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Detection of mRNA Transcripts for Retinoic Acid, Vitamin D_3, and Thyroid Hormone (c-*erb*-A) Nuclear Receptors in Human Skin Using Reverse Transcription and Polymerase Chain Reaction

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Differentiation of keratinocytes involves both non-genomic and genomic events. The genomic effects are regulated by ligand-dependent transcription factors, e.g. the steroid/thyroid superfamily of nuclear receptors. In the present study we examined mRNA expression of receptors for retinoic acid, thyroid hormone, and vitamin D_3 in normal human skin and cultured keratinocytes using reverse transcription coupled to the polymerase chain reaction. The vitamin D_3 receptor and the retinoic acid receptor (RAR) γ together with the more distantly related RXRα were amplified extensively in skin and cultured keratinocytes. RARα was amplified at a lower level, and RARβ was almost undetectable. The thyroid hormone receptors α1 and β1 were weakly amplified, but to comparable levels. Because receptors for retinoic acid, thyroid hormones, and vitamin D_3 are all expressed in human epidermis differentiation of keratinocytes is probably regulated at transcriptional level by these molecules. It remains to be seen whether alterations in the expression of the nuclear receptors occur in certain skin disorders. ***Key words: RAR; RXR; THR; VDR; Epidermis; Keratinocytes.***

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Small molecules such as retinoids, vitamin D_3, and triiodothyronine affect epidermal differentiation both *in vivo* and *in vitro* (1–5). All these compounds bind to intracellular receptors belonging to the steroid/thyroid hormone receptor superfamily. To date, this family comprises 3 retinoic acid receptors (RARs) (6–8), 2 thyroid hormone receptors (THRs) (9, 10), the vitamin D_3 receptor (VDR) (11), and the recently described second class of retinoid receptors (RXRs) (12, 13). The endogenous ligand of RXRs has been identified as a stereoisomer of retinoic acid, 9-cis retinoic acid, which directly binds and activates RXRα (14). Besides acting as a homodimer, RXRα greatly enhances the activity of RARs, VDR, and THRs by heterodimer formation (15). Studies on the mRNA expression of nuclear receptor proteins in dermatological disorders are scanty, probably owing to difficulties in performing Northern blot analysis with the limited amount of material available from abnormal skin lesions. However, by the use of reverse transcription (RT) coupled to polymerase chain reaction (PCR) the mRNA expression can be studied in small skin samples such as obtained from suction blister roofs (16).

In the present study we employed RT-PCR for analysis of mRNA expression of nuclear receptors for retinoic acid, 9-cis retinoic acid, vitamin D_3, and thyroid hormone in normal human skin and cultured epithelial cells.

MATERIAL AND METHODS

Biological specimen

Normal human skin was obtained by shave biopsy in connection with mammary reduction surgery. The samples included the whole epidermis and small portions (≤20%) of papillary dermis.

Human keratinocytes were derived from normal mammary skin and cultured in Nunclon 6-well plastic dishes (Nunc, Roskilde, Denmark) using a 1:3 mixture of DMEM:F12 medium (Gibco BRL, Uxbridge, England) containing 5% foetal calf serum (Nordcell, Stockholm, Sweden), 0.4 μg/ml hydrocortisone, 5.3 μg/ml insulin, 10 ng/ml epidermal growth factor, 0.1 nM cholera toxin, and antibiotics. The calcium concentration was 1.56 mM as determined by atom absorption spectrometry. Primary cells were established in a humidified incubator at 36°C containing 5% CO_2 and 95% air. Growth medium was changed 3 times per week and confluent cultures were harvested by scraping on day 14.

HeLa cells (from human cervix carcinoma) were cultured in DMEM containing 10% FCS and antibiotics.

Reversed transcription (RT)

Total RNA was extracted from normal skin and cultured cells by the guanidinium-phenol-choloroform method (17). Three μg of total RNA was reversedly transcribed into cDNA in a 30 μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM of each dNTP (Pharmacia-LKB Biotechnology, Sollentuna, Sweden), 2 U RNase Block II (Stratagene, La Jolla, CA), 10 μg/ml of oligo-d$(T)_{15}$ as primer, and 200 U M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD). After incubation at 37°C for 60 min, the reaction was stopped by heating at 75°C for 10 min and the mixture stored at −70°C until cDNA amplification was performed.

Oligonucleotide primers

Transcripts of RARα$_1$, RARβ$_2$, RARγ$_1$, RXRα, VDR, THRα1, and THRβ1 were studied by amplification of transcribed RNA using different primer pairs (see Table I). We designed specific primers of 20–23 nucleotides in length with 48–70% GC composition. The calculated melting temperature (>66°C) allowed a stringent annealing temperature in the PCR cycle. The cDNA amplification products, spanning over almost the entire coding sequence, were predicted to be 1261 bp (RARα$_1$), 1300 bp (RARβ$_2$), 1256 bp (RARγ$_1$), 1355 bp (RXRα), 1245 bp (VDR), 1159 bp (THRα1), and 1309 bp (THRβ1) in length (the distance between primers plus primer length).

Polymerase chain reaction (PCR)

The following PCR mixture (48 μl) was prepared immediately before use: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of

Table I. *Nucleotide sequences from the different cDNAs used for amplification by RT-PCR*

Target	Sequence		Bp in ref	Product size/Bp	Reference
RARα$_1$	F	5′AGGCGCTCTGACCACTCTCCA	340–360	1261	(6)
	R	5′GGCCGGGCTGCTTCTGTTGG	1600–1581		
RARβ$_2$	F	5′TGGATGTTCTGTCAGTGAGTCCT	335–357	1300	(36)
	R	5′TTTTCCACTGAGCTGGGTGAGAT	1634–1612		
RARγ$_1$	F	5′GCCACCAATAAGGAGCGACTCT	418–439	1256	(8)
	R	5′TCAGGGTTCTCCAGCATCTCTC	1673–1652		
RXRα	F	5′CTGCCGCTCGATTTCTCCACC	94–114	1355	(12)
	R	5′GGCGCCTCCAGCATCTCCATA	1448–1428		
VDR	F	5′CCAGCACTTCCCTGCCTGACC	17–37	1245	(11)
	R	5′ACTTCGAGCACAAGGGGCGTT	1261–1241		
THRα	F	5′AGAAGCCAAGCAAGGTGGAATGT	474–496	1159	(37)
	R	5′TTCATGTGGAGGAAGCGGCTG	1632–1612		
THRβ	F	5′CAGAAAATGGCCTTACAGCTTGG	305–327	1309	(9)
	R	5′TTCATGTGGAGGAAGCGGCTG*	1613–1593		

* The THRβ primer contains a mismatch (a G instead of a C at position 9) because this primer was designed to be used as reverse primer for both THRα and THRβ.

each dNTP, 0.001% gelatin, and 1.25 U AmpliTaq DNA polymerase (Perkin-Elmer-Cetus Corp., Emeryville, CA). Primers (1 μl) were added to a final concentration of 0.5 mM. The total PCR volume was 50 μl, including 1 μl of the reverse transcription reaction mixture which is equivalent to 100 ng total RNA. The reaction mixture was overlaid with 1 drop of mineral oil (Perkin-Elmer-Cetus Corp.) to prevent evaporation during heating. The tubes were placed in a Thermal cycler (Perkin-Elmer) programmed as follows: (a) 94°C for 60 s (initial melting); (b) 35 cycles of the following sequential steps: 60 s at 94°C (denaturation), 60 s at 61°C (annealing), 120 s at 72°C (extension); and (c) 7 min at 72°C (final extension).

PCR product analysis

Ten μl of the reaction mixture was mixed with loading buffer on a strip of Parafilm. Amplified products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide, in parallel with a Hae III digest of ΦX174 serving as molecular weight markers (Promega), and visualized by UV transillumination.

Restriction enzyme mapping

In certain experiments the amplified products were precipitated overnight in the presence of sodium acetate and ethanol at −20°C. After centrifugation at 15,000 X g for 20 min at +4°C the amplified cDNA was redissolved in distilled water. The cDNA was digested with diagnostic restriction enzymes (Promega, Table II) for 2 h before gel analysis. The specific endonuclease cleavage sites were obtained from published sequences.

RESULTS

To examine the mRNA expression of nuclear receptors in

Table II. *Predicted cleavage sites of PCR products*

Substrates	Enzyme	Number of sites	Fragment sizes (bp)
RXRα	SacI	1	147, 1207
RARα	SacI	1	446, 814
RARβ	KpnI	1	259, 1040
RARγ	SacI	1	562, 693
VDR	Pst I	1	171, 1074
THRα	Pst I	2	207, 457, 495
THRβ	Pst I	1	594, 714

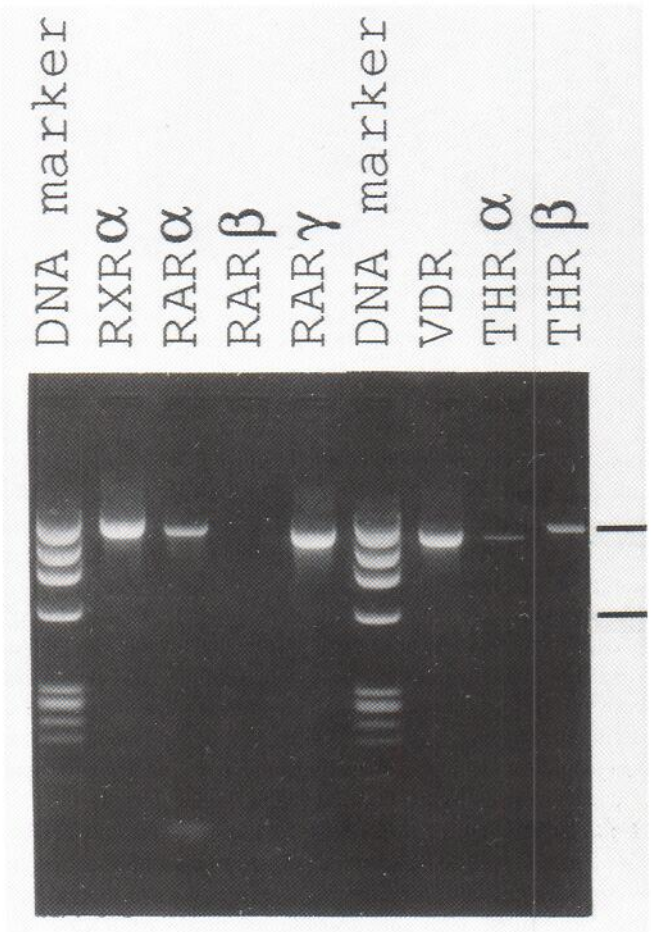

Fig. 1. Detection of mRNAs for nuclear receptor proteins in human skin by RT-PCR, using ethidium-bromide-stained agarose gel for detection of nuclear receptor proteins. The molecular weight marker is a Hae III digest of ΦX174 consisting of fragments of the following sizes (in base pairs): 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, (72). The 1353 and 603 bp fragments are outlined to the right. Similar results have been obtained using cDNA from three different samples.

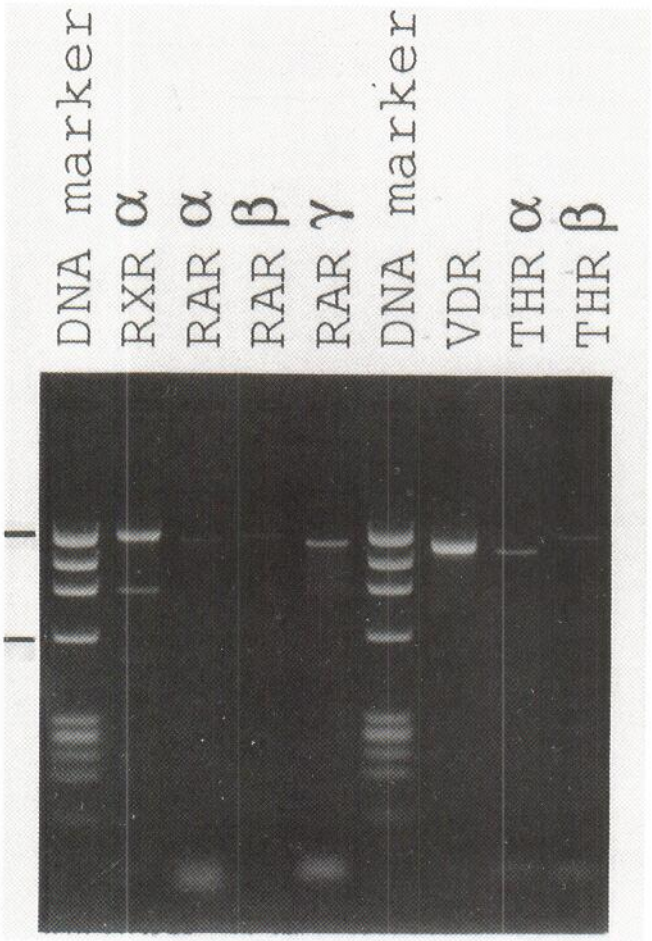

Fig. 2. Detection of mRNAs for nuclear receptor proteins using RT-PCR in cultured epidermal keratinocytes. The molecular weight markers are as shown in Fig. 1. The experiments have been conducted twice with similar results.

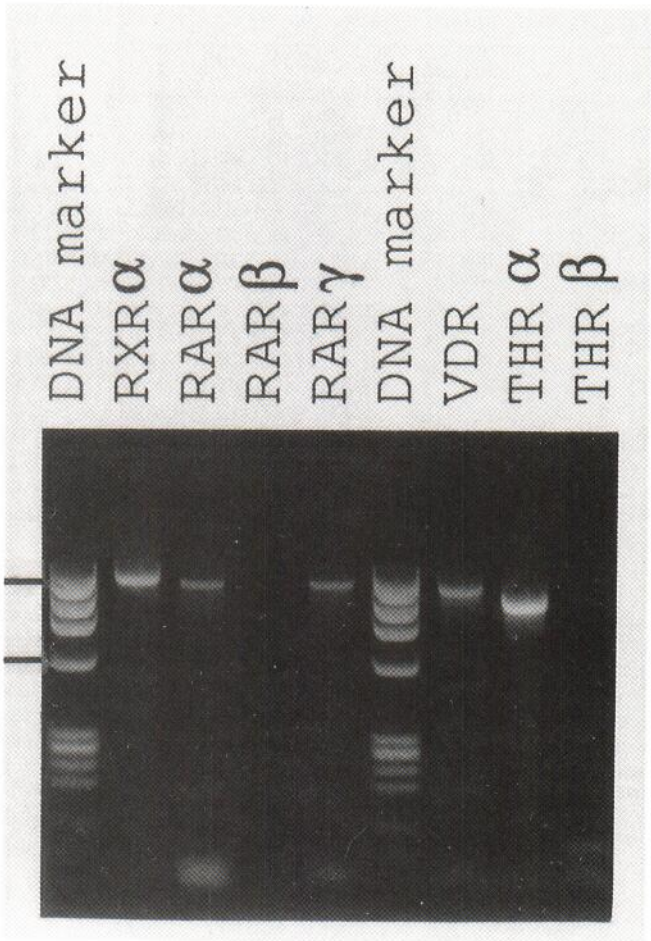

Fig. 3. Detection of mRNAs for nuclear receptor proteins using RT-PCR in cultured HeLa cells. The molecular weight markers are as shown in Fig. 1. Similar results were obtained from two different preparations of cDNA from HeLa cells.

human skin we reversedly transcribed total RNA into cDNA and amplified the cDNA by PCR (RT-PCR) using specific primers (Table I). As shown in Fig. 1, transcripts for RXRα, RARα, and RARγ, but not for RARβ, were found. Similar results were obtained when amplifying cDNA from cultured keratinocytes (Fig. 2) and HeLa cells (Fig. 3). Expression of RARβ was routinely found in identically prepared cDNAs from human liver or placenta, showing that the RARβ primers were working (data not shown). The bands were identified by restriction enzyme mapping. When amplified products from a skin sample were cut with restriction enzymes, new products of expected sizes were obtained (Table II and Fig. 4).

We also studied the mRNA expression of VDR and THRs. As seen in Figs. 1 and 2, the degree of VDR amplification in human skin and cultured keratinocytes was greater than for all other receptors, with the possible exception of RXRα. In HeLa cells the amplification of VDR was less pronounced than in the skin-derived samples (Fig. 3). The two receptors for triiodothyronine (THRα and THRβ) were amplified from cDNAs from skin and cultured keratinocytes, with similar degrees of amplification for the two receptors. Amplification of cDNA from HeLa cells generated THRα but not THRβ (Fig. 3). The identities of the transcripts for VDR and THRs were analysed by restriction enzyme mapping of the amplified products from a skin sample. As seen in Fig. 4, the fragments obtained after restriction enzyme mapping correlated to the expected sizes (Table II).

DISCUSSION

We used RT-PCR to detect the nuclear receptors for retinoids (all-trans retinoic acid and 9-cis-retinoic acid), vitamin D_3, and triiodothyronine in human skin and cultured epithelial cells. RT-PCR is more sensitive than Northern blot, requires less material, and is less time-consuming if many transcripts are studied simultaneously. Amplification of reversedly transcribed RNA without using internal control cDNA has the disadvantage of not allowing exact quantitation of a given mRNA species, but then this piece of information is usually of little value when investigating a heterogenous tissue such as skin. Our data based on RT-PCR confirm some of the previous results obtained with Northern blot technique, showing that human skin, cultured keratinocytes, and HeLa cells all express the nuclear retinoid receptors RARα, RARγ and RXRα. (18, 19). Recently, we succeded in detecting also

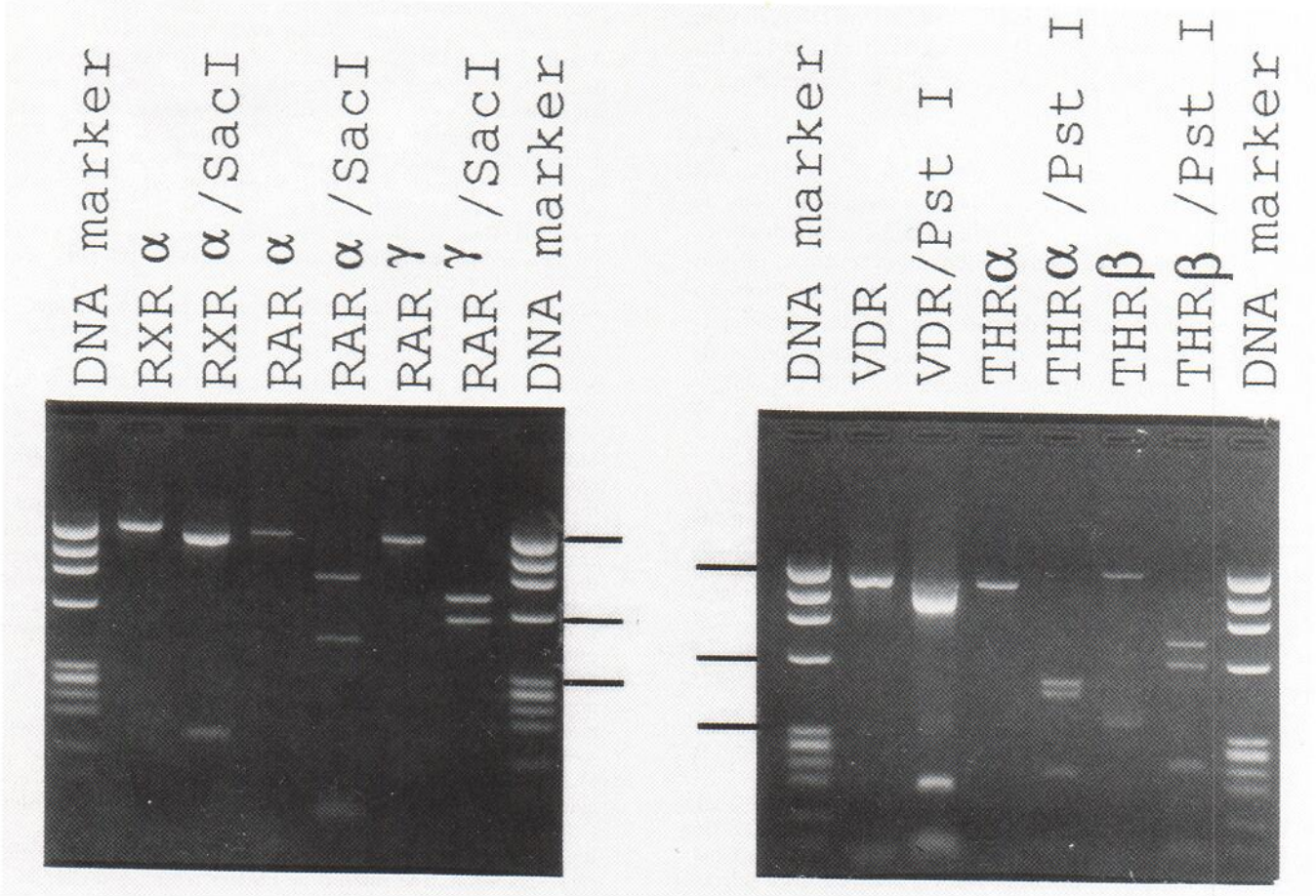

Fig. 4. Detection of mRNA for nuclear receptor proteins following restriction enzyme cutting of the corresponding RT-PCR products (see Material & Methods for details). The molecular weight markers are the same as shown in Fig. 1. The 1353, 603, and 310 bp fragments are outlined.

RXRβ (Törmä et al., unpublished observation). The RXRβ transcript was originally found by Northern blot analysis of human skin and cultured keratinocytes (13). The third member of the RXR family, RXRγ, has not been found to be expressed in human skin or cultured keratinocytes (13).

The expression of vitamin D_3 and thyroid hormone receptors was found in skin specimens, cultured keratinocytes, and HeLa cells. VDR has previously been demonstrated immunohistochemically, in normal and psoriatic skin by Milde et al. (20). They found no differences in the epidermal expression of VDR in normal and non-lesional psoriatic skin, but lesional skin expressed large amounts of the receptors. We look forward to learning how these findings relate to mRNA expression of VDR.

To our knowledge, nothing has been published about the mRNA expression of thyroid hormone receptors in human skin. In the present study, we found the two isoforms THRα1 and THRβ1 to be similarly expressed in skin samples and cultured keratinocytes. We excluded other isoforms, such as THRα2 and THRβ2 because THRα2 does not bind any thyroid ligand (21), and because THRβ2 has been reported to be exclusively expressed in the anterior pituitary gland (22).

The study of transcripts for RARs, VDR, and THRs in skin disorders is interesting because their ligands influence epidermal differentiation both *in vitro* and *in vivo* (1–5). For example, keratin expression, which differs between basal cells and differentiated keratinocytes, is modulated by retinoids and triiodothyronine (23, 24), and certain keratin genes are under the direct control of RARs and THRs (5) but not of VDR (25). Further, retinoids inhibit the expression of epidermal transglutaminase at pre-translational level (26). Transglutaminase catalyses the formation of cornified envelopes in the upper parts of the epidermis. Vitamin D_3 also influences epidermal transglutaminase, an effect apparently mediated via a VDR-receptor (27). Also, the expression of the osteocalcin gene is regulated by vitamin D_3 and VDR at the transcriptional level, at least in skeletal tissue (28). The regulatory region of the osteocalcin gene binds both VDR and RARs, suggesting "cross-talk" between these receptors in the regulation of osteocalcin transcription (28). It is thus possible that several of the proteins involved in epidermal differentiation are regulated at the transcriptional level by the combined effect of retinoids, triiodothyronine, and vitamin D_3.

The second class of retinoid receptors, the RXRs, has not yet been found to regulate expression of any genes involved in keratinocyte differentiation, but in other cell systems RXRs form homodimers that recognize specific response elements, for example, in the genes for CRBPII and apolipoprotein A1 (29–31). Unlike other receptors, RXRs bind to DNA with increased affinity in the presence of its natural ligand, 9-cis-

retinoic acid (31). Further, RXRs stabilize the DNA binding of RARs, VDR, and THRs by forming heterodimers (15, 32), which suggests that RXR plays a central role in a multitude of hormone signalling pathways.

By using RT-PCR it is thus possible to amplify mRNA transcripts for nuclear receptors in small skin samples and cultured cells. The technique will make it possible to study the role of these receptors, for example, in skin diseases characterized by disturbed epidermal differentiation. Further, the amplified products can be sequenced to study possible chromosomal translocations, such as described for RARα in acute promyelocytic leukaemia (33), or point mutations as described for THRβ in generalized thyroid hormone resistance (34, 35).

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