Application of the differential display RT-PCR strategy for the identification of inflammation-related mouse genes

A.M. Silva[1,2], E.G. Pires[1], E.F. Abrantes[1,3], L.R.P. Ferreira[3], R.T. Gazzinelli[3] and L.F.L. Reis[1]

[1]Laboratório de Inflamação, Instituto Ludwig de Pesquisa sobre o Câncer, São Paulo, SP, Brasil
Departamentos de [2]Microbiologia and [3]Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Abstract

Correspondence
L.F.L. Reis
Laboratório de Inflamação
Instituto Ludwig de Pesquisa
sobre o Câncer
Rua Prof. Antônio Prudente, 109
01509-010 São Paulo, SP
Brasil
Fax: +55-11-270-7001
E-mail: lreis@node1.com.br

The inflammatory response elicited by various stimuli such as microbial products or cytokines is determined by differences in the pattern of cellular gene expression. We have used the differential display RT-PCR (DDRT-PCR) strategy to identify mRNAs that are differentially expressed in various murine cell types stimulated with pro-inflammatory cytokines, microbial products or anti-inflammatory drugs. Mouse embryonic fibroblasts (MEFs) were treated with IFNs, TNF, or sodium salicylate. Also, peritoneal macrophages from C3H/Hej mice were stimulated with *T. cruzi*-derived GPI-mucin and/or IFN-γ. After DDRT-PCR, various cDNA fragments that were differentially represented on the sequencing gel were recovered, cloned and sequenced. Here, we describe a summary of several experiments and show that, when 16 of a total of 28 recovered fragments were tested for differential expression, 5 (31%) were found to represent mRNAs whose steady-state levels are indeed modulated by the original stimuli. Some of the identified cDNAs encode for known proteins that were not previously associated with the inflammatory process triggered by the original stimuli. Other cDNA fragments (8 of 21 sequences, or 38%) showed no significant homology with known sequences and represent new mouse genes whose characterization might contribute to our understanding of inflammation. In conclusion, DDRT-PCR has proven to be a potent technology that will allow us to identify genes that are differentially expressed when cells are subjected to changes in culture conditions or isolated from different organs.

Introduction

The identification of genes that are differentially expressed in cells exposed to microbial products, pro-inflammatory cytokines, or anti-inflammatory drugs can help us understand the molecular basis of the inflammatory response and may point out potential new targets for drug therapy. We have used the inflammatory response as a model to evaluate the differential display RT-PCR (DDRT-PCR) (1) as a tool to compare the mRNA content of cells exposed to different stimuli that mimic a stage of the inflammatory response. Current methods for comparing the gene expression profile of different

cell types or of cells that are kept under different conditions include, among others, the DDRT-PCR (1,2), RAP-PCR (3), subtractive cDNA libraries obtained by differential screening (4), SAGE (5), RDA-analysis (6), and more recently cDNA microarray analysis (7,8). A review of the literature shows that all of these procedures have intrinsic advantages and disadvantages that have to be balanced before a method is chosen (9,10). Normally, a gain of sensitivity will imply an increased background of fragments that do not represent a bona fide differentially expressed gene. Here, we show that, after detailed optimization, the DDRT-PCR is a simple, fast, and reliable technique that can identify a high percentage of genes whose pattern of expression can be predicted based upon the pattern of the corresponding fragment.

A variety of pro-inflammatory cytokines like interferons (IFNs), interleukins (ILs), or tumor necrosis factor (TNF) are pleiotropic cytokines that play an important role as antiviral agents, as immune modulators, and in the regulation of cell growth and differentiation (11). In order to exert their biological activities, these cytokines bind to specific cell-surface receptors, leading to the activation or inactivation of the expression of a large set of genes (11).

Interferons bind to two distinct cellular receptors, one specific for the type I IFN family (comprising IFN-α, -β, and -ω) and one specific for the type II IFN or IFN-γ. Binding of IFNs to their specific receptors will trigger a signaling cascade that is mediated primarily by the activation of tyrosine kinases of the Janus family (JAKs). Activated JAKs will in turn phosphorylate the members of STAT (for signal transducers and activators of transcription) family of proteins that will activate the expression of the so-called IFN-stimulated genes (ISGs). Another transcription factor, interferon regulatory factor-1 (IRF-1), has also been implicated as an important transcription factor for the activation of ISGs. Interferon signaling has been extensively reviewed in recent publications (12-18). The proteins encoded by the different ISGs will mediate most of the biological activities of interferons. Examples of functionally characterized ISGs are the family of 2'5-OAS (19), the dsRNA-dependent serine-threonine kinase PKR (20,21), and the MX protein (22) necessary for resistance to influenza virus. Even though an ever-growing number of ISGs are now being identified, the exact mechanism of action of IFNs remains obscure, with the need to identify new genes that would help us to clarify the cytokine network.

Like IFNs, the actions of TNF are also mediated by several proteins encoded by genes expressed upon binding of TNF to its cellular receptors. In the case of TNF, induction of gene expression is achieved by the activation of the transcription factor NF-κB that exists as a latent form in the cytoplasm, bound to the inhibitory component IκB. Phosphorylation of IκB leads to its dissociation from NF-κB and subsequent degradation, with migration of active NF-κB to the nucleus (23-25). Interestingly, recent evidence has indicated that the treatment of cells with the non-steroid anti-inflammatory compound sodium salicylate leads to inhibition of IκB phosphorylation and degradation, suggesting that blocking of TNF signaling might be an effective approach for anti-inflammatory therapy (26-28).

During the inflammatory response triggered by infectious agents, immune competent cells are activated and secrete IFNs, TNF, and many other pro-inflammatory cytokines. Thus, it is also relevant to compare the mRNA population in cells of the immune system that are exposed to microbial products. One of the factors responsible for macrophage activation during *T. cruzi* infection are glycosylphosphatidylinositol anchored mucin-like glycoproteins (GPI-mucins) (29). Exposure of macrophages to *T. cruzi*-derived GPI-mucin activates the expression of

the genes encoding for IL-12 (29) or nitric oxide synthesis in IFN-γ-primed macrophages (30,31). Hence, one can speculate that, by comparing the mRNA content of control, untreated macrophages versus GPI-mucin-treated macrophages, it might be possible to identify new genes that are activated in response to *T. cruzi* infection.

With the aid of DDRT-PCR, we compared the mRNA content of cells stimulated with various cytokines or microbial products and searched for cDNA fragments that could represent genes that were differentially expressed in response to these treatments. The identification and functional characterization of these genes could identify new aspects of the inflammatory response and, moreover, they could be new targets for drug discovery.

Material and Methods

Cell culture, exposure of cells and/or mice to different stimuli, and total RNA isolation

Heterozygous IRF-1 mice (32) were intercrossed and mouse embryonic fibroblasts were prepared from the resulting 12-14-day-old embryos. Individual embryos were transferred to tissue culture dishes containing 10.0 ml PBS and primary fibroblasts were obtained. Mouse embryonic fibroblasts (MEFs) were allowed to grow to near confluence in DMEM containing 10% FBS. For induction by cytokines, cells were treated with 500 IU/ml of rhuIFN-α2/α1 or 30 ng/ml of rmuTNF-α (all cytokines from R & D Systems, Minneapolis, MN, USA). Sodium salicylate was purchased from Sigma and dissolved to 1 M concentration in deionized water. Peritoneal macrophages from C3H/Hej mice were also cultured in DMEM supplemented with 10% FCS and stimulated with *T. cruzi*-derived GPI-mucin (2 pg/ml) and/or IFN-γ (100 IU/ml) for 6 h. Total RNA extraction from cell cultures under different conditions was performed as described (33).

To determine the *in vivo* expression of ISGs, wild type mice or mice with IRF-1 (32) or STAT-1 (34) deficiency were treated orally with l mg/kg of imiquimod, a potent inducer of IFN synthesis (35,36) (provided by 3M-Pharmaceuticals, St. Paul, MN, USA). After 4 h of treatment, animals were sacrificed and organs were collected and frozen until total RNA extraction as described (33).

Generation of cDNA fragments by DDRT-PCR

Two hundred nanograms of DNase-treated total RNA from cell cultures under different conditions were reverse transcribed in a mix containing 20 μM dNTPs, 10 mM DTT, 200 U MMLV-reverse transcriptase, and RT-buffer in the presence of 25 pmol of one of the anchored oligo $T_{11}VN$ (where V is A, C or G, and, N can be A, C, G or T) at 42ºC for 1 h. One tenth of the cDNA first strand reaction was used as template for the DD-PCR reaction in the presence of PCR-buffer (0.01 M Tris-HCl, 0.05 M KCl, 2.5 mM $MgCl_2$), 20 μM all dNTPs, and [α-^{32}P]dCTP, using the same anchored oligo (2.5 μM) plus a set of three random 10-mer primers: PR13: 5'-ctgatccatg-3', PR14: 5'-ctgctctcaa-3', PR15: 5'-gaactaacgg-3', at a final concentration of 0.5 μM. The conditions for PCR reaction were as follows: 94ºC/4 min for one cycle, 94ºC/30 s, 40ºC/2 min, 72ºC/30 s, for 40 cycles plus a final extension at 72ºC for 10 min. An aliquot of radioactive products was fractionated on 6% acrylamide/8 M urea sequencing gel at 1500 V until xylene-cyanol reached the bottom of the gel. The gel was covered with plastic film (SaranWrap®) and exposed to autoradiogram film.

Sequencing and analysis

The cDNA fragments were recovered from the sequencing gels, eluted, re-amplified with the same set of primers as used in DDRT-PCR and sub-cloned into a pUC18

(Pharmacia, Uppsala, Sweden) or pGEM 7zf(+) vector (Promega, Madison, WI, USA). Plasmids derived from at least two different colonies were sequenced on automated DNA sequencers (A.L.F. Sequencer or ABI Perkin Elmer Cetus). Sequence homology of the cloned cDNA fragments with known cDNAs was determined using the BLAST program (37).

Northern and Southern blot analysis, and RT-PCR assessment

For Northern blot analysis, 15 µg of total RNA were fractionated on 1% denaturing agarose/formaldehyde gel and transferred by capillarity to a Hybond filter (Amersham, Buckinghamshire, UK) as described (38). For Southern blot, 20 µg of digested mouse genomic DNA were fractionated on 0.8% agarose gel as described elsewhere (38). Pre-hybridization, hybridization and washes were performed as described (39). Filters were probed with [α-^{32}P]dCTP-labeled cDNA fragments at a specific activity of 1 x 10^9 cpm/µg for the cDNA probe and exposed to X-ray films. For RT-PCR analysis, 1 µg of DNA-free total RNA was reverse transcribed with 0.5 µg oligo dT$_{12-18}$, 200 µM dNTPs, RT-buffer, 0.01 M DTT, 200 units of SuperScript II (Gibco BRL, Gaithesburg, MD) at 42ºC for 60 min. One tenth of the first strand reaction was used as template for PCR reactions in the presence of specific primers designed for each cDNA fragment.

Results and Discussion

In this report, we describe the application of the DDRT-PCR as a tool to identify genes differentially expressed by cells exposed to different stimuli that mimic the inflammatory response. We performed a total of 84 reactions using three different random primers in combination with different anchored primers (see Material and Methods). On av-

erage, we observed 40-50 individually expressed bands per lane on the sequencing gel. About 100 bands were recovered from the gels and, so far, 28 have been cloned. On average the length of re-amplified cDNA fragments varies from 100 to 900 bp. In our model, we show that, when optimized, DDRT-PCR can yield a high percentage of cDNA fragments that indeed correspond to mRNAs. Of the 28 cloned and sequenced cDNA fragments, 21 (78%) correspond to bona fide mRNAs and 5 of 16 sequences (31%) tested by Northern blot or RT-PCR were proven to be differentially expressed. Of the 21 fragments that correspond to an mRNA, 8 (38%) represent newly identified mouse sequences and some of them have no significant homology with other sequences deposited in the GenBank (Table 1).

We have partially characterized 13 expressed cDNA fragments that hybridize with mRNAs in Northern blots, 3 that are amplified by RT-PCR, and 4 that have significant homology with known sequences, mainly ESTs deposited in GenBank. Among the five mRNAs that are differentially expressed, two are new ISGs, one is a new TNF-stimulated gene, one is stimulated by *T. cruzi*-derived GPI-mucin, and one mRNA is modulated by sodium salicylate.

FRAG-6 is one of the new genes that we have shown to be differentially expressed on IFN-treated cells (Figure 1). The probe of 576 bp strongly hybridizes with two mRNA transcripts of 4 and 7-9 kb, that are up-regulated in various organs of imiquimod-treated wild type or IRF-1$^{0/0}$ mice. We have also found that the expression of the FRAG-6 gene is abrogated in STAT-1-deficient mice, suggesting that regulation of FRAG-6 gene expression is regulated by the JAK/STAT pathway (40).

Since the sensitivity of Northern blot is lower than that of DDRT-PCR, it is often observed that DDRT-PCR-derived probes give no detectable hybridization in Northern

Table 1 - Generation of expressed cDNA fragments by DDRT-PCR, their sequence similarities having sequences deposited in non-redundant and ESTs Genbank data, and assessment of their expression.

Note that for some cDNA fragments there is no similarity, but their gene expression is confirmed by Northern blot analysis. NaSal: Sodium salicylate; N: Northern blot analysis; S: Southern blot analysis; N/S: both Northern and Southern analyses; nd: not determined.

|	Clones	Length (bp)	Sequence homology from BLAST search	Accession number	Stimulation with	Expression assessed by	Differential expression
1)	059A14+	253	None	–	TNF	N	TNF-induced
2)	13A10b	361	None	–	NaSal/TNF	nd	–
3)	13A3a	206	Mouse ribosomal protein S3a	emb/Z83368	NaSal/TNF	N	Not altered
4)	13C2R	367	Human vinculin mRNA	gb/M33308	NaSal/TNF	N	Not altered
5)	13G1	242	None	–	NaSal/TNF	nd	–
6)	13T1	357	Mouse lymph node EST	gb/AA186096	NaSal/TNF	N	Not altered
7)	13T5	309	Rat mevalonate kinase	gb/M29472	NaSal/TNF	N	Not altered
8)	13T6	311	Rat mevalonate kinase	gb/M29472	NaSal/TNF	N	Not altered
9)	13T8	262	Mouse MHC class III	gb/AF030001	NaSal/TNF	RT-PCR	Not altered
10)	13VC3	189	Mouse EST similar to protein carboxyl methyl-transferase	gb/AA266007	NaSal/TNF	nd	–
11)	15VC1	183	None	–	NaSal/TNF	nd	–
12)	CTR-3	223	Mouse embryo EST	gb/AA030676	NaSal/TNF	RT-PCR	NaSal-inhibited
13)	FRAG-1	845	Mouse type V alpha-2 collagen	gb/L02918	IFN	N	Not altered
14)	FRAG-3	509	None	–	IFN	S	–
15)	FRAG-6	576	None	–	IFN	N/S	IFN-induced
16)	GPI-3	200	Mouse 28S ribosomal RNA	emb/X00525	GPI-mucin	N/S	Not altered
17)	GPI-7	269	Chinese hamster protein kinase	gb/M63312	GPI-mucin	RT-PCR	Not altered
18)	GPI-8	193	None	–	GPI-mucin	nd	–
19)	GPI-9	105	Mouse SAA3 mRNA	emb/X03506	GPI-mucin	N/S	GPI-mucin-induced
20)	GPIg-3	151	Human fetal brain EST	gb/U50573	GPI-mucin	nd	–
21)	KOI-3	180	Human fetal heart EST	gb/W93552	IFN	nd	–
22)	KOI-4	140	Mouse embryo EST	gb/AA840201	IFN	N/S	IFN-induced
23)	KONI-1	158	None	–	IFN	nd	–
24)	NASA4a	294	Rat mevalonate kinase	gb/M29472	NaSal/TNF	nd	–
25)	NASA4b	167	Mouse EST	gb/AA169064	NaSal/TNF	nd	–
26)	NASAC-3	275	Mouse GAPDH	gb/M32599	NaSal/TNF	N	Not altered
27)	SALI-1	169	None	–	NaSal/TNF	nd	–
28)	WTI-2	199	Rat EST from PC-12 cells	gb/AA684985	IFN	N/S	Not altered
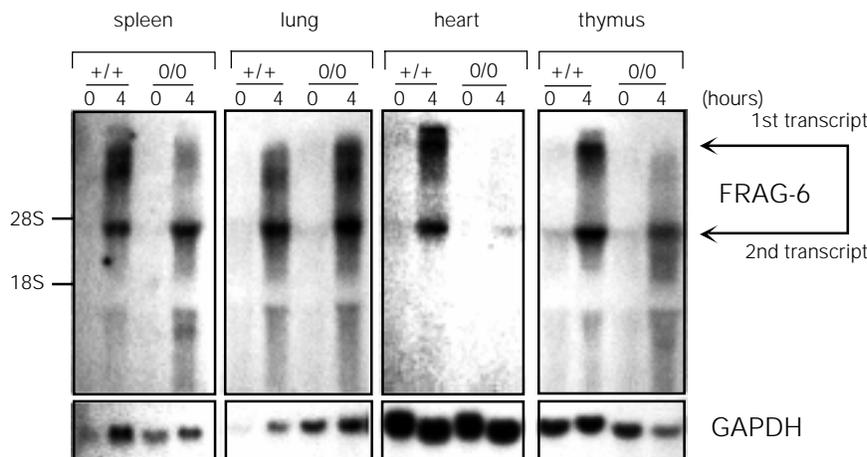
Figure 1 - In vivo induction of FRAG-6, a new mouse interferon stimulated gene identified by DDRT-PCR. Wild type (+/+) or IRF-1$^{0/0}$ (0/0) mice were treated orally with imiquimod, a potent inducer of interferon synthesis, for 4 h or left untreated (0 h). Animals were sacrificed, organs were collected, and total RNA was isolated. Fifteen micrograms of total RNA was fractionated on 0.8% denaturing-agarose gel, transferred to a nylon membrane, and hybridized with a [α-^{32}P]dCTP-labeled FRAG-6 cDNA fragment. In order to ensure equal RNA loading, the filter was stripped and re-hybridized with a [α-[α-^{32}P]dCTP-labeled mouse GAPDH.
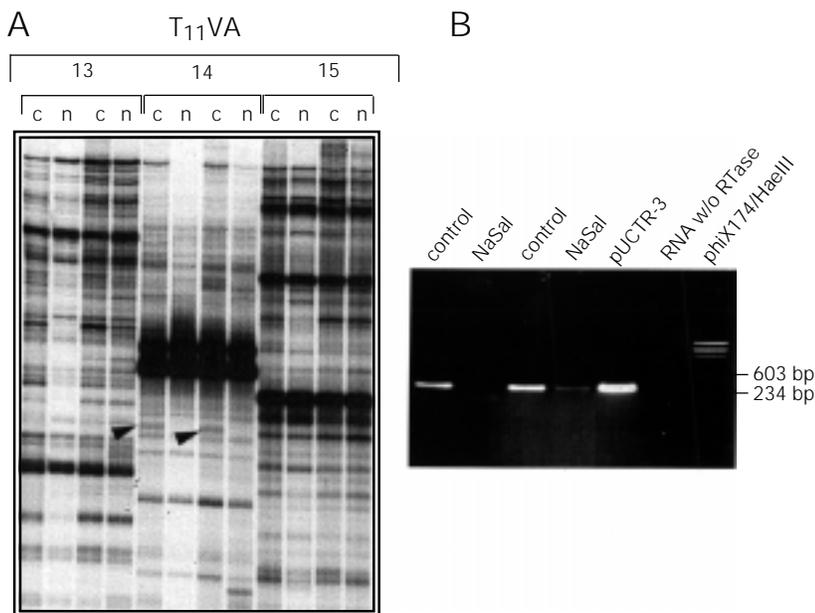
Figure 2 - DDRT-PCR analysis and identification of a sodium salicylate (NaSal)-inhibited gene. A, Total RNA was extracted from near confluent control untreated cells (c) or after 4 h of stimulation with sodium salicylate (20 mM) (n). The cDNA first strand was obtained from DNase-treated total RNA with the anchored oligo $T_{11}VA$ in the presence of dNTPs and MMLV-reverse transcriptase. One tenth of the first strand reaction was amplified by PCR using a combination of different random primers in the presence of [α-^{32}P]dCTP and dNTP mix. The products were analyzed on a 6% sequencing gel. Duplicate samples from control (c) or sodium salicylate-treated (n) cells were obtained from independent cDNA first strand and PCR reactions. Arrowheads indicate a putative differentially expressed band named CTR-3. B, Assessment of the differential expression of CTR-3 by RT-PCR. After recovering from the sequencing gel, elution, re-amplification, cloning and sequencing, specific primers were designed for the CTR-3 cDNA fragment. The two control and NaSal lanes represent independent RT-PCR products obtained from cDNA first strand reactions derived from different mRNA preparations. Lanes indicating pUCTR-3 and RNA w/o RTase are positive and negative controls, respectively. The products were fractionated on 1.2% agarose gel, and stained with ethidium bromide (0.5 µg/ml).

blot analysis. In these cases, we have investigated the expression of the putative differentially expressed mRNA by RT-PCR. In Figure 2A, we show a DDRT-PCR sequencing gel with a differentially represented band (arrowhead). When the cDNA fragment was used as a probe in Northern blot we did not detect any specific hybridization. However, by RT-PCR (Figure 2B), we can see that CTR-3 mRNA is detectable in control, untreated cells but, upon treatment with sodium salicylate, its steady-state level is significantly diminished. We also found that the TNF-induced expression of Cdc2 kinase is inhibited when cells are treated simultaneously with TNF and sodium salicylate (data not shown). We are now using this approach to gain some insight into the molecular mechanism of action of sodium salicylate as an anti-inflammatory drug (Silva AM and Reis LFL, unpublished data). It will be interesting to compare the promoter regions of the Cdc2 kinase and CTR-3 genes to determine the molecular bases of transcription inhibition by sodium salicylate. Finally, a new TNF-stimulated gene named TSG-5 has also been identified and is currently under functional characterization by our group (Pires EG, Abrantes EF, Carvalho AF and Reis LFL, unpublished data).

It is important to state that DDRT-PCR is quite reproducible. By comparing the duplicate lanes in Figure 2A, one will observe a significant degree of similarity in the band pattern. Nevertheless, it should be pointed out that reproducibility is not a central issue in this methodology and ultimately only Northern blot or RT-PCR analysis will confirm the differential expression of a given gene.

Thus, we can conclude that, under optimal conditions, DDRT-PCR is in fact a powerful approach for the identification of genes whose expression is modulated by changing stimuli, culture conditions, or even different stages of pathology. We are currently applying the DDRT-PCR technique to compare and identify new genes that are expressed in normal gastric mucosa and gastric tumors.

Acknowledgments

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