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## Abstract

**Background:** Retinoids are very potent inducers of cellular differentiation and apoptosis, and are efficient anti-tumoral agents. Synthetic retinoids are designed to restrict their toxicity and side effects, mostly by increasing their selectivity toward each isotype of retinoic acids receptors (RARα,β, γ and RXRα, β, γ). We however previously showed that retinoids displayed very different abilities to activate retinoid-inducible reporter genes, and that these differential properties were correlated to the ability of a given ligand to promote SRC-1 recruitment by DNA-bound RXR:RAR heterodimers. This suggested that gene-selective modulation could be achieved by structurally distinct retinoids.

**Results:** Using the differential display mRNA technique, we identified several genes on the basis of their differential induction by natural or synthetic retinoids in human cervix adenocarcinoma cells. Furthermore, this differential ability to regulate promoter activities was also observed in murine P19 cells for the RARβ2 and CRABPII gene, showing conclusively that retinoid structure has a dramatic impact on the regulation of endogenous genes.

**Conclusions:** Our findings therefore show that some degree of selective induction or repression of gene expression may be achieved when using appropriately designed ligands for retinoic acid receptors, extending the concept of selective modulators from estrogen and peroxisome proliferator activated receptors to the class of retinoid receptors.

## Background

Retinoic acids exert profound effects on cellular differentiation and proliferation. In many cases, retinoids display anti-tumoral activities [1,2] which are characterized by a retinoid-induced cell cycle arrest in the $G_0/G_1$ transition phase [3] These biological properties are either due to transcriptional upregulation of target genes through a well defined mechanism [reviewed in [4]] or/and mediated through the ability of retinoids to interfere with the activation of transcription factors controlling proliferative responses of cells to mitogenic stimuli such as AP-1. Transcriptional activation by retinoids is mediated through two families of nuclear receptors, *all-trans* retinoic acid (RARs) and 9-cis retinoic acid receptors (RXRs),

whereas interference with AP-1 is likely to be due to the inhibition of signalling pathways controlled by membrane receptors [5,6] or to protein:protein interactions [7,8] Modification of the *all-trans* retinoic acid structure to improve the specificity and/or the potency of naturally occuring molecules led to the synthesis of a number of compounds characterized by the cyclization of the polyenic chain of *all-trans* retinoic acid and the addition of various groups at different positions. These conformationally restricted retinoids are now used to achieve selective activation of RAR isotypes α, β or γ *in-vitro* and *in-vivo*, which reduces side-effects in therapeutical applications. Synthetic retinoids mimic some of *all-trans* retinoic acid biological effects *in-vivo*, but interact differently with the ligand binding domain of RARα and induce distinct structural transitions of the receptor [9,10]. We have demonstrated that RAR-selective ligands have distinct quantitative activation properties which are reflected by their ability to promote interaction of DNA-bound hRXRα/hRARα heterodimers with the nuclear receptor coactivator (NCoA) SRC-1*in-vitro*[11] The hormone response element core motifs spacing has a determining influence of RXR:RAR DNA-binding activity, by defining the relative affinity of liganded heterodimers for NCoAs. hRXRα AF2 was critical to confer hRARα full responsiveness, but not differential sensitivity of hRARα to natural or synthetic retinoids. These findings suggested that the use of physically distinct NCoA binding interfaces may be important in controlling specific genes by conformationally restricted ligands and may affect the overall activity of synthetic retinoids *vs* natural molecules.

Previous studies have demonstrated that synthetic retinoids can not only be isotype-selective, but also display a certain degree of selectivity toward defined receptor-RARE combinations [12,13]. The role of the ligand structure is emphasized by our recent observations [11], which suggested that further refinement in gene selectivity could be achieved by altering NCoA interaction surfaces. Selective recruitment of p300 or CBP has indeed been shown to be required for selective activation of $p21^{Cip1}$ and of $p27^{Kip1}$ genes respectively [14]. Since transcriptional activation is the end result of multiple interactions between the receptor, its dimerization partner, DNA and ligand, one may speculate that conformationally restricted retinoids with highly selective biological activities may be designed. Beside the tremendous interest for therapeutical applications, this raised the possibility that such retinoids display distinctive abilities to activate endogenous target genes. To further test this hypothesis, we have used the differential display technique as described by Liang and Pardee [15] to investigate the differential regulation of genes by natural and synthetic retinoids in a human cervical carcinoma cell line (HeLa). A first screening allowed to isolate and to clone 140 ESTs that were differentially induced or repressed by retinoids. In this paper, we report the characterization of two genes which are down-regulated by retinoids, and show that differential regulation is observed in different cell types.

## Results

### *Expression of retinoic acid receptors and of nuclear corepressors and coactivators in HeLa cells*

HeLa cells are known to express low levels of endogenous *all-trans* (RARs), 9-cis retinoic acid receptors (RXRs) and nuclear coactivators and corepressors. However, relative levels of expression of these proteins have not been monitored in this cell line and thus a comprehensive study was carried out to characterize mRNA levels coding for each protein. Using RT-PCR amplification of specific transcripts from total RNA, we observed that hRARα, hRXRα and hRXRβ were predominantly expressed in this cell line (Figure 1A). Using nested PCR primers, trace amounts of hRARβ were detected (Figure 1B), whereas hRARγ and hRXRγ were not detectable in these conditions (note that amplification products in the corresponding lanes are non specific amplification products and did not match the predicted size of amplified cDNA). We then assayed similarly expression levels for nuclear coactivators AIB1 [16], CBP [17], p300 [18], p/CIP [19], RAC3 [20], RIP140 [21], SRC1 [22], TIF1 [23], TIF2 [24] and TRIP1 [25]. As shown in Figure 1C, each coactivator mRNA was detectable in HeLa cells, and we also observed by Western blot analysis that members of the DRIP/TRAP coactivator family [26] are also expressed in this cell line (see panel E). Expression levels of nuclear corepressors N-CoR [27] and SMRT [28] were also assessed (Figure 1D). Among these two corepressors, only SMRT/TRAC2 was found to be significantly expressed. Two amplification products were characterized, reflecting the occurrence of two transcripts of SMRT as described previously [29].

Thus HeLa cells express most of coactivators described so far, and SMRT was found to be expressed at significant levels. Retinoic acid receptors hRARα, hRXRα and hRXRβ were predominantly expressed, with trace amounts of hRARβ. We note that the expression of this receptor was barely inducible upon atRA treatment (data not shown), suggesting that cell-specific features may condition the responsiveness of endogenous genes to retinoids. However, we also observed that transiently transfected reporter genes bearing consensus retinoic acid response elements (RARE) are fully inducible in this cell line [11], raising the possibility that chromatin assembly on DNA templates strongly regulates retinoid responsiveness [30]. The expression level of SMRT and of several coactivators was also confirmed by western blot analysis of HeLa whole cell extracts (Figure 1E). This analysis confirmed that most of coactivators are coexpressed in this cell line, as well as very

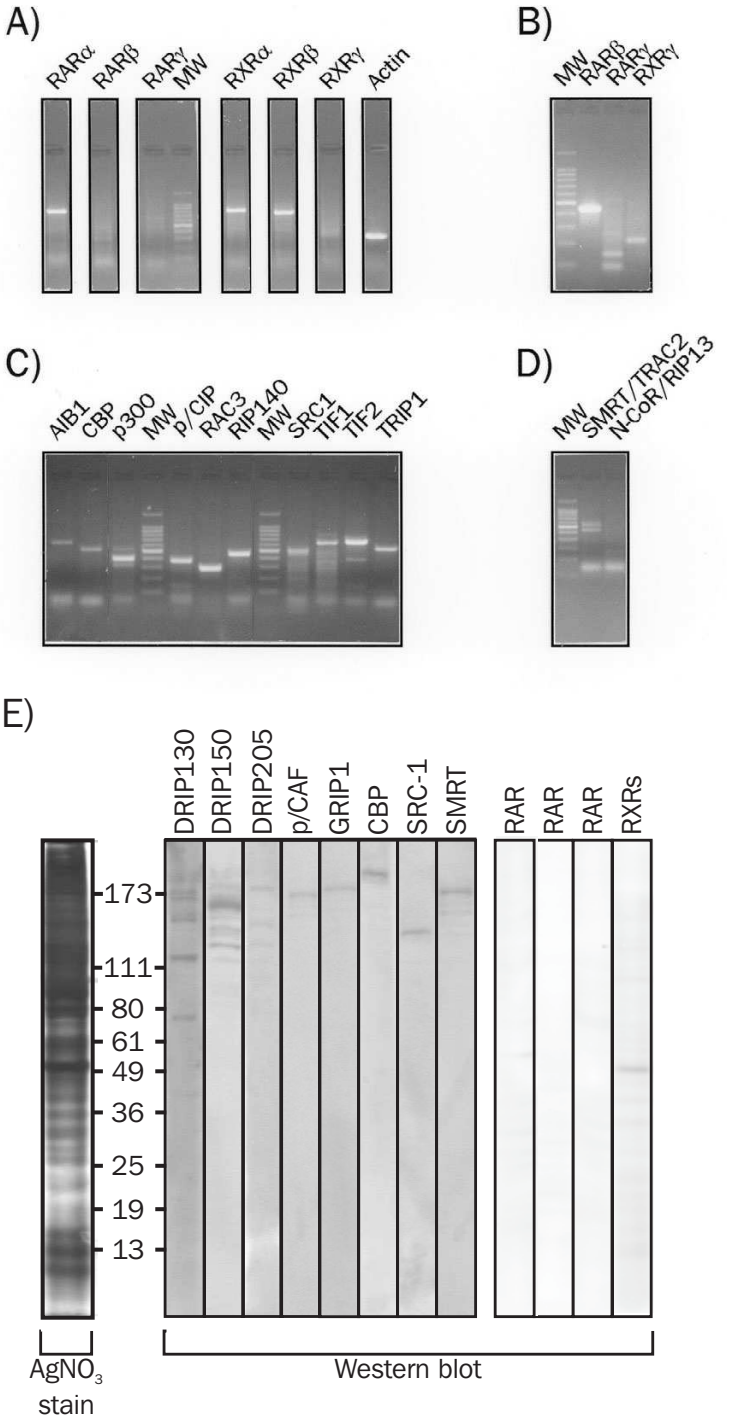


**Figure 1**

*Expression levels of retinoic acid receptors and of transcriptional intermediary factors in HeLa cells.* HeLa cells mRNA was extracted and analyzed by RT-PCR using specific primers to detect (A) hRARα, hRARβ, hRARγ, hRXRα, hRXRβ and hRXRγ transcripts; to confirm the lack of expression of hRARβ, hRARγ and hRXRγ by nested PCR (B) to characterize expression levels of nuclear coactivators (C) and nuclear corepressors (D). E) Western blot analysis of HeLa whole cell extracts. 100 μg of proteins were resolved by 8% SDS-PAGE and blotted onto a nitrocellulose membrane. This membrane was probed with antibodies specific for each indicated receptors, coactivators or corepressor. The left panel shows a silver-stained gel on which 10 μg of cell extract has been separated. Molecular masses are indicated in kDa.

low amount of RARα and detectable amounts of RXRs (available antibodies are not isotype-selective).

### *Identification of genes differentially expressed in response to retinoid treatment*

Thus modulation of gene expression by RAR-specific retinoids may be expected to be mostly dependent upon ligand binding to hRARα and dimerization with either RXRα or RXRβ

Total RNA was then extracted from HeLa cells treated for 4 hours with 1μM atRA, our reference compound, 1μM CD3106/AGN 193109, a RAR-specific antagonist [31], 1μM TTNPB and 20 nM Am580, two synthetic RARα-specific agonists, 1μM CD367, a RAR-specific agonist [32] and 1μM CD2425, a RXR-specific retinoid. mRNAs were then randomly amplified using various combinations of oligodT anchored primers (see legend to figure 2 for more details) and arbitrary chosen primers in the presence of α-[$^{33}$P]-labelled dATP. Products were visualized by autoradiography of high resolution sequencing gels. Examination of autoradiographies allowed the identification of several mRNAs species that were specifically induced upon treatment with some of the retinoids described above. Typical results are shown in Figure 2 for four different sets of primers which allowed the amplification of differentially expressed genes. This differential expression was noted for about 60% of the primer sets, while others did not show any significant variations. Based on visual examination of 40 sequencing gels, cDNAs were extracted from the gel, reamplified by PCR using the same set of primers than that used in the initial RT-PCR reaction, cloned in the PCR-Trap vector by T/A cloning, and sequenced. 140 cDNAs were identified (hereafter noted synthetic retinoid-induced genes or SRIG) following this procedure and could be classified into three categories: (i) sequences with no homology with any known human genes; (ii) sequences overlapping with previously identified ESTs and (iii) sequences homologous to genes with known function(s). These results are summarized in Table 1 and Table 2. 28 new ESTs were identifed and sequences were deposited in GenBank.

To further validate our initial screening, probes ranging in size from 200 to 350 bp were obtained by PCR using primers flanking the insertion site of the cDNA. 96 probes were thus synthesized, spotted onto a nylon membrane to generate cDNAs arrays. Total RNAs extracted from HeLa cells treated for 4 hours by the indicated retinoid (see Figure 3) was reverse-transcribed in the presence of α-[$^{32}$P] dCTP and hybridized to membranes. Twenty genes showed marked differential regulation in this secondary screening, exhibiting various pharmacological profiles (data not shown). Of interest were genes found to be induced upon treatment with the RAR antagonist CD3106 (SRIG 61, 62 and 150). Two genes were repressed upon atRA treatment, but induced in the presence of other synthetic agonists (SRIG 146 and 177) and three were activated to various extent by retinoid agonists (SRIG 126, 148, 169). Other genes followed a more complex pattern which does not match a simple relationship between RAR transcriptional activation, pharmacological properties of retinoids and gene expression levels, reflecting a likely involvement of multiple, retinoid-induced steps in gene regulation.

### *Characterization of genes differentially regulated in response to retinoid treatment*

Search against EMBL/GenBank databases using the Blast server identified the human initiation factor 4B (hIF4B, SRIG 69), human apoferritin H (SRIG 157) and human TAXREB 107 (SRIG 177). We also noted that the human plasminogen activator was identified in this screen, which is a gene known to be activated by retinoids [33], as well as the ubiquitin conjugating enzyme UCE, which was identified recently by a similar approach as an atRA-inducible gene in acute promyelocytic leukemia (APL) cells [33,34]. Northern blot analysis of HeLa cells RNA was thus used to assay the rate of expression of several genes after retinoid treatment and results are shown for ferritin H and TAXREB 107 (Figure 3 and 4).

Homeostasis of iron is mostly dependent on two cellular proteins, ferritins H and L. Ferritin L has been shown to be regulated in HeLa cells by iron, while ferritin H is not regulated by this metal [35]. Northern blot analysis of HeLa cells RNA showed that the SRIG 157 clone, identified as ferritin H in our screen, was clearly down-regulated (Figure 3) by some retinoids. The most active were Am580, CD367, and the RXR-selective ligand CD2425. Thus these retinoids, which do not share receptor binding properties, repressed apoferritin H expression, in contrast to atRA which was inactive in this test.

TAXREB107 binds to tax-responsive elements and thus have functions in the context of HIV infection, by mediating the DNA binding of the HTLV-1 transactivator Tax. It was also identified as a ribosomal protein, and we noted that the ribosomal protein L27a was also identified several times in our screening procedure (Table 2 and data not shown). Again, this mRNA was submitted to a reproducible, selective down-regulation in the presence of TTNPB, CD367 and CD2425 (Figure 4). Thus although no prediction could be drawn from the selectivity of a given ligand for a RAR isotype, this result establishes again that retinoids have distinct abilities to modulate the rate of expression of several cellular genes.

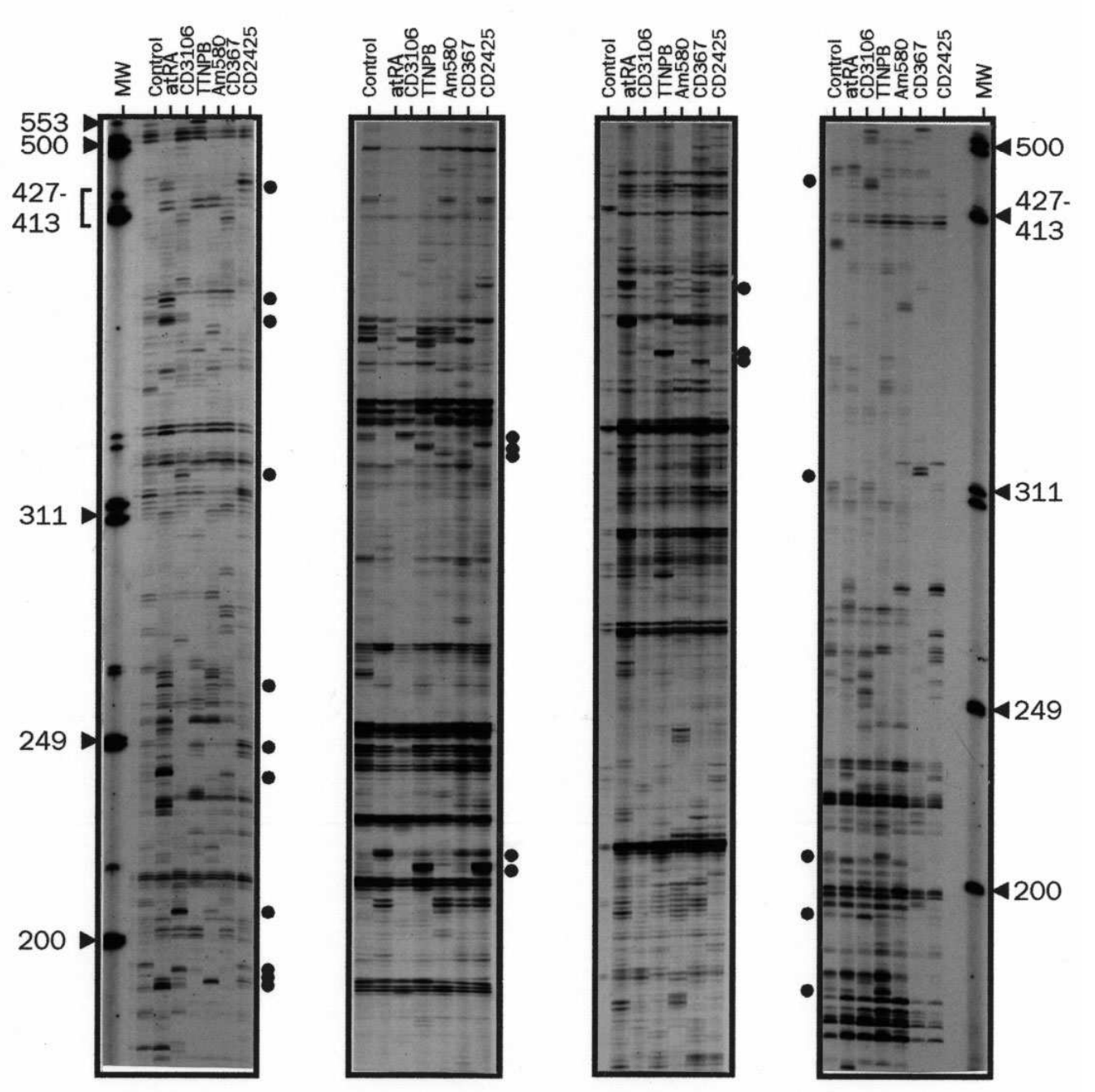


**Figure 2**
Differential expression of mRNA species in HeLa cells treated by retinoids. Differential display RT-PCR analysis of HeLa transcripts obtained from cells treated with 1μM of the indicated retinoid for 4 hours. Total RNA was extracted and purified from HeLa cells and reverse-transcribed with the H-$T_{11}$G (AAGC$T_{11}$G, left panel), H-$T_{11}$A (AAGC$T_{11}$A, middle panel) or H-$T_{11}$C (AAGC$T_{11}$C, right panel) primers. PCR amplification of cDNAs was carried out using the same 3' primer and the H-AP3 primer (AAGCTTTGGTCAG), the H-AP6 primer (AAGCTTGCACCAT), the H-AP12 primer (AAGCTTGAGTGCT) and the H-AP15 primer (AAGCTTACGCAAC) (from left to right) in the presence of α-[33P] dATP. Amplified cDNA fragments were analyzed on 6% sequencing gels and visualized by autoradiography. Typical lanes are shown, with size markers appearing on the left. Selectively regulated mRNAs are indicated by dots. These materials were extracted from the gel, re-amplified by PCR with the same set of primers, cloned into the pCR-TRAP vector (GenHunter) and sequenced.

**Table 1: Summary of newly identified ESTs and of known ESTs potentially regulated by retinoids.**

**A) New sequences**

| Name | Homology | GenBank Accession Numbers | Northern |
|---|---|---|---|
| SRIG 23*, SRIG 28*, SRIG 33.1*, SRIG 53*, SRIG 56*, SRIG 61, SRIG 74, SRIG 81, SRIG 86, SRIG 89, SRIG 90, SRIG 102, SRIG 105, SRIG 107, SRIG 118*, SRIG 119, SRIG 124, SRIG 131, SRIG 144, SRIG 145, SRIG 148, SRIG 154, SRIG 160, SRIG 164, SRIG 178, SRIG 179, SRIG 181, SRIG 185-1. | No significant homology | AI374463*, AI374461*, AI374438*, AI376338*, AI374462*, AI374456, AI374464, AI374455, AI376315, AI374453, AI37441, AI374452, AI374451, AI374450, AI374449*, AI374448, AI374447, AI374446, AI376322, AI376323, AI376324, AI374443, AF096777, AI376330, AI374465, AI376335, AI374444, AI374445 | No signal*, other clones were not tested. |

**B) Identified ESTs**

| | | | |
|---|---|---|---|
| SRIG 8 | 100% with EST AA931835 | AI376308 (Unigene Hs 181165) | No signal |
| SRIG 29 | 100% with EST AA027854 | AI376309 (Unigene Hs 8117) | No signal |
| SRIG 30 | 100% with EST AA534569 | AI376310 (Unigene Hs 13836) | No signal |
| SRIG-62 | 100% with KIAA0043 gene | | |
| SRIG 63 | 74% with EST AA699895 | AI376312 (Unigene Hs 117353) | N.D. |
| SRIG 67 | 90% with EST AA826918 | AI376313 | N.D. |
| SRIG 80 | 89% with EST AA568770 | AI376314 | N.D. |
| SRIG 100 | 97% with EST R02820 | AI376317 (Unigene Hs 31921) | N.D. |
| SRIG 101 | 99% with EST AA449652 | AI376318 (Unigene Hs 11803) | N.D. |
| SRIG 108 | 80% with EST AA205076 | AI374442 (Unigene Hs 17872) | N.D. |
| SRIG 123 | 94% with EST C75518 | AI376319 (Unigene Hs 61184) | N.D. |
| SRIG 134 | 96% with EST AA983976 | AI376320 (Unigene Hs 127105) | N.D. |
| SRIG 135 | 96% with EST AA093075 | AI376321 (Unigene Hs 49015) | N.D. |
| SRIG 150 | 100% with EST AA768579 | AI376326 (Unigene Hs 22549) | N.D. |
| SRIG 153 | 97% with EST AA768579 | AI376327 (Unigene Hs 112227) | N.D. |
| SRIG 156 | 100% with EST AA505468 | AI376329 (Unigene Hs 58609) | N.D. |
| SRIG 165 | 99% with EST AI097038 | AI376331 (Unigene Hs 156103) | N.D. |
| SRIG 173 | 99% with EST AA838424 | AI376333 (Unigene Hs 110978) | N.D. |

A) Sequences were searched against GenBank and no significant homologies were found. Sequences were deposited in GenBank and accession numbers are indicated. B) Identification of previously identified ESTs as potential targets for retinoid modulation. GenBank accession numbers are given, as well as the Unigene family number. ND: not determined.

**Table 2: Summary of genes with known functions as potential targets for retinoid modulation.**

| Name | Homology | Northern |
|---|---|---|
| SRIG 1 | 100% homology with ZNF beta | No signal |
| SRIG 14 | 99% homology with human ribosomal protein L27a | N.D. |
| SRIG 15 | 98% homology with human nuclear protein 55 | No differential regulation |
| SRIG 16 | 100% homology with human fibrilline-2 | No signal |
| SRIG 19 | 100% homology with cytochrome oxydase II | No differential regulation |
| SRIG 24 | 100% homology with human ubiquitin conjugating enzyme | No differential regulation |
| SRIG 45 | 100% homology with human thymidilate synthase | No differential regulation |
| SRIG 52 | 91% homology with human phosphate cyclase | No signal |
| SRIG 62 | Brd3-human bromodomain-containing protein 3 (RING3-like protein) | N.D. |
| SRIG 69 | 97% homology with human initiation factor 4B | Differential regulation |
| SRIG 71' | 77% homology with human spermine/spermidine acetyl transferase | N.D. |
| SRIG 76 | 97% homology with human 5T4 oncofetal antigen | No signal |
| SRIG 93 | 98% homology with human histone H2B.2 | No differential regulation |
| SRIG 96 | 79% homology with human TRIP7 | No signal |
| SRIG 106 | 99% homology with epilepsy holoproencephaly candidate protein-1 | No signal |
| SRIG 112 | 97% avec protein phosphatase | No signal |
| SRIG 113 | 97% with human NaCl electroneutral thiazide-sensitive transporter | No signal |
| SRIG 114 | 98% human 60S ribosomal protein | N.D. |
| SRIG 120 | 99% homology with EST similar to human TRAM protein | N.D. |
| SRIG 121 | 98% homology with human aspartyl beta hydroxylase | N.D. |
| SRIG 126 | 100% with human protein kinase C binding protein Nel | No signal |
| SRIG 128 | 96% homology with human enolase | N.D. |
| SRIG 142 | 85%homology with human carbamyl phosphate synthase | No signal |
| SRIG 157 | 95% homology with human apoferritine H | Differential regulation |
| SRIG 158 | 99% homology with human cytochrome B | No differential regulation |
| SRIG 165' | 96% homology with human CG1 | No signal |
| SRIG 169 | 100% homology with human duplicate spinal muscular atrophy | N.D. |
| SRIG 174 | 99% homology with spermidine acyl transferase | No signal |
| SRIG 177 | 99% homology with human TAXREB 107 | Differential regulation |
| SRIG 185-2 | 98% homology with human plasminogen activator | N.D. |

Sequences were identified according to their homology with previously identified genes. The degree of homology is indicated for each mRNA, as well as the name of the gene. Northern blot analysis results are indicated when available. ND: not determined.

### *The RARβ2 and CRABPII promoters respond differentially to retinoids in murine embryonal carcinoma cells*

Murine pluripotent P19 cells can differentiate into endodermal and mesodermal cells after retinoic acid treatment (reviewed in [36]) and have a number of characteristics which make them suitable for analysis of RA-mediated gene induction [37]. Retinoic acids receptors RARα, RARγ and RXRγ are constitutively expressed in this cell line (data not shown), whereas RARβ is induced upon atRA treatment (Figure 5A). The cellular retinoic acid binding protein type II (CRABPII) gene expression is also regulated by retinoids (Figure 5B and [38]). To further investigate the possibility that retinoids have differential abilities to stimulate gene expression in a similar cellular background, we monitored RARβ and CRABPII expression by Northern blotting and RT-PCR upon stimulation by limited concentrations (2x Kd) of several natural or synthetic retinoids. atRA turned out to be a good inducer of the RARβ promoter, with transcripts becoming detectable after 4 hours of induction. mRNA accumulation reached a plateau after 8–10 hours of induction, after which a clear, specific down-regulation was observed. (Figure 5A). Other retinoids could be distinguished on the basis of the rate of induction and of their efficiencies to promote RARβ mRNA accumulation. TTNPB was a strong inducer in our system, as well as CD367. Am580 caused a time course of mRNA accumulation slower when compared to that observed with atRA and other retinoids, and yielded maximal mRNA levels increased by 3-fold when compared to atRA. The CRABPII gene transcriptional regulation by retinoids exhibited a very different time-course, characterzed by a very slow accumulation of mRNA at early time points (<8 hours) with synthetic retinoids (Figure 5B). atRA, on the contrary, induced a moderate but rapid induction of the CRABPII mRNA synthesis, reaching a plateau in less than 8 hours, whereas all other agonists were weak inducers at early time points, and were much more potent at 24 and 48 hours.

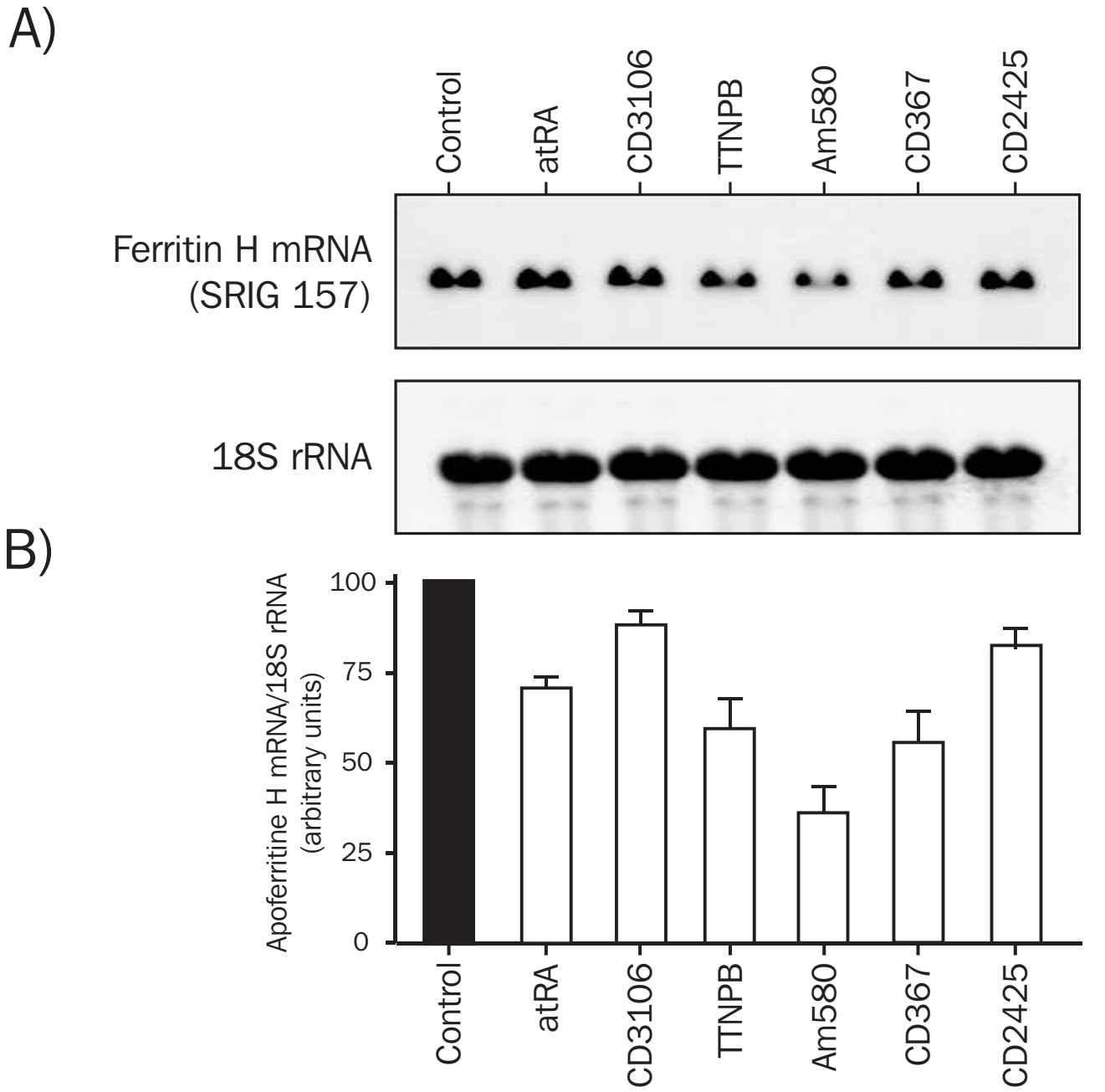


**Figure 3**
Expression of ferritin H mRNA in HeLa cells treated with different ligands of the retinoic acid receptor. HeLa cells were treated with the different ligands for four hours. Total RNA (20 μg) was probed sequentially with fluorescein-labeled partial human ferritin H cDNA and 18S rRNA probes. Blots were quantified using a Storm™ apparatus. Values for the ferritin H transcript were normalized to the 18S rRNA level. *A) Upper panel:* ferritin H transcript in HeLa cells treated with various retinoids. *Lower panel*: 18S rRNA. B) Quantification of ferritin H expression. Results are presented as the mean +/- S.E.M. of three different experiments. Cells were treated with 25nM atRA, 30nM CD3106, 80nM TTNPB, 20 nM Am580, 10nM CD367 and 100nM CD2425 for 4 hours.

Ranking of retinoids for their potency is therefore similar when assessing both RARβ2 and CRABPII mRNA accumulation at 48 hours (TTNPB>CD367>Am580>atRA). However, this order of potency may considerably vary when

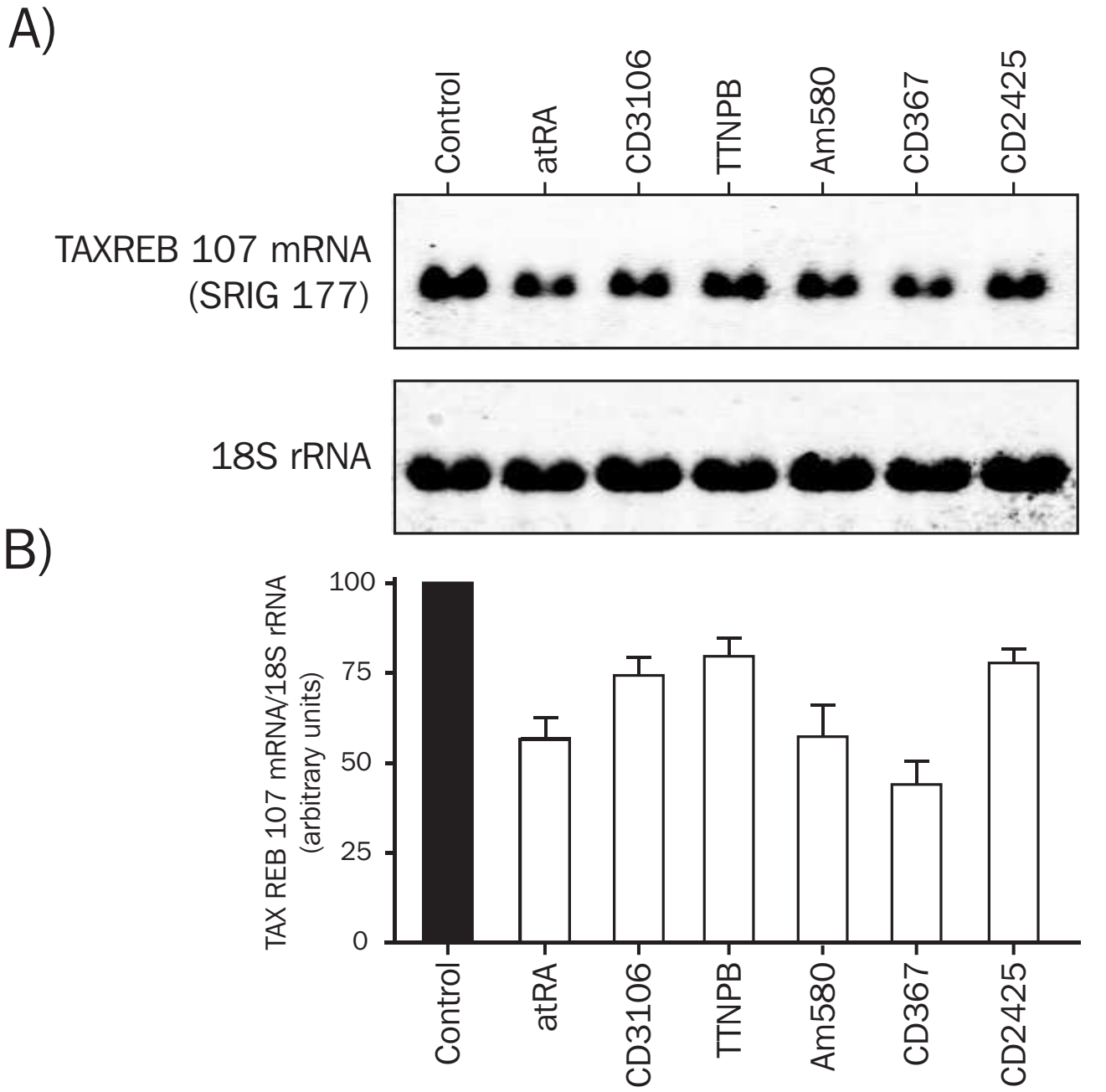


**Figure 4**
Expression of TAXREB107 mRNA in HeLa cells treated with different ligands of the retinoic acid receptor. HeLa cells were treated with the different ligands for 4 hours. Total RNA (20 μg) was probed sequentially with fluorescein-labeled partial human TAXREB cDNA and 18S rRNA probes. Blots were quantified using a Storm™ apparatus. Values for the TAXREB transcript were normalized to the 18S rRNA level. *A) Upper panel:* TAXREB107 transcript in HeLa cells treated with various retinoids. *Lower panel*: 18S rRNA. B) Quantification of TAXREB107 expression. Results are presented as the mean +/- S.E.M. of three different experiments. Retinoid concentrations were as in Figure 3.

considering earlier time points: a 2-hours induction, more likely to reflect transcriptional processes, yields the following ranking for the RARβ2 gene: CD367>atRA>TTNPB>Am580, and a 8-hours induction for the CRABPII gene gives the following ranking: atRA>CD367>TTNBP=Am580.

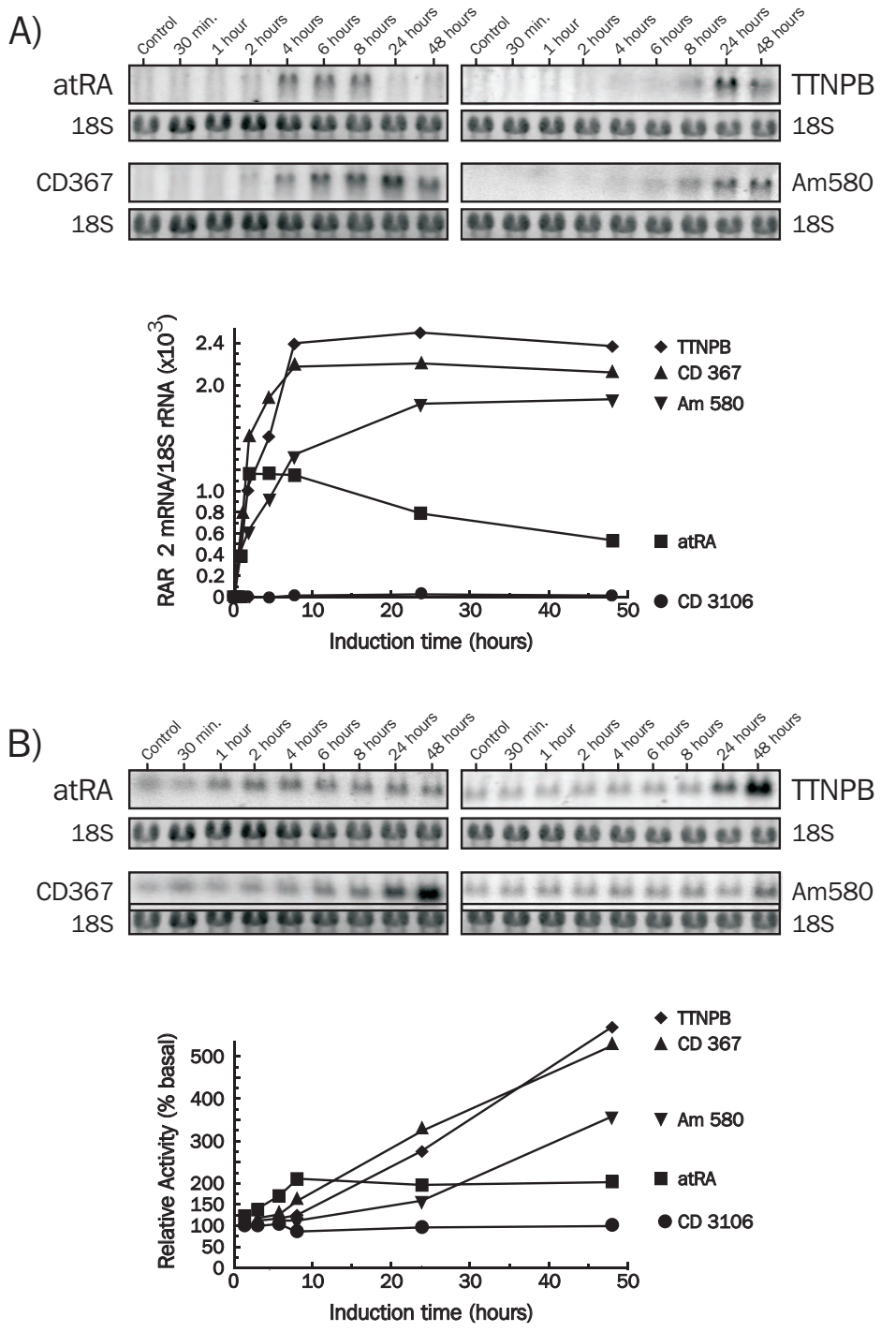


**Figure 5**
Expression of RARβ and of CRABPII in murine P19 cells treated with different ligands of the retinoic acid receptor. A) Time-course analysis of RARβ transcripts. RARβ transcripts were assayed by Northern blotting (upper panel) and quantified by densitometry. A more rigorous measurement of each time point was carried out by submitting the same sample to real time PCR quantification, using, as for the Northern blot analysis, 18S RNA as an internal standard. All results are expressed relative to RARβ level of expression in non stimulated cells. Representative autoradiograms and PCR quantification are shown here, but have carried out 3 times with similar results. B) Time-course analysis of CRABPII transcripts. Assays were carried out as described in A), setting the reference (non stimulated cells) to 100%. Retinoid concentrations were as in Figure 3.

## Discussion

Pursuant to our discovery that natural and synthetic retinoids possess distinct abilities to activate a reporter gene in an identical cellular background, and irrespective of their affinity for their cognate receptor [11], we set up a differential display approach to extend this observation to the regulation of chromatin-organized, endogenous genes. Selective induction or repression of tens of mRNAs species was observed during the initial screening, revealing that genes involved in multiple aspects of cellular regulation could be potentially regulated specifically by one or several retinoids. Only a few genes are known to be regulated by retinoids, and identification of new targets for these molecules is critical for a better understanding of the pharmacology of retinoids.

As stated by a number of investigators, the mRNA differential display method yields false positives and also identified cDNAs which were not detectable by northern blotting. However, it is worth noting that we restricted our study to genes that are induced very early by retinoids by using induction times of 4 hours, and this may be an explanation for not reaching an intracellular concentration allowing further detection. In addition, intracellular retinoid uptake and metabolic transformation of each compound may vary, and thus introduce variations which are not related directly to transcriptional regulation. Indeed, retinoid-regulated genes follow various kinetics of induction and reaching a steady state may necessitate up to 24–48 hours in P19 cells, as noted very clearly for the CRABPII gene ([39] and Figure 5). Within this cellular context, we further show that retinoids have also differential abilities to promote both RARβ and CRABPII gene transcription, giving support to our working hypothesis. This regulation appeared to vary according to the promoter.

Interestingly, we identified several genes that were down-regulated by retinoids. Apoferritin H and TAXREB107 were clearly inhibited by a specific set of retinoids (40–50% inhibition), to an extent which is considered to be highly significant in pathological states. This may also be relevant in normal conditions, when considering that a biological response is very likely to be part of an integrated signaling pathway, in which a decrease of 50% of a given step may strongly alter the end result of the activation of this pathway. For example, vascular endothelial growth factor (VEGF) has been shown to be down-regulated by retinoids by two-fold in human keratinocytes and this may be related to the therapeutic effects of retinoids in diseases such as psoriasis and Kaposi' sarcoma [40].

Retinoids have numerous side effects which severely limit dosage in clinical trials. They include skin irritation and inflammation, elevation of serum triglycerides, hypothyroidism and others such as headache (reviewed in [41]). Given the potential of retinoids in treating various disorders such as skin hyperproliferation and photoaging, cancer therapy and in metabolic disorders, it is of interest to identify systematically target genes in altered tissues. Our approach identified such candidates genes, and the availability of DNA microarrays and of the human genome sequence allows now a genome-wide search of directly or indirectly regulated genes. Combined with appropriate structure-activity relationships studies, retinoids define a very promising field in medicinal chemistry. Used alone or in combination with other powerful molecules such as histone deacetylase inhibitors, one may think of achieving a high degree of selectivity, and thereby reduce toxicity and other side effects of these promising therapeutic agents.

## Materials and methods

### *Materials*

All-trans retinoic acid was purchased from Sigma (Saint Quentin Fallavier, France). Synthetic retinoids CD3106, TTNPB, Am580, CD367 and CD2425 were obtained from Galderma Inc. (Sophia-Antipolis, France). 10 mM stock solutions were prepared in DMSO and stored at -20°C in the dark. Dulbecco's modified Eagle's medium, fetal calf serum and penicillin/streptomycin mix were purchased from Biowhittaker (BioWhittaker, Verviers, Belgium). Oligonucleotides were purchased from Eurogentec (Le Sart-Tilman, Belgium).

### *RNA preparation*

Human HeLa cells were grown in DMEM medium supplemented with 10% fetal calf serum and 1000 U/mL of penicillin and 10 μg/mL of streptomycin. Cells were treated with the indicated retinoic acid receptors ligands for 4 hours. Total RNA was prepared using RNAble reagent (Eurobio, Les Ulis, France) according to the manufacturer's protocol. Total RNA (50 μg) was then treated with 10 U RNase-free DNaseI (Genhunter, Nashville, TN, USA) for 1 hour at 37°C to digest genomic DNA. The purified RNA was adjusted to 1 μg/μl and checked for integrity by standard agarose gel electrophoresis.

### *Differential display PCR*

The differential display reaction was performed using the RNAimage™ kit as indicated by the manufacturer (Genhunter, Nashville, TN, USA). Briefly, reverse transcription was performed using 1 μg RNA and an oligodT anchored primer. The PCR reaction was carried out with the anchored oligodT primer used in all possible combinations with sixteen different arbitrary primers (HAP-1/HAP-16) in presence of [α-$^{33}$P]dATP (2000 Ci/mmol, Amersham, Les Ulis, France). Reactions mixes were submitted to 40 cycles of PCR as follows: 94°C for15 sec, 40°C for 2 min and 72°C for 30 sec followed by an elongation step at 72°C for 5 min. PCR products were then fractionated on a

8 M urea-6% polyacrylamide gel and visualized by autoradiography. Differentially expressed cDNAs were extracted, purified and reamplified under similar PCR conditions with radioinert deoxyribonucleotides. Amplified cDNAs were then cloned into the pCR-TRAP vector (Genhunter, Nashville, TN, USA) as indicated by the manufacturer. The size of the cloned insert was checked from 3 to 4 colonies and sequenced. Sequence homologies were established using Basic Local Alignement Tool against the GenBank databases. Identified ESTs were then searched against the UniGene (NCBI) database.

#### *Western-blot analysis*

Western blotting and antibodies: Whole cell extracts were prepared as follows: $5.10^6$ cells cells were grown, and monolayers were scraped rapidly in ice-cold 1× Phosphate Buffered Saline (PBS). Cells were lysed in one volume of SDS-PAGE loading buffer and briefly sonicated. Western blotting was carried out as described [11]. The DRIP205, 130 and 150 anti sera were a gift from Drs C. Rachez and L.P. Freedman. Peroxidase-coupled anti-mouse, anti-goat or anti-rabbit IgGs were from Sigma. All other antibodies were purchased from SantaCruz Biotechnology (SantaCruz, CA.).

#### *Reverse transcription and amplification (RT-PCR) of retinoic acid receptors, coactivators and corepressors mRNAs in HeLa cells*

RNA was extracted and submitted to reverse transcription as described above. Primers were designed to amplify cDNAs fragments ranging in size from 300 to 600 bp and were as follows: hRARα, 5'-CCATTGAGACCCAGAGCAGC-3' and 5'-TGTGTCCATGTGGCGTGGGC-3'; hRARβ, 5'-CAATTGAAACACAGAGCACC-3' and 5'-CCACCAAGTGGTGACTGACTG-3'; hRARγ, 5'-TGGAGACACAGAGCACCAGC-3' and 5'-GTCAGTCTGCTGCCTGAAGC-3'; hRXRα, 5'-CTCCTCAAGCAAGCACTATG-3' and 5'-AGAGCTTAGCGAACCTTCCC-3'; hRXRβ, 5'-TCAGGCAAACACTACGGGGT-3' and 5'-GCATACACTTTCTCCCGCAG-3'; hRXRγ, 5'-CTCAGGAAAGCACTACGGGG-3' and 5'-CCGGATACTTCTGCTTGGTG-3'; AIB1, 5'-GAGCCGACAGGCACTTGAAT-3' and 5'-CCACTGCTGCCATTCATGTG-3'; CBP, 5'-CGCTCAGATGGGACAGCTTG-3' and 5'-ACTTCTCTAGCGTGTCCCCC-3'; p300, 5'-TGGGGTCCCCTGTTCAGC-3' and 5'-GTTATCGGTGCTGAGTCCCAGG-3'; p/CIP, 5'-AAGCCCCTCCACAACAGTTT-3' and 5'-CAGCAGTATTTCTGATCGGG-3'; RAC3, 5'-CCAGATCCAGCCTTTGGTCG-3' and 5'-ATGCCAGACATGGGCATGGG-3'; RIP140, 5'-TCAGCCCAGCAGTTGCATGG-3' and 5'-TCCATTTGCGCTGTGTGGGC-3'; SRC1, 5'-AATGTGTTCAGTCAAGCTGTCCAG-3' and 5'-TGGTTATTCAGTCAGTAGCTGCTG-3'; TIF1, 5'-CCAATGAGGACTGGTGTGCAG-3' and 5'-GCTTTTGAGGCGTTTCTTCCG-3'; TIF2, 5'-CTGAACCAGCATCTTCGAACA-3' and 5'-ATTTCCGTGTTGTGTCTCCC-3'; TRIP1, 5'-GGCTGTGGCTCATCATACGG-3' and 5'-TGAGTGACATGGACTCGCCG-3'; SMRT, 5'-TGACCTATAGAAGCCAGGC-3' and 5'-GAGAGTGTCTCGTACTGCG-3'; N-CoR, 5'-GATCATGGTGTTGTCATGTCC-3' and 5'-AGACAGTGTCTCATACTGCGC-3'. Actin primers were, 5'-ATCATGTTTGAGACCTTCAA-3' and 5'-CATCTCTTGCTCGAAGTCCA-3'. Linearity was assessed as described above.

#### *Reverse transcription and amplification (RT-PCR) of CRABPII and RARβ2 transcripts in P19 cells*

Reverse transcription was performed using oligodT primers as recommended by the manufacturer (Promega, Charbonnières, France). Primers were designed as follows:RARβ 5'-AAGTGGTAGGAAGTGAGCTG-3' and 5'-CTACATTGAGCAGTATGCCG-3 and CRABPII 5'-CCAGGTGGAAGGATCTGTTC-3' and 5'-ATTGGTCAGTTCTCGGCTCC-3'. PCR conditions were 40 cycles of 30 sec at 94°C, 1 min at 58°C and 1 min 30 sec at 72°C followed by an elongation step at 72°C for 7 min (RARβ) and 30 cycles of 30 sec at 94°C, 1 min at 56°C and 1 min 30 sec at 72°C followed by an elongation step at 72°C for 7 min (CRABPII).

#### *Northern Blot analysis*

20 μg of total RNA were separated by electrophoresis through a 1% agarose gel containing 0.62 M formaldehyde. RNA was then transferred to a Hybond-$N^+$ membrane (Amersham, Les Ulis, France). Membranes were probed sequentially with cDNA of interest and an 18S rRNA probe. Probes were labeled with Fluorescein-dUTP using the Random Prime labelling module (Amersham, Les Ulis, France). Prehybridization and overnight hybridization at 65°C were performed in 5X SSC (1X SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7), 0.1% SDS, 5% dextran sulfate and 5% liquid block (Amersham, Les Ulis, France). After hybridization, membranes were washed at 65°C successively in 2X SSC, 0.1% SDS, 1X SSC, 0.1% SDS, 0.5X SSC, 0.1% SDS, and 0.1X SSC, 0.1% SDS.

Hybridized probes were revealed using the ECF amplification system (Amersham, Les Ulis, France), and visualized using a Storm™ phosphofluoroimager (Molecular Dynamics, Sunnyvale, CA). Bands intensities were quantified using the ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA). Values for mRNA of interest were normalized to values for the 18S rRNA.

#### *Real-Time PCR*

After purification of RNAs and reverse-transcription as described above, the synthesized cDNAs were analyzed by PCR amplification using the TaqMan PCR master mix (Applied Biosysytems, Foster City, CA.) and the appropri-

ate mix of primers. Typically, a mix of 18S mRARβ promoter primers was used. 18S primers were purchased from Applied Biosystems. The FAM/TAMRA probe, forward and reverse primers for the mRARβ transcript were CAGCACCGGCATACTGCTCAA, TCAGTGGATTCACCCAGGC (RAR468F) and TCGGGACGAGCTCCTCAG (RAR557B). Reactions (40 cycles) and data analysis were carried out on a ABI Prism 7700 (Perkin-Elmer).

## Authors' contributions

Céline Brand and Pascaline Ségard carried out the differential display screening, analysis of clones and P19 experiments. Pascal Plouvier, Pierre Formstecher and Pierre-Marie Danzé participated to the HeLa cell characterization (receptors and coactivators) and Philippe Lefebvre conceived and coordinated the study.

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