Nucleic Acids Research, 1999, Vol. 27, No. 10 **2211–2218**

Identification of genes regulated by muscarinic acetylcholine receptors: application of an improved and statistically comprehensive mRNA differential display technique

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Received December 7, 1998; Revised and Accepted March 24, 1999

ABSTRACT

In order to identify genes that are regulated by muscarinic acetylcholine receptors, we developed an mRNA differential display technique (DD) approach. By increasing redundancy and by evaluating optimised reagents and conditions for reverse transcription of total RNA, PCR and separation of PCR products, we generated a DD protocol that yields highly consistent results. A set of 64 distinct random primers was specifically designed in order to approach a statistically comprehensive analysis of all mRNA species in a defined cell population. This modified DD protocol was applied to total RNA of HEK293 cells stably expressing muscarinic m1 acetylcholine receptors and cells stimulated with the receptor agonist carbachol were compared to identical but non-stimulated cells. In 81 of 192 possible PCR experiments, 38 differential bands were identified. Sequence analysis followed by northern blot analyses confirmed differentially expressed genes in 19 of 23 bands analysed. These represented 10 distinct immediate-early genes that were up-regulated by m1AChR activation: *Egr-1*, *Egr-2*, *Egr-3*, NGFi-B, ETR101, c-*jun*, *jun*-D, Gos-3 and *hcyr61*, as well as the unknown gene *Gig-2*. These data show that this improved DD protocol can be readily applied to reliably identify differentially expressed genes.

INTRODUCTION

Metabotropic cell surface receptors including neurotransmitter receptors couple extracellular signals to specific cellular responses that are mediated by changes in protein synthesis and gene expression. Therefore, comparative studies of either mRNA or protein levels are a key to understanding these processes. In order to identify changes in gene expression in different cellular populations or developmental stages, classical methods including differential screening (1) and subtracted cDNA libraries (2) are used. These techniques were supplemented recently by the introduction of PCR-based methods. Among these, differential display of RNA (DD), invented by Liang and Pardee (3), is the most commonly used one (4,5). The obvious benefits of this method are that several samples can be analysed simultaneously and that both up- and down-regulated genes can be identified in the same experiment. Therefore, DD has become one of the favoured approaches for the discovery of novel pharmaceutical targets (6–8).

Muscarinic acetylcholine receptors (mAChRs) are G protein-coupled cell surface receptors with the classic seven transmembrane domain topology. In addition to short-term cellular effects these receptors regulate gene expression in response to external stimuli. In brain, mAChRs are involved in long-term potentiation and synaptic plasticity, as well as in higher cognitive functions including learning and memory (9). Such plastic alterations in neuronal structure and function have been proposed to be associated with the rapid and transient transcription of activity-dependent genes such as the immediate-early genes c-*fos*, *jun*-B, *Egr-1* and *Egr-2* (10–12). As a corollary, regulated *Egr-1* expression in brain cortex is related to visual memory in primates (13).

In order to identify genes that are regulated by mAChRs, we improved the DD technique. Although appearing straightforward at first glance, achieving reproducible performance of the DD method is far from simple. Many laboratories have realized various limitations of DD, including irreproducible patterns, poor resolution of fragments, co-migration of bands, contamination and large numbers of false positive hits (14,15). Although several adjustments and refinements of each individual step in the DD method as well as modifications of the technical paradigms have been described since its original publication (as reviewed in 5,7,16), some of these technical shortcomings still remain. In this study, we identified and solved critical issues related to equipment, reagents and protocols and we designed a set of 64 different random primers to approach a statistically comprehensive screen of all possible mRNA species. By using this modified strategy, we performed a screen for genes induced by m1 mAChRs and initial results were used to evaluate this DD protocol.

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Table 1. A set of 64 primers was designed to enable a complete screen with the odds of 1.5–6.2 to match a target gene

Primer	5′-sequence	3′-end	Primer	5′-sequence	3′-end
(A) Set of primers for a comprehensive DD-PCR screen					
DD1	HP5/EP5	AAA	**DD33**	HP5/EP5	GAA
DD2	HP5/EP5	AAC	**DD34***	HP3/EP3	GAC
DD3	HP5/EP5	AAG	**DD35***	HP3/EP3	GAG
DD4	HP5/EP5	AAT	**DD36***	HP5/EP5	GAT
DD5	HP5/EP5	ACA	**DD37**	HP1/EP1	GCA
DD6	HP3/EP3	ACC	**DD38***	HP1/EP1	GCC
DD7	HP3/EP3	ACG	**DD39***	HP1/EP1	GCG
DD8	HP5/EP5	ACT	**DD40***	HP1/EP1	GCT
DD9	HP5/EP5	AGA	DD41	HP3/EP3	GGA
DD10*	HP3/EP3	AGC	DD42	HP3/EP3	GGC
DD11	HP3/EP3	AGG	DD43	HP1/EP1	GGG
DD12	HP5/EP5	AGT	DD44	HP3/EP3	GGT
DD13	HP5/EP5	ATA	DD45	HP5/EP5	GTA
DD14	HP5/EP5	ATC	**DD46***	HP3/EP3	GTC
DD15	HP3/EP3	ATG	DD47	HP3/EP3	GTG
DD16	HP5/EP5	ATT	DD48	HP5/EP5	GTT
DD17	HP5/EP5	CAA	DD49	HP5/EP5	TAA
DD18*	HP3/EP3	CAC	DD50	HP5/EP5	TAC
DD19*	HP3/EP3	CAG	DD51	HP5/EP5	TAG
DD20*	HP3/EP3	CAT	DD52	HP5/EP5	TAT
DD21	HP3/EP3	CCA	DD53	HP5/EP5	TCA
DD22	HP1/EP1	CCC	**DD54***	HP3/EP3	TCC
DD23*	HP1/EP1	CCG	DD55	HP3/EP3	TCG
DD24	HP3/EP3	CCT	DD56	HP5/EP5	TCT
DD25	HP3/EP3	CGA	**DD57**	HP2/EP2	TGA
DD26*	HP1/EP1	CGC	**DD58**	HP2/EP2	TGC
DD27*	HP1/EP1	CGG	**DD59**	HP2/EP2	TGG
DD28	HP3/EP3	CGT	**DD60***	HP2/EP2	TGT
DD29	HP5/EP5	CTA	DD61	HP5/EP5	TTA
DD30	HP3/EP3	CTC	DD62	HP5/EP5	TTC
DD31*	HP3/EP3	CTG	DD63	HP5/EP5	TTG
DD32*	HP5/EP5	CTT	DD64	HP5/EP5	TTT
(B) Different 5′-sequences of DD primers					
HP1	TGCCG**AAGCTT**GATT				
HP2	TGCCG**AAGCTT**CGAC				
HP3	TGCCG**AAGCTT**TGGT				
HP5	TGCCG**AAGCTT**GGAG				
EP1	TGCCG**GAATTC**GATT				
EP2	TGCCG**GAATTC**CGAC				
EP3	TGCCG**GAATTC**TGGT				
EP5	TGCCG**GAATTC**GGAG				

MATERIALS AND METHODS

Cell culture

HEK293 cells stably transfected with the m1 mAChR were grown in DMEM/F12 medium supplemented with 10% foetal calf serum and 500 µg/ml geneticin (G418; Life Technologies). The cells were maintained in a 5% CO_2 atmosphere at 37°C. For experiments, the cells were grown to an approximate density of 80–90%. Sixteen hours before experiments, growth medium was replaced by serum-free DMEM/F12 without G418. Incubation of cells with test substances were performed by adding concentrated stock solutions of carbachol (Sigma) to a final concentration of 1 mM, or carbachol together with 10 µM atropine (Sigma) to the culture medium. Identical cells generated in parallel from the same passage were used as unstimulated controls. Effective receptor stimulation and transduction responses were verified by measuring carbachol-induced β-amyloid precursor protein ecto-domains released into the conditioned medium as described (17).

RNA extraction

Total RNA was prepared from the cells by using the RNeasy kit (Qiagen) according to the manufacturer's instructions. The RNA preparations were subjected to DNase I digestion (Boehringer Mannheim) in the presence of recombinant ribonuclease inhibitor RNase OUT (Life Technologies) for 30 min. RNA was extracted with phenol and precipitated in ethanol.

Differential RNA display (DD)

Equal amounts of 0.2 µg RNA each were transcribed to cDNA in 20 µl reactions containing 20 µM dNTP, 10 µM DTT, 2 µl Expand™ Reverse Transcriptase (Boehringer Mannheim), 1 µl RNase OUT (Life Technologies™) and 1 µM either one base anchor oligonucleotides $HT_{11}A$, $HT_{11}G$ or $HT_{11}C$ (18). Reverse transcription was performed for 60 min at 42°C with a final denaturation step at 95°C for 3 min. Aliquots of 2 µl of the obtained cDNA each were subjected to PCR employing the corresponding one base anchor oligonucleotide (1 µM) along with either one of the DD random primers (1 µM; Table 1), 1.8 mM $MgCl_2$, 0.66 µM each dGTP, dCTP and dTTP (Amersham Pharmacia Biotech), 1 U AmpliTaq polymerase (Perkin-Elmer Corp.) and 0.5 µM [α-^{35}S]dATP (2000 Ci/mmol; NEN) in a 20 µl final volume. PCR conditions according to Zhao *et al.* (19) were modified and performed as follows: one round at 94°C for 1 min for denaturing; cooling at 1°C/s down to 40°C; 40°C for 4 min for low stringency annealing of primer; heating at 1°C/s up to 72°C; 72°C for 1 min for extension. This round was followed by 35 high stringency cycles: 94°C for 45 s; cooling at 1°C/s down

In addition to their combinatorial maximal sequence variation of the 3 bases 3′-end (A), the set of 64 DD primers possesses a common structure of distinct 15 bases 5′ sequences (B). HP1, HP2, HP3 and HP5 represent *Hin*dIII restriction site-containing 5′-ends of random primers with a 5 bases sequence upstream of the restriction site as well as a 4 bases downstream spacer sequence between the restriction site and the 3′-end. EP1, EP2, EP3 and EP5 represent alternative *Eco*RI-restriction site-containing 5′-ends of random primers with otherwise identical structure. DD primers used in this study are indicated by bold type. Primers that resulted in the generation of a differential band are marked by an asterisk (A).

to 60°C; 60°C for 2 min; heating at 1°C/s up to 72°C; 72°C for 1 min. One final step at 72°C for 5 min was added to the last cycle. PCR products were dried in a SpeedVac concentrator (Savant), resuspended in 10 µl DNA loading buffer, boiled for 5 min, centrifuged for 2 min at 14000 r.p.m. (Eppendorf 5415 centrifuge) and 3 µl each were separated on 0.4 mm thick, 6% polyacrylamide/7 M urea sequencing gels in a temperature-regulated LKB 2010 Macrophore Sequencing System (Amersham Pharmacia Biotech) at 55°C and at 2000 V. Gels were dried on Whatman 3MM paper at 80°C for 1 h and X-ray films (DuPont) were exposed for 8–16 h.

Cloning and sequencing of differential RNA display products

Differential bands were excised from the gel, boiled in 200 µl H_2O for 10 min and cDNAs were precipitated from the supernatant fluids using ethanol and glycogen/sodium acetate, followed by dialysis against 10% glycerol for 1 h on a 0.025 µm VS membrane (Millipore). The obtained preparations were used for re-amplification by 40 high stringency cycles in 50 µl PCR mixtures containing the corresponding primer pairs as used for the DD-PCR and identical conditions, with the exception of the initial round for non-specific annealing and replacing [α-^{35}S]dATP with unlabelled dATP. Re-amplified cDNAs were purified by agarose gel electrophoreses, gel extraction using the Qiaex II kit (Qiagen) and were ligated into pBluescript IIKS+ (Stratagene) using the *Hin*dIII restriction sites. Cloned cDNA fragments were sequenced with an ABI 377 DNA sequencer (Perkin Elmer) using T3- and T7-derived sequencing primers.

Northern blot analysis

Aliquots of 5–20 µg total RNA each were separated by agarose formaldehyde gel electrophoresis and blotted onto Hybond™-N$^+$ nylon membranes (Amersham Pharmacia Biotech) as described (20). Membrane filters were probed with [α-^{32}P]dCTP random primed cDNA probes prepared from cDNA clones or from PCR products obtained in the differential display. Hybridized filters were washed under high stringency conditions and were subjected to autoradiography. Equal RNA loading was confirmed by probing the identical blots with a glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) probe. A human cDNA probe of ETR101 was obtained as an EST (IMAGp998N14274) from the Research Center/Primary Database of the German Genome Project in Berlin, Germany (21).

RESULTS AND DISCUSSION

By using a novel, modified DD technique, we identified differentially expressed genes induced by mAChRs. Our DD protocol was designed to fulfil two major requirements: reproducibility for many paradigms of differential gene expression without the need to re-improve individual steps and, second, to provide a standard set of primers coupled to a sufficient number of DD reactions in order to perform a statistically comprehensive DD study.

Improvement of the differential display protocol

Redundancy is a main advantage of the DD technique and extensive use of it helps prevent false positive results (22). In this study parallel cell culture experiments were done in triplicate followed by duplicate PCR reactions generating 6-fold redundancy for each experimental condition. In addition to optimal redundancy, using cell-based systems for the analysis of receptor function in transcriptional regulation enables the carrying out of plain stimulation of the receptors. In tissues, receptors are constitutively active and pharmacological stimulation may not further increase this activity. In addition, assuring proper receptor function is readily possible in cell culture. On the other hand, a disadvantage of many cell-based systems is that they may have limited physiological relevance. Tissues typically contain multiple cell types with possibly distinct gene expression profiles and two independent tissue samples may contain different ratios of cell types. Therefore, using tissues as the RNA source for the analysis of differential gene expression requires careful selection of comparable tissue samples.

In this study, as in most described DD protocols, total RNA was used as the starting material. Although some protocols suggest the use of poly(A)$^+$ RNA (23,24), it is important to note that poly(A)$^+$ RNA may carry over oligo(dT) from the purification matrix and confound the subsequent PCR (25). Moreover, stoichiometric extraction of poly(A)$^+$ RNA from total RNA derived from distinct sources is impossible. To exclude partial degradation, we tested total RNA preparations by northern blotting before using it for DD. RNA was treated with DNase to reduce artefacts caused by possible contamination with genomic DNA. Reverse transcription was done with one base anchored oligo(dT) primers (19). Among four commercial reverse transcriptases tested for the yield of cDNA (26), for reproducibility and for equal distribution of bands on DD gels, Expand™ Reverse Transcriptase (Boehringer Mannheim) and SuperScript™II RNase H$^-$ Reverse Transcriptase (Life Technologies) worked best.

Reproducible, semi-quantitative PCR is crucial for the DD technique. Since its invention in 1992 (3), several improvements of the PCR conditions were established for primers, concentrations of templates and nucleotides and annealing temperatures, as well as cycle design (22,25,27–32). We tested all of these parameters in this study and generated an optimised PCR protocol (Materials and Methods). This protocol achieved a reproducible pattern of DD-PCR products over the entire length of a 52 cm sequencing gel after electrophoresis (Fig. 1). The choice of PCR reagents and reaction tubes was critical for consistent results. AmpliTaq DNA polymerase (Perkin Elmer), freshly diluted preparations of dNTPs and primers and thin-wall reaction tubes (Biozym) worked best. In addition, the choice of thermal cycler proved critical. Among seven tested cyclers, only three produced consistent banding patterns [Multi Cycler® PTC 200 (MJ Research); GeneAmp® PCR System 2400 (Perkin Elmer); Robocycler® (Stratagene)].

To reduce the rate of mismatches that occur with low stringency annealing during subsequent PCR cycles, we followed the strategy published by Zhao *et al*. (19): only one initial cycle with low stringency annealing (40°C) was followed by 35 high stringency PCR cycles with annealing temperatures of 60°C. This protocol significantly improved the reproducibility of band patterns. Similar previously published modifications included three to four low stringency cycles followed by 18–22 high stringency cycles (33,34).

The choice of the low stringency annealing temperature significantly affected the numbers of both PCR products and differential bands. The optimal temperature of 40°C generated homogeneous distributions of PCR products of 50–450 bp in

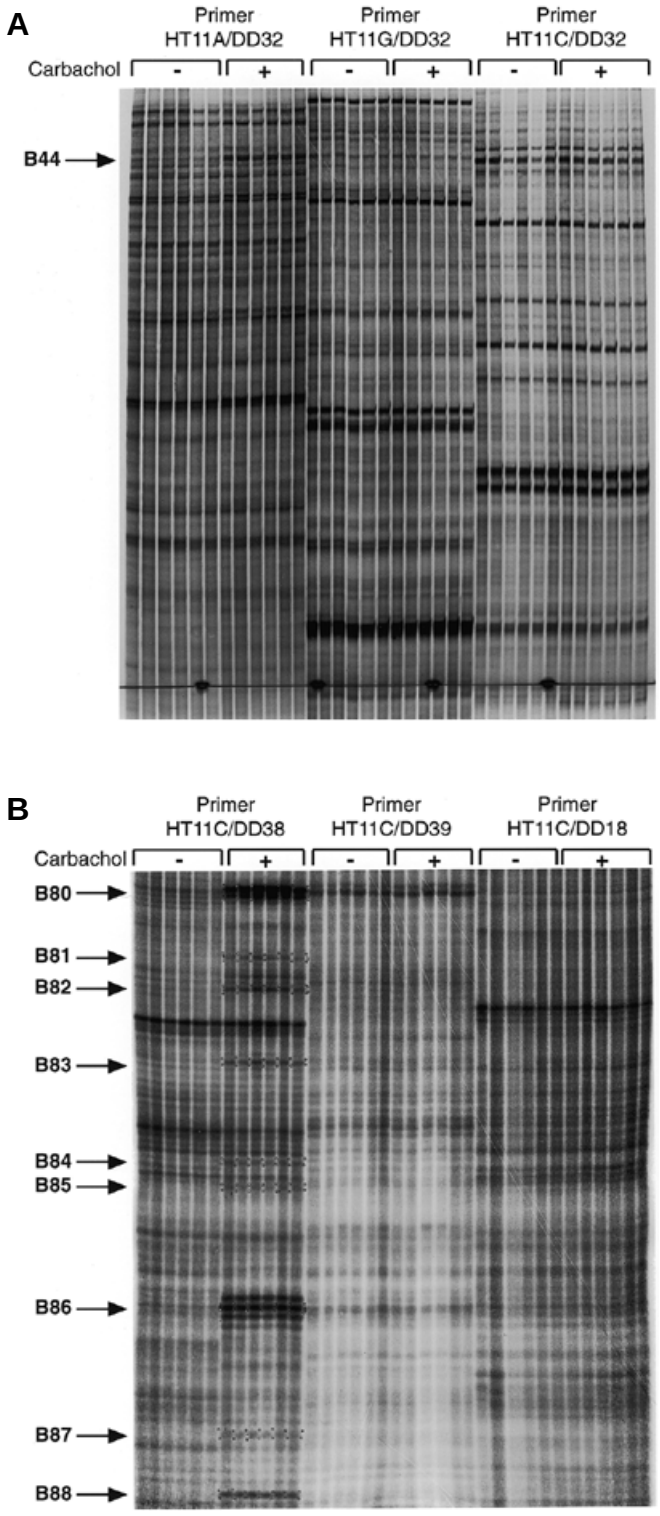

Figure 1. Two representative differential display autoradiographs. Three HEK293 cell cultures stably transfected with m1 mAChR were treated with carbachol and RNA of these was used for DD-PCR in duplicate experiments each and compared with identical cell cultures that were not treated with carbachol. Differential bands are indicated by arrows. (**A**) Three DD analyses after 1 h of m1 stimulation using one random primer and three distinct one base anchor primers. (**B**) Three DD analyses after 3 h of m1 stimulation using one one base anchor primer together with three distinct random primers.

length that are readily separated on a 52 cm sequencing gel. Lower temperatures, including 38 and 36°C, yielded preferentially shorter PCR products of 50–150 bp. Higher annealing temperatures, including 42 and 44°C, yielded lower amounts of PCR products that were, however, homogeneously distributed over the entire size range.

The temperature ramping speed of 1°C/s between both denaturation and annealing, and annealing and polymerisation, generated the optimal homogeneity of product size distribution in the PCR. Higher ramping speeds of 1.5–2.5°C/s generated preferentially short PCR products of <200 bp. This was in contrast to a recent study that found no effect of various ramping speeds on band patterns (35). Such differences likely depend on distinctions among thermal cyclers.

The choice of radiolabel also affected our results. Using [^{35}S]dATP resulted in better resolution of PCR products on the sequencing gels, as compared to [^{33}P]dATP. In agreement with the observation by Tokuyama and Takeda (36), incorporation of [^{33}P]dATP resulted in bands with significantly smaller sizes. Because ^{35}S-labelled compounds release radioactive vapours during PCR (37,38), we positioned the thermal cyclers and gel dryers under a hood.

The detection of differential cDNAs requires the use of high resolution polyacrylamide gels (39). For optimised separation, we used denaturing 6% polyacrylamide/urea sequencing gels. These were run at a water flow controlled temperature of 55°C to minimise gel smiling effects. Commercially available high resolution polyacrylamide stock solutions (Long Ranger™; FMC) improved resolution as compared to self-made polyacrylamide mixes. Sharpness of band patterns was dependent on sample purity. Artefacts caused by precipitates including smears were avoided by centrifugation before loading and subsequent loading of supernatant fluids.

Using differential display for statistically comprehensive screens

Under numerical considerations, a minimum number of primers was suggested for use in DD which should be sufficient to detect a maximum number of possible differential expressed messages (18,33,40). These suggestions were based on the initial DD strategy of using 10mer random primers in combination with two base anchored oligo(dT) primers introduced by Liang and Pardee (3). Optimisations of DD protocols using such primers were published recently (23,35). Zhao *et al*. (19) modified a strategy using one base anchored primers (18); it significantly improved both the reproducibility and the sensitivity of the technique. We tested the specificity of annealing stringency using such primers by cloning and sequencing of DD-PCR products and by comparing resulting sequences with GenBank entries. In 16 identified known cDNA sequences, the stringency of random primer annealing at 40°C was limited to the last 7 bases at the 3′-end. The stringency ranged from 5/7 (n = 2), 6/7 (n = 7) up to 7/7 (n = 7). In each case the 2 bases at the 3′-end matched exactly. Most mismatches where found at positions 6 and 7 upstream from the 3′-end. Bertioli *et al*. (41) found no mismatches in the last four 3′-end positions in 23 different cDNA fragments analysed. Linskens *et al*. (33) reported that mismatch frequencies in 34 cDNAs occurred more frequently towards the 5′-end of the last eight 3′-end bases using a 41°C annealing temperature, although they also detected mismatches at the last two 3′-end positions.

These results indicate that the specificity of DD primers is limited to the most 3′-end positions. Because redundancy may occur if the last 3′-end bases of the primers are identical, we designed these positions as diversely as possible. Our data guided the design of a set of 64 primers that generated reproducible DD-PCR products. This set of primers is suited for statistically comprehensive DD screens (Table 1).

The above stringency analyses led to the following statistical calculations. The length of cDNAs that can be separated unequivocally ranged from 50 to 450 bp. The use of oligo(dT)-based anchored primers generates cDNAs which represent sequences within positions –50 to –450 upstream of their 3′-end. The odds of a random 7 bp sequence hybridising within a specific 400 bp sequence equals 0.024 for a stringency of 7/7 ($1/4^7$ bases × 400 bases = 0.024) or 0.098 for a stringency of 6/7 ($1/4^6$ bases × 400 bases = 0.098). Using 64 different random primers with all possible nucleotide combinations at the last three 3′-end positions generates the odds of: $1/4^4$ bases × 400 bases = 1.563 (for a stringency of 7/7) or $1/4^3$ bases × 400 bases = 6.250 (for a stringency of 6/7). A screen with all 64 random primers in combination with three anchor primers should suffice for a statistically comprehensive screen.

We therefore constructed a set of 64 primers that included a common structure composed of three parts. The first part consists of a 6 bases *Hin*dIII or alternatively *Eco*RI restriction site (designated H or E for the nomenclature in Table 1), with a 5 bases overhang at the 5′-end. The 5′ overhang was necessary both for later cloning and for preventing competition due to self-annealing of restriction site-specific sequences. The second part consists of a 4 bases spacer sequence between the restriction site and the variable 3′ sequence; it was chosen out of four different 4 bases sequences according to the GC content of the following variable third part of the primers. The third, 3′-terminating part is variable, consisting of all 64 possible sequences for 3 bases.

Analysing differential muscarinic m1 receptor-inducible genes by the optimised differential display protocol

We used this differential display protocol to analyse mRNAs generated in response to m1 mAChR stimulation. By using 81 of 192 possible combinations of the extended DD primer set, we obtained 38 differential bands. Only bands that were clearly different in at least five of six lanes as compared to six control lanes were defined as positive hits and were used for further analyses (Fig. 1A). From these 38 differential bands, 37 showed an increased band density whereas density of one differential band was decreased in comparison to the corresponding band in control conditions. The three distinct one base anchor primers yielded similar numbers of differential bands (Table 2). Protocols with two base anchor primers reportedly result in major differences among primers in numbers of differential bands (23,33).

More than one cDNA sequence was identified in most differential bands, with a range of 1–10 and a mean of 4.5 distinct cDNA species per band, and the ratio of individual cDNA species over all cDNAs in a band was highly variable among bands. Moreover, we found that additional rounds of re-amplification of bands with low cDNA yields prior to cloning significantly increased the number of false positive cDNA amplicons with each round of re-amplification. It should be avoided.

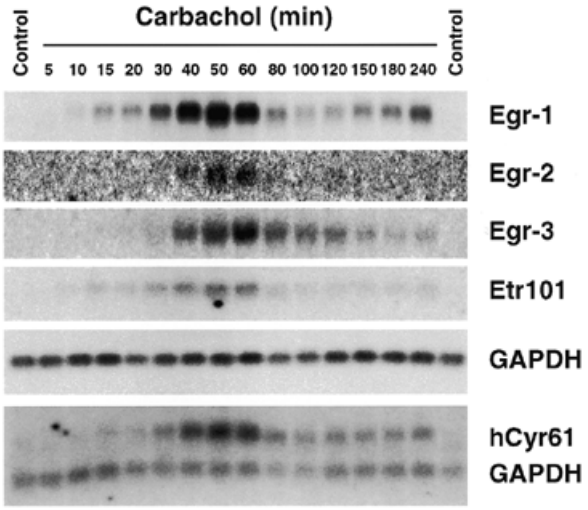

Figure 2. Northern blots of mAChR-induced mRNAs identified by differential display. Five micrograms of total RNA of m1 cells from different time points after m1 stimulation were each loaded and transferred to nylon membrane (Materials and Methods). As a control total RNA of non-stimulated cells was used. Detection of mRNAs was done with ^{32}P-labelled DNA probes of *Egr-1*, *Egr-2*, *Egr-3*, ETR101 and *hCyr61* cDNA as indicated. As a loading control for RNA, membranes were hybridized with ^{32}P-labelled DNA probe from GAPDH cDNA.

To guide our strategy for validation, we ranked the differential bands according to differences in signal intensities. The most frequent clone caused a differential signal predominantly in bands with large differences in signal intensities. Bands with less obvious differences in signal intensities failed to contain a predominant clone, and more often, less frequent clones corresponded to the differentially expressed gene. Our results demonstrate that it is highly unlikely to identify a differentially expressed gene by direct sequencing of cDNA extracted from a differential band without prior separation of individual species. Moreover, the results suggest that the technique described here produces generally one differential clone per differential band. Future improvements of the DD technique should therefore involve an additional step of separating the cDNA species of identical length prior to cloning or sequence analysis. Direct sequencing prior to separation unequivocally results in a high rate of false positive DD bands (>80%) and to a frequent failure to identify the differentially expressed clone.

Table 2. Efficiency of one base anchor primers

Anchor primer	No. of differential bands (1 h screen)	No. of differential bands (3 h screen)
$HT_{11}C$	7 (n = 27)	16 (n = 11)
$HT_{11}G$	12 (n = 27)	2 (n = 10)
$HT_{11}A$	19 (n =27)	5 (n = 6)

Evaluation of differential bands using the three distinct one base anchor primers in combination with a different number of DD primers using RNA from m1 cells after 1 h of m1 induction (n = 81) as well as 3 h induction (n = 27). DD autoradiographs were evaluated as described in the text.

Table 3. Summary of data revealed by performed DD analyses

Time after mAChR activation	No. of primer combinations used	No. of differential bands	No. of bands analysed	No. of verified bands
1 h	81 (42%)	38 (0.47/primer combination)	23	19
3 h	27 (14%)	23 (0.85/primer combination)	–	–

Data represent numerical results from DD screens for m1 mAChR-inducible genes after 1 and 3 h of receptor activation. Percentages of primer combinations used were calculated from a theoretically comprehensive screen with 192 possible primer combinations as described.

Table 4. Characterization of 19 analysed differential display bands

DD band	Primer (anchor/random)	Gene product, protein function
B29	$HT_{11}G$/DD38	Egr-1, transcription factor
B32	$HT_{11}A$/DD38	Egr-1, transcription factor
B33	$HT_{11}A$/DD38	Egr-1, transcription factor
B34	$HT_{11}A$/DD60	Gig-2, unknown
B37	$HT_{11}G$/DD19	c-jun, transcription factor
B38	$HT_{11}A$/DD19	c-jun, transcription factor
B39	$HT_{11}C$/DD23	NGFi-B, transcription activator
B40	$HT_{11}G$/DD46	Etr101, unknown function
B41	$HT_{11}G$/DD46	Etr101, unknown function
B43	$HT_{11}G$/DD38	Egr-1, transcription factor
B44	$HT_{11}A$/DD32	hCyr61, growth promoter
B47	$HT_{11}C$/DD32	c-jun, transcription factor
B53	$HT_{11}A$/DD18	Egr-3, transcription factor
B54	$HT_{11}A$/DD20	hCyr61, growth promoter
B55	$HT_{11}A$/DD20	Egr-2, transcription factor
B56	$HT_{11}G$/DD18	Egr-3, transcription factor
B57	$HT_{11}G$/DD18	Gos-3, transcription factor
B58	$HT_{11}G$/DD18	Egr-3, transcription factor
B69	$HT_{11}G$/DD26	jun-D, transcription factor

DNA sequencing and database analysis identified genes and novel gene tags. Verification of regulation of identified genes was done by northern blot analyses.

For the selection of cDNA clones for further validation, we excluded PCR products with anchor primer sequences on both ends. Our protocol did not generate clones with random primer sequences at both ends; these should be included in the analyses, however, should they occur. Random primers without anchor primers are used for arbitrary primed PCR in RNA fingerprinting (42) and this strategy was also proposed for DD (28).

Northern blot analyses showed that 19 of 23 analysed differential bands carried a differential clone (Table 3 and Fig. 2). Each one of these contained both random and anchor primer sequences at the 5′- and 3′-ends, respectively (Table 4). Three of 23 differential bands contained genomic adenovirus DNA sequences derived from transformation of the HEK293 cells used in this study. These were obtained with anchor primer $HT_{11}A$ and random primer DD39. One of 23 bands contained only $HT_{11}C$ anchor primer sequences at both ends and was therefore excluded from northern analysis.

Five of the differentially expressed genes were found repeatedly (Table 4). This redundancy falls within the calculated odds range. It proved beneficial for confirming positive hits. On some occasions, distinct anchor primers generated redundancy, as shown for c-*jun* and *Egr-1*. This may be related to the known variability of polyadenylation start sites behind distinct polyadenylation signals (43). Variable polyadenylation is further underscored by different entries for cDNA sequences in the databases: for human *Egr-1*, for example, three distinct cDNA entries (accession nos X52541, M62829 and M80583) are accessible in the GenBank database, with G, A or T as the 3′-terminating nucleotide, respectively. Our data are less compatible with primer mismatch, because sequence analyses showed that the PCR conditions used here did not produce mismatches at the two initial bases at the 3′-end, at least for the random primers used.

Identification of several immediate-early genes

Most of the genes induced within 1 h of m1 mAChR activation encoded transcription factors, including c-*jun*, *jun*-D and Gos-3, as well as *Egr-1*, *Egr-2* and *Egr-3*, members of a transcription factor family with a zinc-finger DNA-binding domain structure (Table 4). Muscarinic AChR-induced expression of transcription factor gene families *jun*, *fos* and *Egr* via intracellular signals are known (44–52; reviewed in 53,54). A more detailed study of the mAChR-coupled regulation of the *Egr* family showed that mAChRs can regulate *Egr-1*, *Egr-2*, *Egr-3* and *Egr-4* and that *Egr*-dependent transcription of downstream target genes is also readily inducible as a result of receptor stimulation (55).

Additional genes induced by m1AChR included NGFI-B, a transcription activator of the nuclear factor superfamily (56), and ETR101, an immediate-early gene with unknown function (57). Carbachol induction of gene expression was shown for NGFI-B in rat astrocytes (58) and in PC12 cells (47) and for ETR101 in a 2PK-3 B lymphoma cell line transfected with m1 mAChR (59). In addition, we identified the immediate-early effector gene *hCyr61*, a member of the insulin-like growth factor-binding protein superfamily (60). *hCyr61* is a known growth factor-inducible gene (61), with physiological roles in proliferation, migration and adhesion (62). In addition, a previously unknown human gene, *Gig-2* (for G-protein-coupled receptor-induced gene 2), was identified; *Gig-2* transcript levels increased within 40 min of stimulation, and reached a maximum intensity within 60–100 min of stimulation. Because mAChR-coupled *Gig-2* expression was not blocked by cycloheximide, it fulfils the criteria of an early-immediate gene, too.

Taken together, all identified mAChR-inducible genes were immediate-early genes, and for some of these genes, signal-regulated inducibility was previously known. The physiological roles of these genes in signal-regulated cell functions need further study. As an example, we showed mAChR-coupled transcription

from *Egr*-dependent promoters including the human acetylcholinesterase promoter (55,63).

In order to identify additional mAChR-inducible target genes and late response genes, we analysed cells 3 h after stimulation by DD (Fig. 1B). Initial experiments with 27 of 192 possible primer combinations revealed 23 differential bands. This rate was twice as high as compared to our 1 h screen. Comparisons of DD band patterns from the 1 and 3 h screens with identical primer combinations showed that *hCyr61* (primer $HT_{11}A$ and DD32) and *Egr-1* (primer $HT_{11}G$ and DD38) were elevated in both conditions. c-*jun*, *Egr-3* and Gos-3, however, were no longer increased 3 h after stimulation. Many additional differential bands in the 3 h screen were not yet elevated after 1 h of stimulation.

DD is an efficient method to identify differential gene expression

Our results demonstrated that the modified DD protocol is useful to identify both known and novel genes that are under the control of cell surface receptors. This protocol generates a high number of differential bands, with <10% false positive bands. The number of false positive bands can be greatly reduced with increased redundancy in the tissue culture manipulations and PCR reactions and by rational analysis of differential bands that most likely contain several different PCR products, only one of which represents the differential cDNA.

On the basis of the data generated by this DD screen for mAChR-inducible genes, we calculated that a complete screen of RNA expressed within 1 h of receptor stimulation can yield approximately 80 differential bands. Anticipating increasing redundancy related to the number of primer combinations used, 25–40% of these bands are likely to represent different cDNA species; this may lead to the identification of 20–32 mAChR-inducible genes. Most of these will be immediate-early genes, as suggested by the genes characterized so far. Three hours after mAChR activation, a significantly higher number of differential bands was evident, some of which are identical to genes already induced within 1 h of stimulation, but another set of genes represent late effector or downstream target genes.

To establish an inventory of mAChR-inducible genes several time points after receptor activation need to be analysed. Therefore, it may be helpful to accompany the DD by other gene expression profiling techniques, including subtractive hybridization, subtractive cDNA libraries or the use of DNA microarrays (64). In the absence of a complete inventory of human genes, DD has the advantage over DNA chip technology of identifying novel genes that are exclusively expressed under specific conditions. Electronic databank comparisons like electronic subtraction (ES) (65), including serial analysis of gene expression (SAGE) (66), need extensive sequencing and they detect predominantly abundant mRNA. DD is a widely applicable method, needs less amount of starting material and can generate useful information even when performed only partially (67). The DD protocol as worked out and evaluated in this study should short-circuit starting this technique for new applicators and give the opportunity to carry out a statistically complete screen for given paradigms.

ACKNOWLEDGEMENTS

Funded by the BMBF, DFG and AFI.

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