in fact, people have actually shown, RXR itself in order to activate transcription in response to 9-cis-retinoic acid has to bind to promoters containing a DR1 kind of a motif. So, you can see, RXR not only serve as a heterodimeric partner for a number of other receptors, it can also bind to its own response elements as homodimers containing a DR1 kind of a sequence and activate transcription response to 9-cis-retinoic acid. So, RXR became a very unique receptor among all the receptors discovered so far in the sense that it has the quality of both steroid hormone receptors and type II receptors. As I told you, steroid hormone receptors primarily bind as homodimers; type II receptors primarily bind as heterodimers. But, RXR is one unique example, where it can not only bind as heterodimers with the other nuclear receptors, it can also form homodimers and activate transcription with specific response element. So, RXR is a very important discovery in

the area of nuclear hormone receptors. And, in all these heterodimeric binding, the RXR binds the first half-site or the 5 prime half-site; and, the partner whether it is thyroid hormone receptor, vitamin D receptor, RAR, binds to the 3 prime half-site. And, that is how they bind and then activate transcription.

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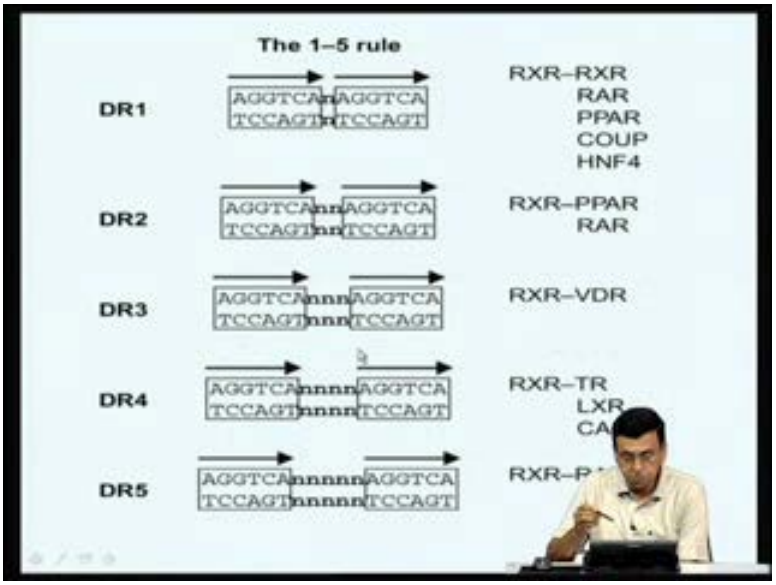

So, this is what is known as the 3-4-5 rule, which was discovered by Kazuhiko Umesono; soon became a 1 to 5 rule, because two more response elements: DR1, DR2 were discovered, which also requires RXR as a binding partner. So, this is the summary of all the receptor that went on into this to actually show RXR-RXR homodimers can bind to promoters containing a DR1 kind of a motif, where AGGTCA AGGTCA separated by 1 base-pair rule on 1 base-pair spacer.

In addition to RAR-RXR, RXR can also form a heterodimer RAR, another receptor called peroxisome proliferator-activated receptor, hepatocyte nuclear factor 4, chicken ovalbumin upstream promoter transcription factor. So, all these receptors along with RXR as a heterodimeric partner bind to promoters containing a DR1 kind of a response element; whereas, RXR-PPAR heterodimer as well as RXR-RAR heterodimers can also bind to promoters containing a DR2 kind of a sequence, where the two half-sites are separate by 2 base-pair spacer; whereas, the DR3 response element is a unique response element for RXR-VDR, vitamin D receptor heterodimer; whereas, the DR4 type of a response element is the response element for RXR thyroid hormone receptor and later

the same DR4 sequence was also found be recognized by RXR-LXR heterodimer and also another novel receptor called as CAR.

And, the DR5 response element, where the two half-sites are separated by 5 base-pair spacer became the response element for RXR-RAR as well as another transcription factor called NGF-IB. So, you can see, how RXR became a common heterodimeric partner for a number of other receptors. And, when binding to different response elements, where the two half-sites separated by different lengths of spacer, different physiological responses can be brought about.

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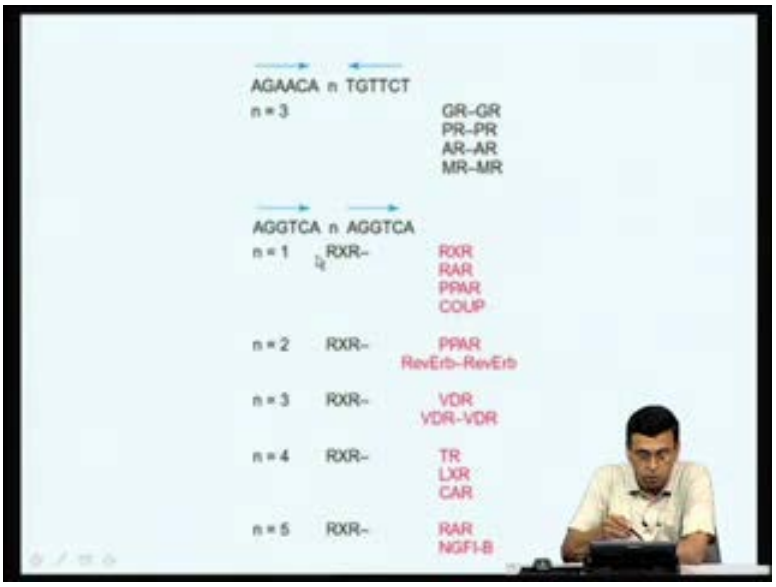

So, the story be the summary of what we have discussed so far. The steroid hormone receptor binds to inverted repeat sequences separated by 3 base-pair sequence. And, we had a number of other receptors that we have discussed here, where the two direct repeats are separated by either 1, 2, 3, 4 or 5 base-pair spacers. And, if it is separated by 1 base-pair, these are the receptors, which go and bind. If it is separated by 2 base-pair spacer, these are the receptors, which will bind in combination with RXR. Similarly, the receptor is indicated red here along with a common heterodimeric partner, can bind to different direct repeat sequences.

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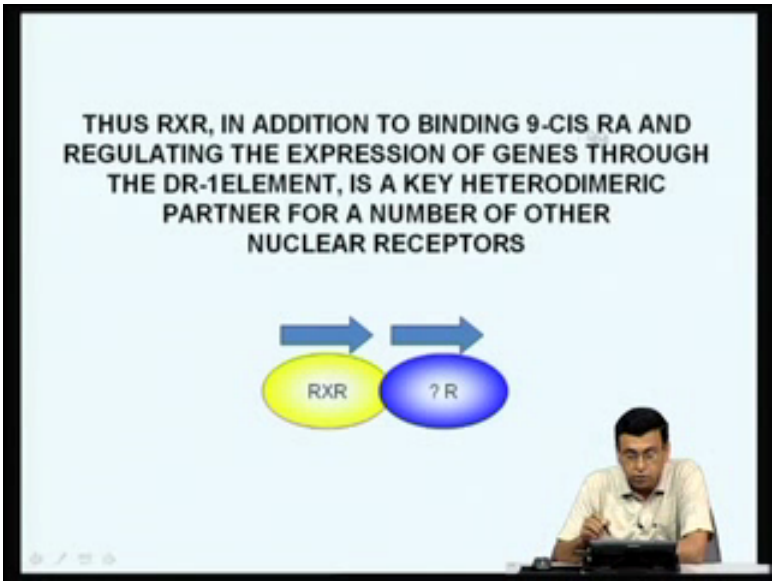

So, the RXR, in addition to binding to 9-cis retinoic acid and regulating the expression of genes through the DR-1element, is also the key heterodimeric partner for a number of other nuclear receptors. This is the paradigm that emerged out of all these studies. So, RXR became a very important molecule along with the various nuclear receptors.

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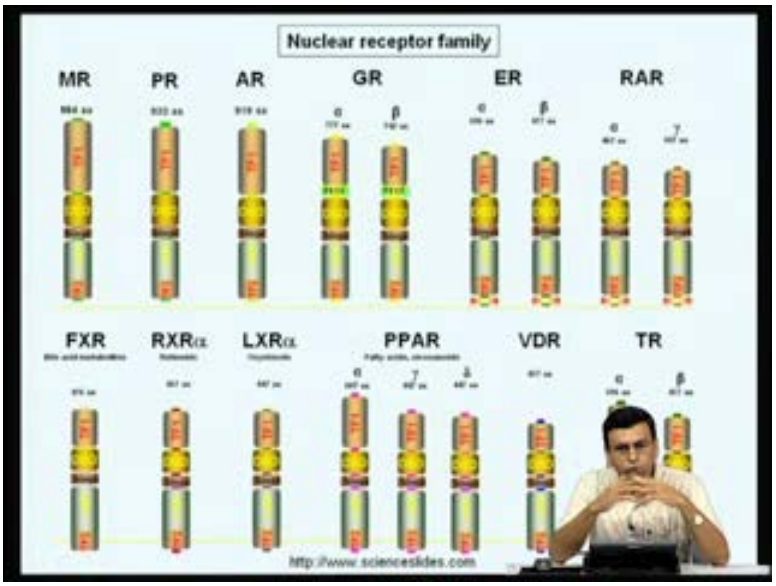

Today, we have a number of nuclear receptors and this just shows you some of them, which have been very well-characterized. The panel here actually shows the steroid hormone receptors; we have MR – mineralocorticoid receptor, progesterone receptor,

androgen receptor, glucocorticoid receptor, estrogen receptor, so on and so forth. Again, you can see that all these receptors share the same structural features – amino terminal domain, DNA binding domain, and transcription activation domain. The amino terminal domain is supposed to encode what is called as a ligand independent transcription activation function. Therefore, it is called as TF1; whereas, the ligand binding domain encodes what is called as the ligand-dependent transcription activation function.

In the next class, we will discuss exactly how the ligand actually activates transcription; how the ligand binding domain results in the activation of transcription. For example, FXR – the farnesoid X receptor the ligand actually was found to be bile acid metabolize. The RXR – as I said, 9-cis retinoic acid is the ligand for RXR. Many oxysterols were found to be ligands for liver X receptors. Peroxisome proliferator-activated receptor – a number of fatty acids and icosanoids were found to be ligands for the PPAR go with molecules. And, all these are very important targets for a number of drugs now for number of diseases like arthritis, diabetes and so on and so forth, obesity. All these receptors seem to play a very important role in activating the expression of genes involved. And, if you have improper activation, it results in disease like obesity, diabetes and so on and so forth. So, there are very important molecules as far as drug targets and drug discovery is concerned.

Vitamin D receptor in thyroid hormone we already saw. So, the story started from the steroid hormone receptors led to the discovery of retinoic acid receptor, thyroid hormone receptor, vitamin receptor. Now, we have peroxisome proliferator receptor and many other receptors, which are binded in number of important molecules like bile acid metabolize, oxysteroids, fatty acids, icosanoids. So, all these molecules seem to be activating expression of target genes by binding to specific intracellular receptors. And, they altogether constitute the nuclear receptor superfamily.

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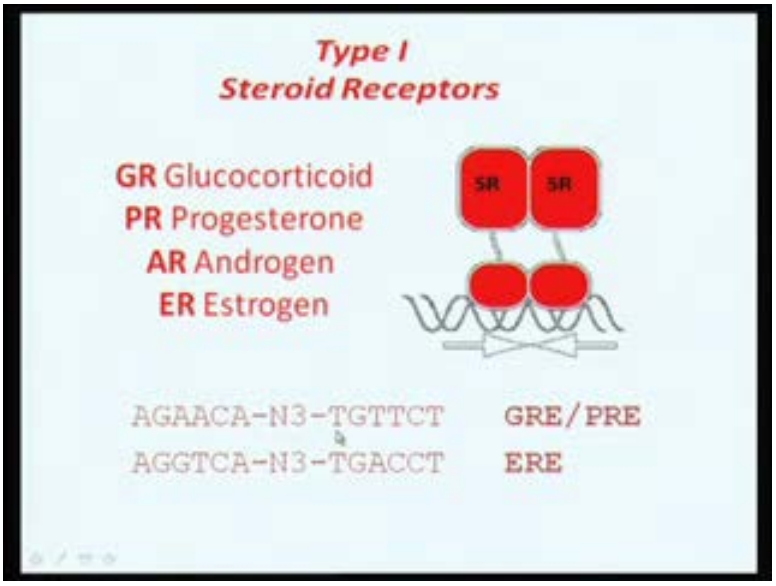

So, this is just the summary of what we discussed so far; I will not discuss again. Just to tell you that the steroid hormone receptor, type I receptors bind as homodimers to inverted repeat sequences like GREs and EREs. And, the only 2 base-pairs distinguish between the GRE and ERE.

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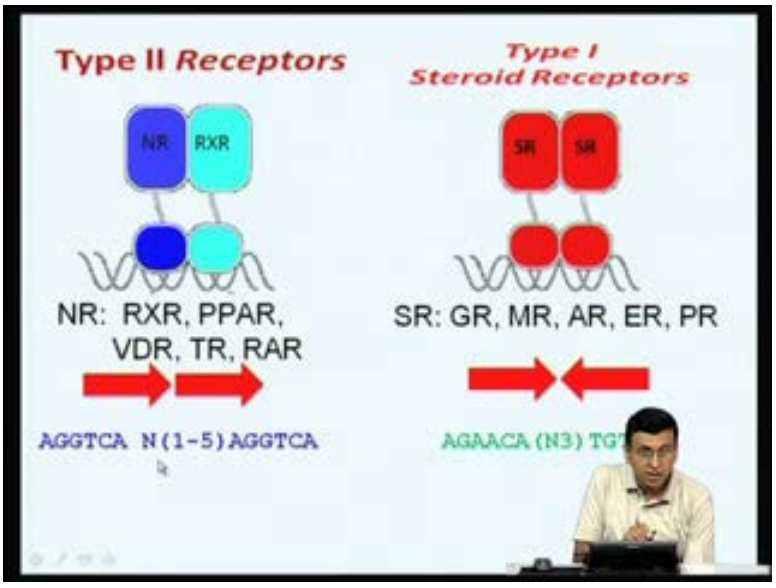

And, the type II receptors, what we discussed today consists of nuclear receptors like RXR, peroxisome proliferator activated receptor, vitamin D receptor, thyroid hormone receptor and retinoic acid receptor. All these things, the RXR serve as a common

heterodimeric partner. This cartoon is a little bit (( )). The RXR binds a 5 prime half-site and the nuclear receptor binds to the 3 prime half-site. So, these are pretty different from the type I receptors, which primarily bind as homodimers. And, while this receptor binds to the inverted repeats or palindromic sequences, these type II receptors bind to direct repeat sequences separated by spacers, which can range from 1 to 5.

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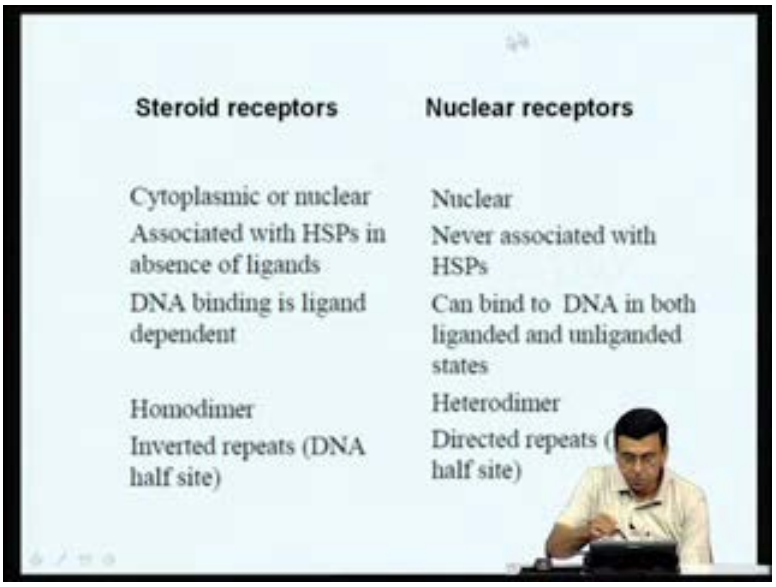

Now, again summarize; the steroid receptors are primarily cytoplasmic; and, the absence of the ligands; and, the DNA binding is ligand dependent; whereas, the nuclear receptors are already nuclear; they do not associate with each of proteins; they can bind to DNA either in the liganded or unliganded form; steroid hormone receptors primarily bind to DNAs as homodimers; nuclear receptors primarily bind as heterodimers with the exception of RXR, which can bind both as homodimer as well as heterodimer. As steroid hormone receptors primarily bind to inverted repeat sequences or palindromic sequences, the other nuclear receptor we discussed today primarily bind to directed repeat sequences.

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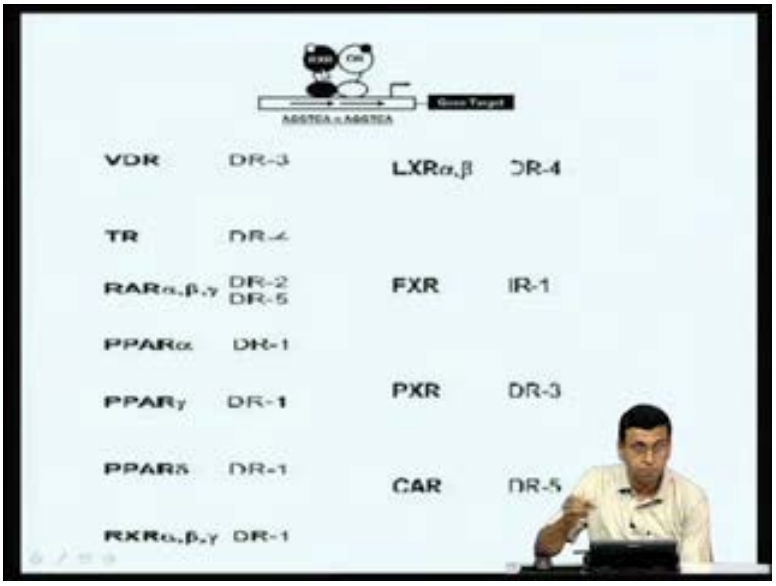

This is again a summary of what we discussed clearly showing that a number of other (( )) In addition to what we have discussed today, a number of other receptors have been discussed and their response elements to which they bind also have been characterized. And, all these receptors, the common heterodimeric partner is RXR.

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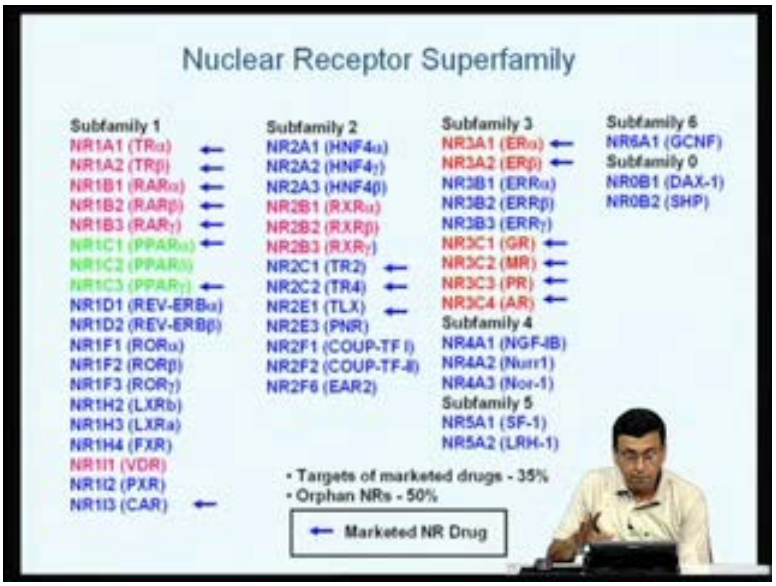

So, this is the summary of nuclear receptor subfamily. Now, a new nomenclature was evolved to make sure that there is no confusion. For example, estrogen receptor alpha is now designated as nuclear receptor 3A1; glucocorticoid receptor has been designated as

nuclear receptor 3C1 and so on and so forth. A new nomenclature was developed to make sure that everybody follows the same nomenclature. And, what we discussed so far in the last two classes is the thyroid hormone receptors, retinoic acid receptors, estrogen receptors, the steroid hormone receptors and RXRs. What I have shown in the red, little bit of PPAR as I just mentioned. But, there are number of other receptors and lot of research is going onto see what kind of ligands they bind, what kind of response elements they bind, and what is the physiological response of these genes. And, what I have indicated here as arrows, all these receptors, which are indicated arrows, are all very important drug targets.

So, I think I will stop here. Just to tell you that the research we started with purification of vitamins, thyroid hormones, ultimately, led to cloning of nuclear receptors and how it became a very important field.

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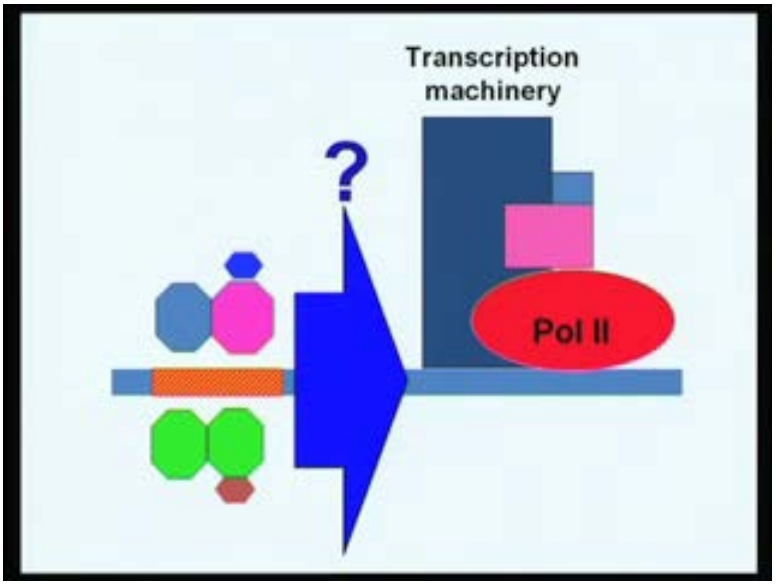

What we discussed so far is how this receptor binds with DNA. And, what we will discuss in the next class is that once they bind to the DNA, how do they activate transcription; how do they interact with various transcription of factors and they activate transcription.

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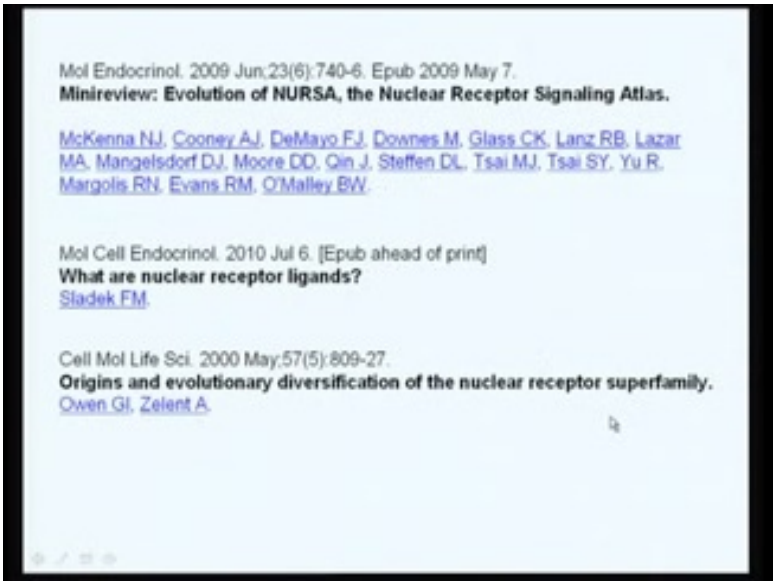

And, these are some of the key references I have indicated here. You can go through.

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And then, there is also a very important website, where you can go. And, there are nice videos, which are actually paid to give you updates of what is going on in this area, is called as nuclear receptor signalling atlas. I think I will stop here.