Genetic analysis of peroxisome proliferator-activated receptor γ1 splice variants in human colorectal cell lines

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Abstract. Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor family. In colon, this transcription factor is involved in differentiation of absorptive cells. PPARγ participates also in colon carcinogenesis and cancer progression. Two isoforms, namely PPARγ1 and PPARγ2, have been described. Recently, new PPARγ1 transcripts whose translation raises PPARγ1 protein have been characterised. They differ from each other by combination of untranslated exons localised in the 5' UTR of the *PPARG* gene. Here, we studied whether such a diversity of PPARγ transcripts occurs in human colon cell models. Based on bioinformatic analysis, putative untranslated exons were identified in the human *PPARG* gene. By RT-PCR analysis, we have demonstrated that several of these untranslated exons are included in PPARγ transcripts from colon-derived cell lines or in those derived from other tissue. Using HT-29 cells, changes in PPARγ1 mRNA levels were observed after treatment with PPARγ agonists such as pioglitazone and troglitazone. These modifications correlated with particular PPARγ transcripts excluding the untranslated exon A2. HT-29 cells treatment with actinomycin D or cycloheximide showed that the presence of PPARγ mRNA including exon A2 was dependent on *de novo* protein synthesis. We concluded that diverse PPARγ1 mRNA exist in colorectal cells. Levels of PPARγ1 transcript varied according to the phenotype of colon cell model used. We suggest that regulation of PPARγ1 mRNA levels could be dependent in part on the composition of untranslated exon(s) in the 5' UTR of PPARγ1 mRNA.

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Abbreviations: PPAR, peroxisome proliferator-activated receptor; *EST*, expressed sequence tagged; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Key words: peroxisome proliferator-activated receptor γ, human colorectal cancer, colon differentiation, splicing

Introduction

Peroxisome proliferator activated receptors (PPAR) belong to the nuclear hormone receptor superfamily (1). There are three isotypes named PPARα, PPARß/δ and PPARγ (2). These nuclear receptors act as heterodimers with the 9-*cis* retinoic acid receptor and bind to a specific DNA sequence, the peroxisome proliferator response element, a direct repeat of the 5'-AGGTCA-3' sequence spaced by one base (3,4). After ligand activation, the heterodimer complex regulates the transcription of specific genes involved in several physiological functions (2). In colon, PPARγ is implicated in differentiation of absorptive cells (5), in carcinogenesis or tumour progression even though contradictory results have been obtained in murine and human models (6).

The PPARγ gene is localised to chromosome 3, band 3p25 (7). The open reading frame consists of exons E1-E6 which encode the different domains of the protein. In human, two PPARγ isoforms have been identified and referred to as PPARγ1 and PPARγ2 (7,8). PPARγ2 differs from PPARγ1 by an additional 28 amino acid NH2-terminal sequence (8). The 5' UTR region of PPARγ transcripts, raising PPARγ1 and -2 proteins, is highly variable and three exons termed A1, A2 and B have been described (9,10). Due to different promoter usage and alternative splicing, two other PPARγ transcripts have been identified and they have been referred to as PPARγ3 and -γ4 (10,11). PPARγ1 mRNA consists of A1-A2-E1-6 exons, whereas PPARγ3 mRNA is composed only of A2-E1-6 exons. Both the exons A1 and A2 are not translated. The PPARγ4 mRNA has a transcription initiation site at the boundary of exon E1 (at position -29 upstream of the start point of exon E1) (11). All these PPARγ transcripts generate the same protein PPARγ1. Other untranslated exons

have also been identified in the 5' UTR of PPARγ mRNA in mouse (12), in monkey (13) and recently in normal human mammary epithelial and breast cancer cells (14). Wang *et al* demonstrated the presence of multiple transcription start sites leading to different PPARγ1 transcripts observed in mammary epithelial cells and breast cancers cells (14). The different promoters are thought to mediate specific PPARγ1 transcript expression in normal and tumoral tissues. In monkey macrophage and human macrophage-derived cell lines, Zhou *et al* (13) and Chen *et al* (15) respectively showed the presence of the so-called untranslated exon C and exon D in addition to the previously identified exons A1, A2 and B. Promoter usage and alternative splicing have raised seven PPARγ transcripts referred to as PPARγ1-7.

Due to the complexity of the PPARγ gene, we postulated that in colon cell models the regulation of *PPARG* gene expression could be the result of multiple promoter usage which in turn generates other transcripts encoding the protein PPARγ1 than the already described PPARγ1, 3 or 4 mRNA. Many studies over the last 20 years have reported cancer-specific alternative splicing in the absence of genomic mutations (16). As well as being associated with cancer, the nature of the alternative gene products is usually consistent with an active role in cancer. Therefore, the alternative splicing process itself is a potential target for gene therapy. Using RT-PCR semi-quantification carried out with polyA$^+$ RNA from human colon cells and 5' extension primer experiments, we have demonstrated the presence of different PPARγ1 transcripts. Treatment of HT-29 cell line, a colon tumour cell model, with PPARγ agonists, actinomycin D or cycloheximide suggested that the variation in PPARγ mRNA levels could depend on the nature of PPARγ mRNA in colon cell model.

Materials and methods

Reagents. Dulbecco's modified Eagle's minimum medium (DMEM), trypsin/EDTA solution, fetal calf serum (FCS), non-essential amino acids solution, and Dulbecco's sodium phosphate buffer (DPBS) were purchased from Eurobio (Les Ulis, France). *Taq* polymerase, RNAsin, reverse transcriptase (M-MLV) and primers were obtained from Invitrogen Corporation (Paisley, Scotland). Restriction enzymes and protease inhibitor ready-made solution were from Sigma Corporation (St. Louis, MO, USA). BM Chemiluminescence revelation kit was from Roche (Mannheim, Germany). Commercial polyclonal rabbit antibodies against PPARγ were from Cayman (Tallin, Estonia). Polyclonal rabbit antibodies against ß-actin were purchased from Sigma. Polyclonal rabbit antibodies against catalase were prepared and characterized as described before (17). Troglitazone was purchased from Cayman; pioglitazone was a gift from Tadeka Chemicals Industries Ltd. (Osaka, Japan). Other reagents were of analytical grade.

Cell culture. HT-29, Caco-2 cell lines were obtained from M. Rousset (INSERM U505, Paris, France). The HCT116 cell line was a gift of J.N. Freund (INSERM U682, Strasbourg, France). SW620 and MRC5 cell lines were obtained from N. Martinet (INSERM 555, Nancy, France). Vero and L293 cell lines were a gift of J.L. Goergen (INPL, Nancy, France). A549 cells were obtained from N. Frossard (EA 3771, Strasbourg, France). HepG2, THLE, MCF-7, HeLa cells are part of the laboratory cell-line collection. Cells, except Caco-2 cells, were cultured in DMEM-25 mM glucose containing 10% (v/v) heat-inactivated (30 min at 56˚C) FCS and 2 mM L-glutamine, and they were seeded at a concentration of 10^5 cells/ml. Caco-2 cells were cultured in the same medium containing 20% (v/v) heat-inactivated FCS and 1% (v/v) non-essential amino acids. Seeding was $6x10^4$ cells/ml. All cells were grown in a 5% CO_2 humidified atmosphere. Medium was changed daily, 48 h after seeding. After 5 and 15 days of culture, cell layers were washed twice with cold-DPBS for 5 min. Cell layers were frozen at -80˚C until RNA extraction or cell homogenate preparation.

Cell treatments. Agonist treatment was carried out 48 h after HT-29 cell seeding for five days. Medium containing 1 or 10 µM troglitazone or pioglitazone and 1 µM 9-*cis* retinoic acid was changed daily. Cell layers were then washed three times with cold DPBS and stored at -80˚C. HT-29 cells were assessed after two and five days for each treatment by using Trypan blue staining at least four times. Results are expressed at mean ± SD. Five and 15-day-old HT-29 cells were treated in the presence of 1 or 10 µg/ml cycloheximide or 1 and 10 µM actinomycin D for 24 h. Thereafter, cell layers were washed twice with cold-DPBS, and stored at -80˚C until total RNA extraction.

RT-PCR analysis and semi-quantification of PPARγ transcripts. Total RNA was phenol/chloroform extracted (18). PolyA$^+$ RNAs were purified on oligo dT cellulose according to standard protocol (19). cDNAs were obtained by reverse transcription reaction according to standard protocol with M-MLV reverse transcriptase (200 U) and 1 µg of total or polyA$^+$ RNA. cDNA amplification was carried out in 20 mM Tris-HCl (pH 8.4) buffer containing 50 mM KCl, 1.5 mM $MgCl_2$, 5 U of RNAsin, 0.2 mM dXTP, 0.4 µM each selected sense and antisense primers, 2 µl of reverse transcription sample and 2.5 U of *Taq* polymerase for 35 cycles (95˚C, 30 sec; annealing temperature at Tm of the primers, 30 sec; 72˚C, 30 sec) followed by an extension step at 72˚C for 5 min. Primers used in this study are listed in Table I. GAPDH was used as an internal control. Amplicons were analysed on a 2% (p/v) TBE-agarose gel. The bands obtained were quantified (Gel Doc, Bio-Rad, Hercules, USA). Results are expressed as the ratio ($PPAR\gamma\ isoform_{human}/GAPDH_{human}$) in arbitrary units. H*inf*I restriction enzyme which specific site is present only in exon A2 was used to verify the presence of exon A2 in amplicons obtained with primer sense A0, A1 or A5 and antisense primer E1. All PCR-products described here were cloned in pBlueScript plasmid and sequenced (INRA, Champenoux, France). We did not find any difference in sequence with those described on the UCSC Genome site. Total RNA from SW480, HCT EB and LS174T cells were prepared by T.L. as decribed above.

5'-Primer extension. The primer extension was carried out according to Domenjoud *et al* (20) starting with 4 µg of polyA$^+$ RNAs purified from HT-29 cells. Primers used are listed in Table I. 5' end primer labeling was carried out with

Table I. Sequences of primers used in this study.

Exon	Sequence primer (5'-3')	Genomic localisation on *PPARG* gene	Length (bp)	Tm (˚C)
Primers used in RT-PCR experiments				
A	ACATCTTGGGAAGACGGCCT	12303981-12304000	20	50
A0	TTGGGTCGGCCTCGAGGACA	12304467-12304486	20	54
A1	GTTAGGCTCCCAGGAGGAGTGGTCA	12305390- 12305416	25	54
A4	CTCTACCTTTAGACCATTCTGCAG	12322341-12322364	24	50
A5	GCAAGAAGGAGATGACATATACTTAAG	12325334-12325360	27	50
A3	CTTCGTGAGGGGTGTGCTG	12328321-12328338	19	50
A2	GAAAGAAGCCAACACTAAACCAC	12328883-12328905	23	48
C	TTCAGGATGATTCTTGTCTCTGGA	12388353-12388376	24	49
E1a[a]	GGGCCAGAATGGCATCTCTGTG	12396219-12396240	22	53
E1b[b]	TGTGGAGTAGAAATGCTGGAGAAG	12396327-12396350	24	51
E4	TCATGCTTGTGAAGGATGCAAGG	12397969-12397991	23	60
E6	TCCAGTGCATTGAACTTCACAGC	12433578-12433600	23	60
GAPDH antisense	GCTGTGATGGCATGGACTGTGGTCAT	6517010-6517033	26	60
GAPDH sense	GACCCCTTCATTGACCTCAACTACATG	6515994-6516020	27	60
Primers used in 5' primer extension procedure				
A1	GTTAGGCTCCCAGGAGGAGTGGTCA	12305366-12305390	25	58
A2	GAGAAAATGGCCTTGTTGTATATTTG	12328905-12328930	26	48
A5	CTTAAGTATATGTCATCTCCTTCTTGC	12325334-12325360	27	50
E1	GGGCCAGAATGGCATCTCTGTG	12396219-12396240	22	53

[a]The E1a antisense primer is localised at the 5' end of exon E1. It was used to amplify the untranslated PPARγ1 exonic regions composed with combined exons A1-A2, A5-A2 and A2 or A1 alone. [b]The E1b antisense primer is localised at the 3' end of exon E1. It was used to amplify the untranslated PPARγ1 exonic regions composed with combined exons A0-A2 or A0 alone.

^{32}P[γ]-ATP using a labelling kit purchased from Amersham-Biosciences (Orsay, France). Lengths of extension products were estimated in 5% acrylamide/7 M urea gel prepared in TBE buffer.

Western blotting. Cell homogenates were prepared in freshly made extraction buffer according to Mansen *et al* (21). Protein (50 μg) was resolved in 10% SDS-PAGE (22). Proteins were transferred onto PVDF membranes (23) and they were further proceeded for chemiluminescence detection according to the manufacturer's protocol. Dilution of the first antibody solution was 1:1,000 for polyclonal rabbit antibodies directed against PPARγ, 1:20,000 for polyclonal rabbit antibodies against ß-actin, and 1:1,000 for polyclonal rabbit antibodies against catalase.

Statistics. All the statistics were computed using SPSS v11.5 software (SPSS Inc., Chicago, IL, USA). For the comparison of the variation of PPARγ transcript contents along cell culture duration, one way ANOVA was performed with Bonferroni *post-hoc* test for the multiple comparisons. Differences were considered significant at $P<0.05$.

Results

Untranslated exons mapping on the PPARG gene. We first compiled the representative sequences of all spliced *EST* present in the data bank resources (Table II). In order to map putative and known untranslated exons of the *PPARG* gene, we looked for: a) cryptic splice sites as 5'-aggu-3' and 5'-agg-3'; b) intronic polypyrimidine sequences between a putative acceptor splice site and an A-branch site were localised in order to define the boundaries of internal exon; c) the presence of a putative *Inr* element was investigated in the genomic region upstream of each known or putative first exon. In addition to the known exons namely A, A0, A1, A2 and B, we found two additional exons A4 and A5 (Fig. 1b). No amplification product was obtained when RT-PCR was carried out with primers designed in exon A or A4 and E1, neither in colon normal-matched and tumour sample, nor in colon adenocarcinoma-derived cell lines (data not shown). Zhou *et al* (13) have suggested that exon C and D identified in monkey macrophages may exist in the human *PPARG* gene. Sequence alignments demonstrated that <50% similarity exists between the sequence of exon D from monkey

Table II. Selected human *EST* used to map the untranslated exons on the *PPARG* gene.

EST	cDNA bank	Untranslated exon recovered[a]	Site or authors
AB107271	Adipocyte	A1 (296), E1	NCBI
AB097931	Adipocyte	A1 (296), E1	NCBI
AL523434	Neuroblastoma	A0 (160), A2, E1-E6	NCBI
AA298089	Ovary	A1 (196), A2, E1	
BM924484	Mix of stomach, colon and kidney	A (287), E1-E2	NIH
BX363320	Uterine	A0, A2, E1-E5	NBCI
CB133173	Liver	A5, A2, E1-E2	NCBI
BM744007	Ascite	A2 (42), E1-E3	NBCI
CN289139	Stem cell	A5, A2, E1-E2	(39)
AL543579	Placenta	A0 (119), E1-E6	NBCI

[a]Numbers represent the sequence length in nucleotides of untranslated exon.

and that of the corresponding human genomic sequence. We decided therefore to exclude exon D from the present study. Likewise, the sequence of exon C is present in the human *PPARG* gene. However, using RT-PCR experiments, we did not observe the presence of any transcript including exon C joined to E1 neither in human colorectal-derived cells (Fig. 2) nor in human colorectal samples (data not shown). In contrast, we amplified the exonic region between exons C and E1 when RT-PCR was performed with total RNA extracted from Vero cells in a control experiment (Fig. 2).

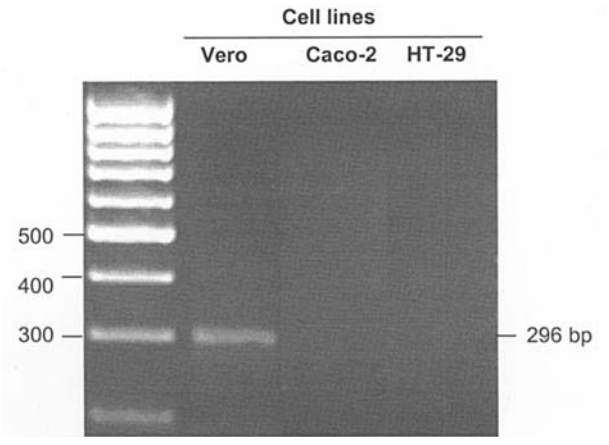


Figure 2. Absence of mRNA containing exon C in colon-derived cell lines. Total RNA was extracted from 5-day-old Vero, Caco-2 or HT-29 cells. RT-PCR experiments were performed with specific primers designed from exon C and exon E1 of the *PPARG* gene.

Presence of PPARγ protein in colon derived cell lines. PPARγ protein content was quantified in four colon adenocarcinoma cell lines namely Caco-2, HT-29, HCT116 and HT-29 MTX 10^{-5} M Rev. Upon cell culture, Caco-2 and HT-29 MTX 10^{-5} M Rev cells differentiated spontaneously as

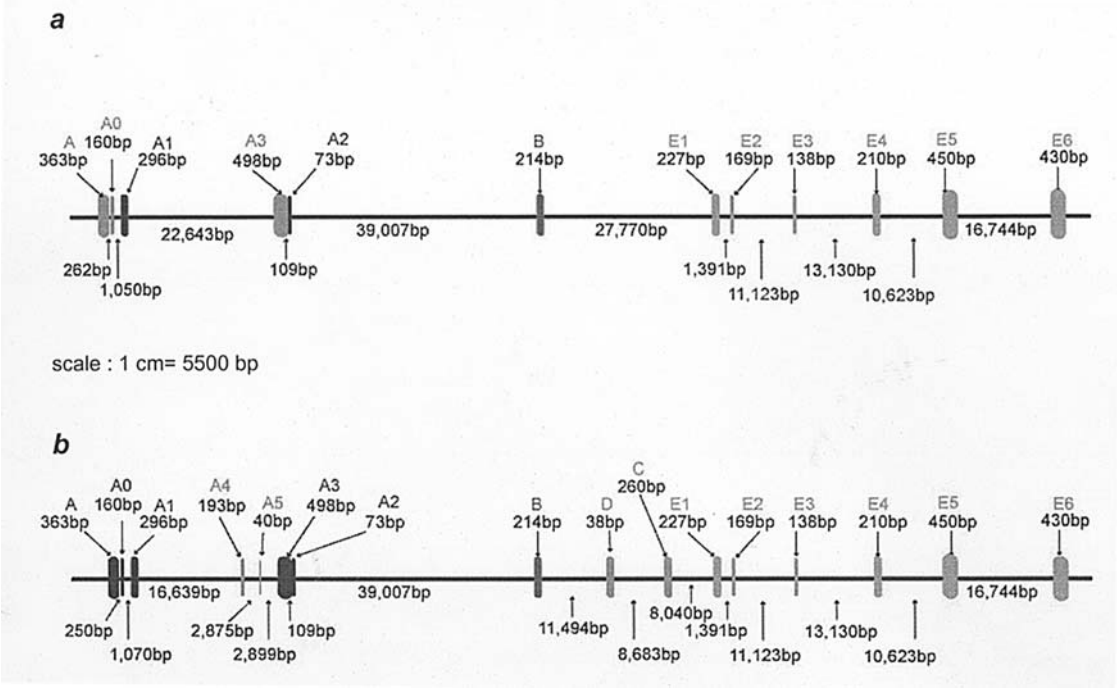


Figure 1. Map of the PPARG gene. (a) Untranslated exons nomenclature is based on published work (9,10,13-15). This scheme (adapted herein) shows *PPARG* gene organization presented by Wang *et al* (14). (b) Predicted organization of the *PPARG* gene from bioinformatic analysis and completed with reference (14). Lengths of known or putative untranslated exons and introns are given.

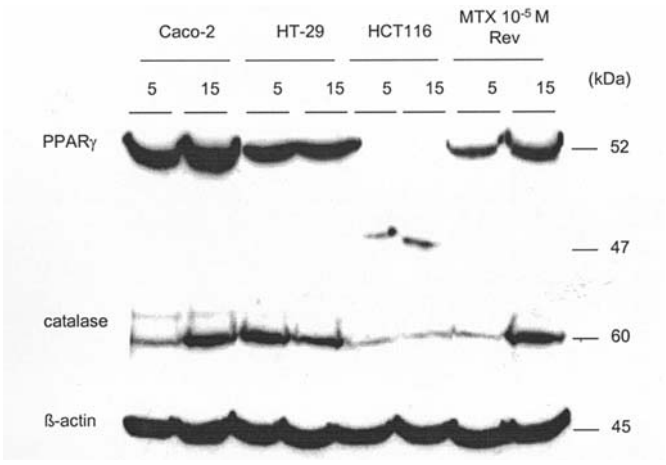


Figure 3. PPARγ protein content in colon-derived cells. Cell homogenates were prepared from 5- and 15-day-old cells. Protein extracts were submitted to Western blotting as described in Materials and methods. Blots were probed with a commercial rabbit polyclonal antibody raised against PPARγ. Control experiments for protein loading were performed with a monoclonal antibody raised against ß-actin and with Coomassie blue coloration of the PVDF membrane (data not shown). A polyclonal antibody against catalase (17) was used to demonstrate spontaneous differentiation occurring in Caco-2 and HT-29 MTX 10^{-5} M Rev cells. Typical results are from at least three independent experiments.

absorptive (24) and mucus-secreting cells (25), respectively. HT-29 and HCT116 cells remained undifferentiated in the same culture delay. As previously shown (26), catalase content used as a marker of differentiation was increased during spontaneous differentiation process occurring in Caco-2 cells, but also in HT-29 MTX 10^{-5} M Rev cells. We demonstrated that PPARγ protein was present in these cells with an estimated M_r at 50 kDa (Fig. 3). In Caco-2 or HT-29 MTX 10^{-5} M Rev homogenates, PPARγ protein content was increased from 5-15 days in culture whereas it decreased in HT-29 during the same culture duration. In HCT116 cells, PPARγ protein corresponded to a band estimated at 47 kDa. The reason for such a difference is not clear. We suggest that PPARγ protein is degraded in this cell line and that the observed band is the result of receptor degradation as previously reported (7,27).

Presence of different PPARγ1 transcripts in colon derived cell lines or from other tissue origins. PPARγ1 mRNA content was quantified by RT-PCR analysis in the selected colon cell lines. As shown in Fig. 4, PPARγ mRNA levels (estimated with primers designed in exon 4 and 6) increased significantly upon culture duration in Caco-2 and HT-29 MTX 10^{-5} M

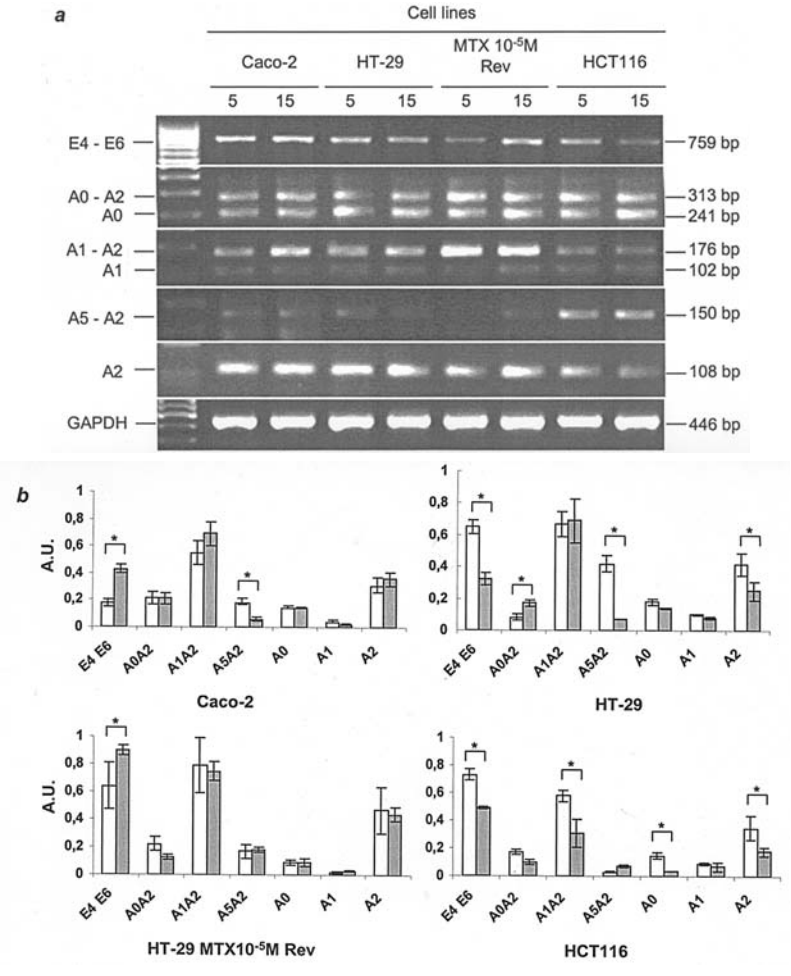


Figure 4. mRNA untranslated exonic region expression in colon cell lines. PolyA+ mRNAs were prepared from 5- or 15-day-old cells and untranslated exonic regions of PPARγ1 transcripts were quantified by semi-quantitative RT-PCR with specific primers from each exon Ai and exon E1 (Table I) or exon E4 and E6 (a). The amplified regions are named according to the composition of untranslated exon fused to exon E1. The intensities of the bands were quantified and the ratio between intensities of PPARγ1 exonic regions and GAPDH were determined at 5 (white bar) and 15 days (grey bar) (b). The bar graphs represent the mean ± SD from 3 independent experiments. Significant difference (*) at P-value <0.05 (ANOVA) are shown in (b).

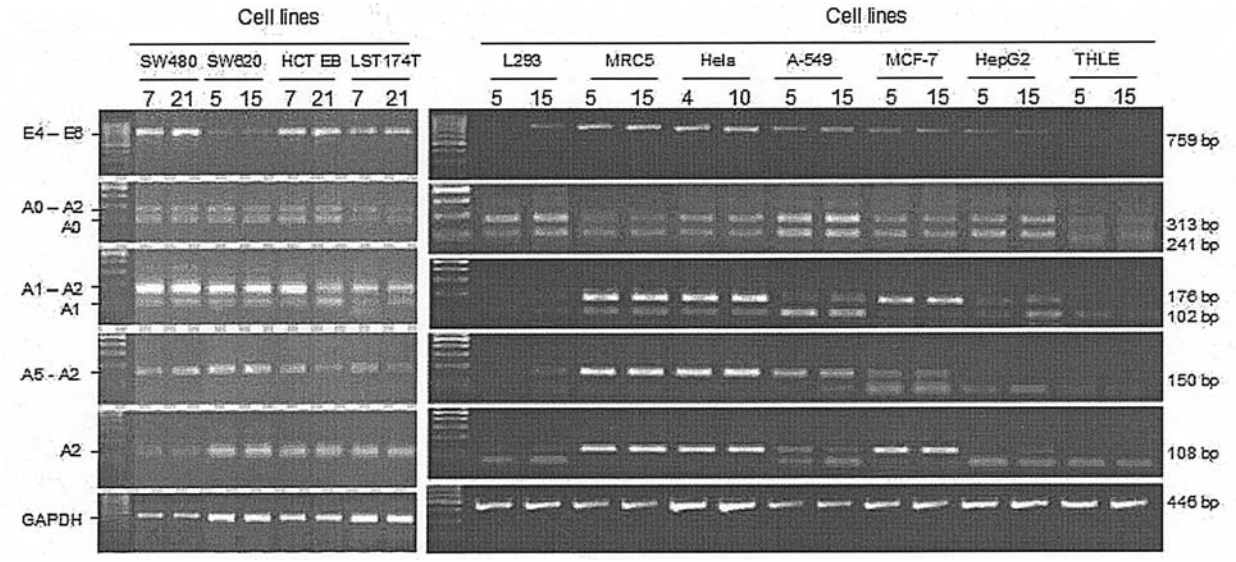


Figure 5. mRNA untranslated exonic region expression in various cell models. Total RNA was prepared from cells harvested during proliferation or stationary culture stage and submitted to RT-PCR with specific primers from each exon Ai and exon E1 or exon E4 and E6. The amplified regions are named according to the composition of untranslated exon linked to exon E1. SW480, SW620, HCT EB and LS 174T are colon adenocarcinoma-derived cell lines. L293 originated from a kidney cell transformed with adenovirus 5 DNA, MRC5 cell line is a fibroblastic-derived cell line, HeLa cells derived from an epidermoid carcinoma, A549 from a lung carcinoma, MCF-7 cells from a breast adenocarcinoma, HepG2 from a hepatocellular carcinoma. THLE cell line was obtained after SV40 large antigen-T immortalization of human hepatocytes (40).

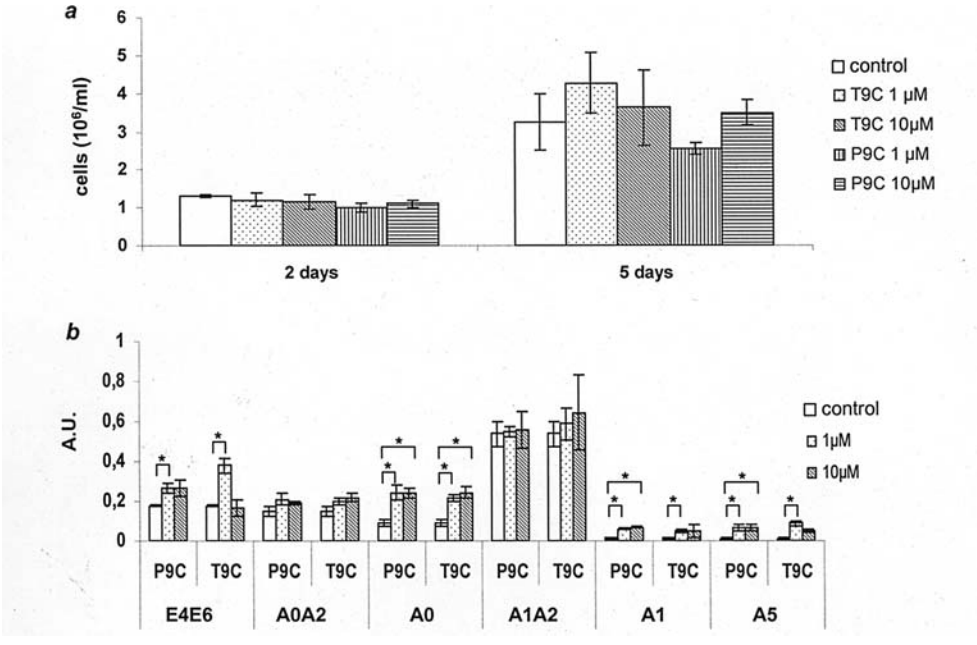


Figure 6. Quantification of PPARγ1 transcripts in HT-29 cells treated with PPARγ agonists. HT-29 cells were treated during 5 days with troglitazone or pioglitazone as indicated. (a) Cell count was carried out with Trypan blue. Cells were numbered at least 4 times for each treatment. (b) Total RNA was extracted from cell layers and submitted to RT-PCR analysis using primers designed in untranslated exon Ai and E1 or in exon E4 and E6. The intensities of the bands were quantified and the ratio between intensities of each PPARγ1 exonic region and GAPDH was calculated. The bar graphs represent the mean ± SD from 3 independent experiments. Significant differences (*) at P-value <0.05 are shown (ANOVA). P9C, T9C: combined treatment respectively with pioglitazone or troglitazone and 9-*cis* retinoic acid (see Materials and methods).

Rev differentiating cells, whereas they decreased in HT-29 and HCT116 cells. We investigated which transcripts may account for the total level of PPARγ1 mRNA. Several exonic regions were amplified with sense primer designed for each untranslated exon and antisense primer designed for exon E1. Since PPARγ was present in the four cell lines tested, semi-quantification of PPARγ1 transcripts were planed using polyA$^+$ RNA prepared from cells cultured for 5 and 15 days (Fig. 4b).

Using exon A0 or A1 sense primer and exon E1 antisense primer (Fig. 4a), two PCR products were obtained with an estimated length of 313 and 241 bp or 176 and 102 bp, respectively. The longest PCR products included exon A2 as demonstrated by digestion of the PCR product with H*inf*I

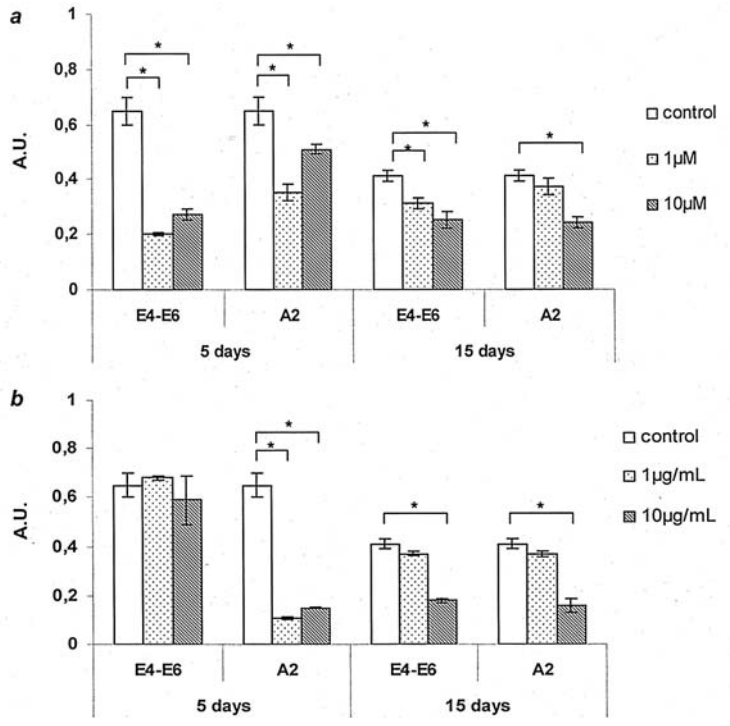


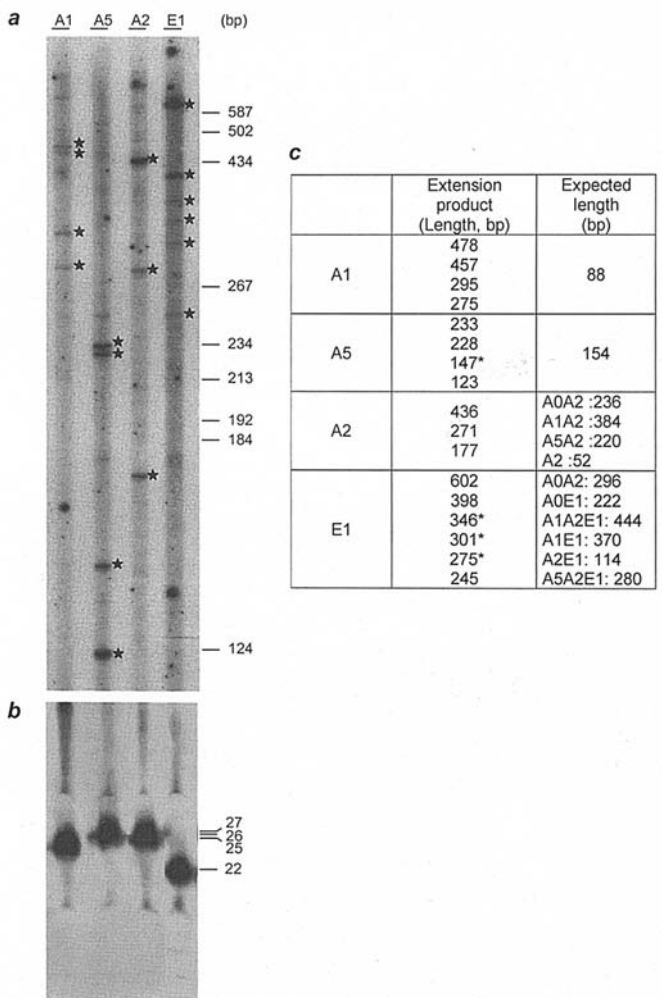
Figure 7. PPARγ1 mRNA levels after drug treatment. Caco-2 and HT-29 cells were cultured during 5 and 15 days and treated with actinomycin D (a) or cycloheximide (b) for 24 h. Total RNA was extracted and submitted to RT-PCR using primers designed in exon E4-E6 and exon A2 and E1, respectively. The intensities of the bands were quantified and the ratios between intensities of each PPARγ1 exonic region and GAPDH were calculated. The bar graphs represent the mean ± SD from 3 independent experiments. Significant difference (*) at P-value <0.05 are shown (ANOVA).

which restriction site is present only in exon A2 (data not shown). Likewise, amplification with sense primer in exon A5 and antisense primer in exon E1 gave a single band estimated at 150 pb which contained exon A2 (Fig. 4a). Amplification was also carried out with sense primer designed in exon A2 (Fig. 4a). The obtained PCR products estimated at 108 bp account for all the mRNA including exon A2 or for the mRNA containing exclusively exon A2 in its 5' UTR, namely PPARγ3 mRNA (10).

Semi-quantification of each exonic region suggested that the transcripts for the so-called PPARγ1 isoform were mainly represented by combination of exons A1-A2-E1-6 as observed previously (9). The other mRNAs containing the untranslated region A0-A2, A5-A2, A0 alone or A1 alone, were less abundant in all colon cell lines tested. However, the relative levels of each mRNA depended on cell line and culture duration. The transcripts containing the A5-A2 region decreased in Caco-2 and HT-29 cells whereas they increased in HCT116 cells (Fig. 4b). Transcripts containing the A0-A2 region decreased in HT-29 MTX 10^{-5} M Rev cells. Furthermore, each PPARγ1 transcript studied here was recovered in the other cell lines tested but with strong differences in the mRNA levels (Fig. 5). For example, transcripts containing A5 were detected in MRC5 and HeLa cells, but we failed to detect them in HepG2 and THLE cells. PPARγ1 transcripts containing the A1-A2 exonic region (band estimated at 176 bp) were more abundant in MRC5, HeLa or MCF-7 than in A-549 cells. At least in HepG2, PPARγ1 level was represented mainly by transcripts that contained untranslated exonic region A0-A2 or A0 alone. In other colorectal cell lines used, higher expression of exonic region A5-A2-E1 was observed in SW480, SW620, HCT EB and LS174T cells (Fig. 5).

Effect of agonist treatment on PPARγ1 mRNA level in HT-29 cells. HT-29 cells were treated with troglitazone or pioglitazone in the presence of 9-*cis* retinoic acid during the proliferation stage of the cells, in order to investigate possible changes in PPARγ1 transcripts levels (Fig. 6b). Since PPARγ agonists have been shown to block cell growth and to increase cell anoikis, we first counted cell populations during dose- (1 or 10 μM agonist and 1 μM 9-*cis* retinoic acid) and time course-treatment (5 days). There were no significant variations in cell counts at whatever the treatment used (Fig. 6a). A significant increase was observed only in PPARγ mRNA content including untranslated exon A1 (3 times), A0 (2 times) or exonic region A5-A2 (5 times). No significant alteration was observed in the mRNA levels of the other transcripts, which included untranslated exon A2 in exonic region A1-A2 and A0-A2 (Fig. 6b).

Effect of actinomycin D and cycloheximide on PPARγ1 mRNA level in HT-29 cells. Since increased PPARγ mRNA levels were observed for transcripts excluding A2 exon (except for the A5-A2 untranslated region), we hypothesised that the presence of A2 untranslated exon may contribute to PPARγ mRNA stability. Therefore, HT-29 cells were cultured for 5 or 15 days, then treated with actinomycin D (1 or 10 μM) or cycloheximide (1 or 10 μg/ml) for 24 h (Fig. 7a and b, respectively). RT-PCR analysis was limited to amplification of E4-E6 and A2-E1 exonic regions. Cells treated with actinomycin D at 5 days of culture exhibited a 70% decrease



	Extension product (Length, bp)	Expected length (bp)
A1	478 457 295 275	88
A5	233 228 147* 123	154
A2	436 271 177	A0A2 :236 A1A2 :384 A5A2 :220 A2 :52
E1	602 398 346* 301* 275* 245	A0A2: 296 A0E1: 222 A1A2E1: 444 A1E1: 370 A2E1: 114 A5A2E1: 280

Figure 8. 5' Primer extension products revealed by urea-sequencing gel. 5' Primer extension was carried out as described in Materials and methods with polyA+ RNA prepared from human HT29 adenocarcinoma-derived cells. (a) The extension products are indicated by black stars. (b) Results of free labelled primers are presented. (c) The table summarizes the length of each primer extension product. The known exonic regions recovered by the procedure are indicated by asterisk.

in PPARγ1 initial level estimated with primers designed in exon E4 and E6 (Fig. 7a). Amplification product of the exonic region containing exon A2 was reduced to 50%. HT-29 cells were less drug-sensitive after 15 days in culture, since mRNA levels were only reduced with high drug concentration (a 40% decrease). We next treated cells with cycloheximide for 24 h in order to verify if PPARγ mRNA levels could be altered by inhibition of protein synthesis (Fig. 7b). In 5-day-treated HT-29 cells, no change was found when PPARγ levels were quantified with primers designed in exon E4 and E6. An 85%-decrease was observed in PPARγ1 mRNA level estimated with primers designed in exon A2 and E1. In contrast, alteration in PPARγ mRNA levels in 15-day-old HT-29 cells was only obtained with high concentrations of cycloheximide (a 60% decrease). Our results suggested that during the proliferation stage of HT-29 cells, two mechanisms regulate changes in PPARγ transcript levels; firstly, whatever the mechanism, one part of PPARγ transcript seems to be dependent on the presence of *de novo* protein synthesis. Secondly, part of the PPARγ transcripts turnover is high as demonstrated by actinomycin D treatment and quantification with primers designed in exon E4 and E6.

5'-Primer extension. We performed 5' primer extension with polyA+ prepared from 15-day-old adenocarcinoma cells (Fig. 8). As shown in Fig. 8a, we failed to detect extension product corresponding to exon A2 alone (estimated length at 52 bp). These results contrasted with previous work demonstrating that exon A2 alone constitutes a specific transcript *id est* PPARγ3 in human cell models (10). Experiments were also extended with antisense primer designed in exon A1, A5 and E1. Surprisingly, several unexpected extension products were obtained starting with A1, A2, A5 and E1 primers. Our results suggested that either unknown exon may exist and participate in the diversity of PPARγ1 transcripts or the lengths of known exons are greater than previously described.

Discussion

PPARγ has been implicated in colorectal cancer and is thought to exhibit a protective action when the receptor is activated with a specific agonist (28). This protective role has been studied in colorectal cancer-derived cells and results suggest that PPARγ agonist treatments lead to inhibition of cell growth, enhancement of protein markers for differentiation and/or induction of cell apoptotic processes. The agonist effect depends largely on the cell line tested and is dose-dependent (29).

In colon and colorectal cell lines, PPARγ1 is the main protein isoform (30,31). Recently, several transcripts resulting from alternative promoter usage and splicing of untranslated exons of the *PPARG* gene have been identified (14). Bioinformatic analysis based on previous data (9,10,13,14) and representative *EST* alignment (Table II) lead us to complete a previous map of known and putative untranslated exons named exon A, A0-A5 (Fig. 1). However, using RT-PCR experiments, we failed to detect A, A3 and A4 and therefore it is likely that these exons are not included in human colon PPARγ1 mRNA. Wang *et al* demonstrated that exons A and A3 are used in normal and tumoral mammary tissues (14). Exon A3 expression is restricted to normal mammary tissue suggesting a tissue specific expression. In the present study, similar results have been obtained with exon A5, whose expression is higher in fibroblast or HeLa cells than in colorectal cancer-derived cells (Figs. 5 and 4, respectively). Our results demonstrated that mRNA composed of the exonic region A5-A2 is higher in proliferating cells in three out of four cell-lines tested (Fig. 4b).

In colorectal cancer-derived cells, we showed that several transcripts encoding PPARγ1 protein are present as demonstrated by RT-PCR and by 5' primer extension experiments (Figs. 4, 5 and 8). Our results support evidence that PPARγ1 transcription depends on multiple promoter usage and alternative splicing leading to fine transcription regulation within the cells as described within macrophages and breast cancer cells (13-15). Likewise, in pre-adipocytes and in mature adipocytes, PPARγ1 mRNA levels remain constant whereas PPARγ2 mRNA levels increase upon adipocyte differentiation (32-34). When colon cancer-derived

cells were treated with PPARγ agonists (Fig. 6b), the PPARγ mRNA level was modified only for the combined A0-, A1-, and A5-A2-PPARγ1 transcripts, the levels of the other transcripts remained constant. It must be noted that we used PPARγ agonist concentrations that did not induce cell apoptosis as it has been demonstrated by others (29,35,36). Additional treatments either with actinomycin D (Fig. 7a) or with cycloheximide (Fig. 7b) suggest that PPARγ transcript content depends on RNA flux controlled in two ways, high mRNA turnover and protein synthesis-dependent stability of mRNA. Moreover, we show here that increases in the PPARγ1 mRNA levels in response to receptor agonist treatment were associated with increases in particular transcripts levels (Fig. 6b). Similar results were obtained recently with human THP-1 macrophages (15). Treatment with agonist increased PPARγ transcript isoform composed of particular exon C and D (and named PPARγ5 isoform), whereas agonist treatment leads to decrease PPARγ1 and γ2 mRNA levels. We suggested that A2 could participate to structural stability of PPARγ mRNA. Taken together, our results did not confirm previous work demonstrating that PPARγ3 is the main isoform in colon cells models (26,37). Using 5' extension experiment, we failed to demonstrate any extension product at the expected length (at 52 bp) when experiments were carried out with antisense primer designed in exon A2 (Fig. 8). Likewise, when HT-29 cells were treated with PPARγ agonist at low concentration, no change was observed in the mRNA isoform levels corresponding to the so-called PPARγ1 or PPARγ3 transcripts, as demonstrated by others using oxidized metabolites of linoleic acids as endogenous ligands for PPARγ (37). Others studies are needed to clarify the impact of specific treatment to the expression of each particular PPARγ1 transcript isoform. The role of each PPARγ1 transcript has to be established in colon cell model, even though the implication of the receptor in intestinal cell regulation begins to emerge (5).

Alternative splicing is a crucial mechanism to explain the diversity of gene expression. This mechanism has been shown to be altered in cancer (16). The involvement of PPARγ mRNA diversity had to be stated during development, proliferation or differentiation in the case of colon cells. As demonstrated recently, alternative splicing could explain the diverse modifications observed in signalling pathway from embryonic stem cells to tissue stem cells (38). In the present work, we suggested that alternative splicing could modify the diversity and the levels of PPARγ transcripts. This process could change the transcription rate in response to a specific signalling pathway or by ligand coactivator-dependent recruitment, as suggested by Chen *et al* (15). Finally, using 5' primer extension (Fig. 8) we detected unexpected 5' mRNA regions, which could not be assigned to association of some known exon A1. It is possible that other exonic combinations may exist as demonstrated recently in human THP-1 macrophages (15). It will be of interest to investigate if other untranslated exons may exist or not. In conclusion, we reported that in colon cancer-derived cells, diverse transcripts encoding PPARγ1 protein exist due to specific promoter usage and alternative splicing. The respective levels of the transcripts depend on the colon cell model used.

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