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Programmed cell death in procyclic *Trypanosoma brucei rhodesiense* is associated with differential expression of mRNAs

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Abstract

Procyclic *Trypanosoma brucei rhodesiense* have a cell death mechanism which can be activated by an external signal, the lectin ConA, *in vitro*. ConA has been shown to cause profound changes in cellular morphology and induce fragmentation of nuclear DNA in *T.b. rhodesiense* which are characteristic of apoptosis, a form of programmed cell death (PCD) in other eukaryotic cells. RNA analysis of trypanosomes induced to undergo PCD revealed that RNA remains intact up to 48 h into the process, a time when nuclear DNA fragmentation has already started. Using the randomly amplified differentially expressed sequences polymerase chain reaction method, ConA-induced cell death in *T.b. rhodesiense* is shown to be associated with differential expression of mRNAs, including up regulation of mRNAs late in the death process. The results demonstrate that trypanosomes actively participate in their own destruction through a PCD process and confirm that cell death in trypanosomes is associated with *de novo* gene expression.

Keywords: apoptosis; *Trypanosoma brucei rhodesiense*; programmed cell death, lectin; differential display; differential gene expression

Abbreviations: bp, base pair(s); ConA, concanavalin A; FBS, foetal bovine serum; EtdBr, ethidium bromide; kbp, kilobase pair(s); PCD, programmed cell death; RADES – PCR, randomly amplified differentially expressed sequences – polymerase chain reaction

Introduction

Until recently it was assumed that apoptosis, the most common form of PCD in multicellular organisms (Wyllie *et al*, 1984), evolved to regulate growth and development in multicellular organisms and that a regularised system of cell death and other essential developmental programmes appeared in phylogeny after the onset of multicellularity (Vaux *et al*, 1994). The finding of apoptosis in a number of parasitic kinetoplastids [*Trypanosoma cruzi* (Ameisen *et al*, 1995); *Trypanosoma brucei rhodesiense* (Welburn *et al*, 1996); *Leishmania (L) amazonensis* (Moiera *et al*, 1996)] which represent some of the first organisms to diverge from the eukaryotic phylogenetic tree, and in two other unicellular organisms, *Dictyostelium discoideum* (Cornillon *et al*, 1994) and *Tetrahymena thermophila* (Christensen *et al*, 1995) which diverged some time later, has important implications for our understanding of the origins of PCD in eukaryotic cell survival (Ameisen, 1996). Such studies may offer new possibilities for controlling the debilitating diseases caused by parasitic kinetoplastid organisms (Welburn *et al*, 1997).

The ability of ConA to stimulate apoptosis in African trypanosomes (Welburn *et al*, 1996) presents an ideal system to examine the regulation of PCD in single celled trypanosomatids *in vitro*, to identify genes implicated in this process and determine whether unicellular eukaryotes share effectors and regulators of cell suicide with multicellular organisms.

To examine whether lectin induced parasite death in *T.b. rhodesiense* is under genetic control and to determine the molecular mechanisms involved, we have examined RNA fidelity in ConA treated cells up to the point at which DNA fragmentation begins in these cells. A differential display method, randomly amplified differentially expressed sequences PCR (RADES – PCR) (Murphy and Pelle, 1994), has been used to further confirm RNA fidelity and to determine whether specific RNAs are up or down regulated during the death process. Unlike other differential display methods, RADES – PCR differential display utilises cDNA as the template and exploits the fixed 5′-specific miniexon and 3′-specific oligo (dT) sequences of all trypanosome mRNAs characterised to date (De Lange *et al*, 1984; Parsons *et al*, 1984). This method is applicable to all kinetoplastids and can be used to identify parasite specific message from material containing vector or host mRNAs.

Results

Cell death is reversible up to 7 h post treatment with ConA

Previously, apoptosis in procyclic forms of *T.b. rhodesiense* was shown to be induced at a concentration of 50 μg/ml of ConA (Welburn *et al*, 1996). To determine the minimum concentration of ConA required to induce apoptosis in these parasites, the concentration added to procyclic cultures was titrated from 50 μg/ml to 0.1 μg/ml. A concentration of 1 μg/ml was found to be equally as effective in inducing apoptosis as the 50 μg/ml previously utilised (Welburn *et al*, 1996) when assessed for DNA fragmentation. Therefore, in all experi-

ments reported here, the lower concentration of ConA was used.

ConA-induced apoptosis in procyclic forms of *T.b. rhodesiense* is a relatively slow process; the characteristic DNA ladder of apoptotic cells beginning to become detectable 48 h into the process but most clearly detectable after 72 h. In deciding on time points to use for the RADES–PCR differential display analysis it was of interest to determine whether trypanosomes could recover from ConA treatment and at which point cells were irreversibly committed to dying. Procyclic cultures of *T.b. rhodesiense* were treated with 1 μg/ml of ConA for 2, 4, 7, 12, 24 and 48 h following which they were harvested, washed and placed in Cunningham's procyclic medium (Cunningham, 1977) lacking ConA. Cells treated with ConA for up to 7 h were recoverable, assumed the normal shape of procyclic form trypanosomes and underwent normal cell division. Cells treated for longer than 7 h remained rounded and agglutinated and did not undergo any further rounds of division but continued to undergo the induced apoptotic programme. It appears therefore that after 7 h of treatment with 1 μg/ml of ConA, procyclic *T. rhodesiense* cells are irreversibly committed to the death process. From these preliminary results, cells treated with ConA for 7, 24 and 48 h, in addition to untreated control cells, were chosen for RNA and RADES–PCR differential display analysis.

RNA remains intact up to 48 h into the apoptotic process

Apoptosis in *T.b. rhodesiense* has previously been shown to require *de novo* protein synthesis (Welburn *et al*, 1996) and it was of interest to determine whether RNA remains intact in the period leading up to DNA fragmentation at 72 h post treatment and whether the regulation of trypanosome gene expression was altered during the process. We have examined RNA fidelity as judged by ribosomal and small structural RNAs up to 48 h into the apoptotic process and, while there is some variation in small tRNAs between samples the higher Mol. Wt. RNAs were not observed to be degraded by ConA treatment and appeared intact, especially at 48 h ConA treatment, at which time DNA fragmentation had already started (Figure 1). Furthermore, there was no detectable difference in the total amount of RNA isolated from treated and untreated cells. Hybridisation of a Northern blot of this RNA with β-tubulin confirmed RNA fidelity showing equal intensity labelling of message for this housekeeping gene over the 48 h period (data not shown).

Alterations in RNA expression including up regulation of gene expression late in the death process and transient expression during the process are clearly visible in RADES fingerprints of dying cells

To determine whether alterations in mRNA expression occur during ConA-induced apoptosis of procyclic form *T.b. rhodesiense*, RNA from untreated control cells and cells treated for 7, 24 and 48 h was converted to cDNA; total cDNA was amplified by PCR with miniexon and oligo (dT) primers. These amplified cDNAs were used as templates for RADES–PCR analysis.

For each RADES–PCR two primers were used, a 13-mer containing the 11 most 3′ nucleotides of the trypanosome miniexon sequence followed by a mixed base and either A, C, G or T, and a 10-mer primer of arbitrary sequence. RADES–PCRs were conducted at two template concentrations (1 ng/μl and 0.2 ng/μl) to distinguish artefactual products (differentially amplified in response to total template concentration, but which are

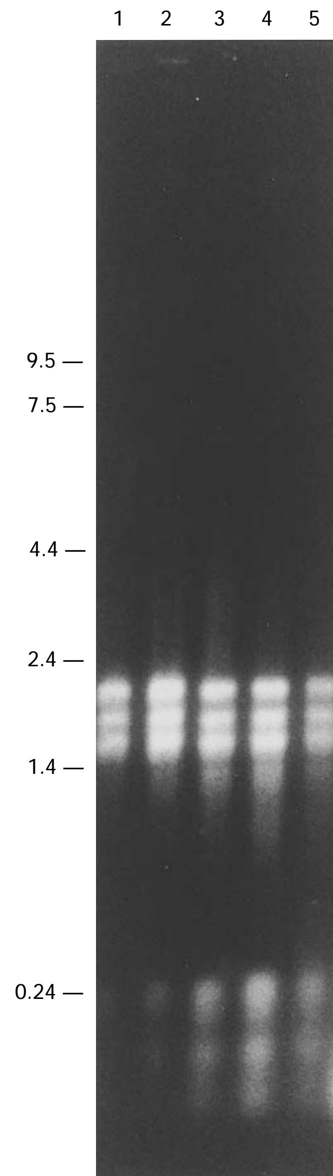

Figure 1 Analysis of fidelity of RNA isolated from *T.b. rhodesiense* parasites undergoing apoptosis 0 (control), 2, 7, 24 and 48 h following treatment with the lectin ConA lanes 1–5 respectively. Note the three distinct *T. brucei* s.l. ribosomal RNA bands (approximately 2.2, 1.7 and 1.5 kb) and smaller RNA transcripts, including tRNA molecules, are clearly visible and distinct for all time points. Position of size markers (BRL) 9.5, 7.5, 4.4, 2.4, 1.4 and 0.24 kb marked to left

not derived from differentially expressed genes) from products that are genuinely derived from differentially expressed genes.

The transcripts observed using RADES–PCR were predominantly similar, only showing differences in one or two transcripts between time points, further confirming fidelity of the RNA in cells undergoing ConA treatment. RADES–PCR analysis at the two different template concentrations generated similar products for the different time points, supporting earlier results of the RNA analysis and confirming that mRNA degradation has not occurred up to 48 h into the apoptotic process (Figure 2).

While some products showed reduced amplification (transcript decreasing in intensity) as the period of ConA treatment increased, others showed either a temporary increase at a specific time point or a steady increase in amount of transcript over time of treatment with ConA. Products which alter according to template concentration and which are not derived from differentially expressed sequences are clearly visible and were readily excluded from further analysis. Differentially expressed sequences which clearly altered at the different time points using both template concentartions are highlighted in Figure 2. For miniexon primer ILO 2452 and arbitrary primer ILO 1499 (Figure 2A) a product of approximately 750 bp was amplified from cells treated with ConA for 7 h but not from the untreated control cells nor from cells treated for 24 and 48 h (Figure 2A, arrow). Amplification of this transcript was confirmed at both template concentrations (Figure 2A lanes 2 and 2′). Since apoptosis induced by ConA is reversible up to 7 h, this product is likely to be derived from a gene involved in the initiation of the process, but whose effect is reversible. Two products each of 850 bp up-regulated late in the death process (48 h) were observed with minexon primer ILO 2453 and arbitrary primer ILO 1501 (Figure 2B, lane 4 and 4′, arrow) and with miniexon primer ILO 2451 and arbitrary primer ILO 1501 (Figure 2C lane 4 and 4′). It is of interest that the only difference between both sets of RADES–PCR is a single nucleotide at the 3′ end of the miniexon primer (a T for ILO 2453 and a C for ILO2451), yet there were significant differences in the patterns of the PCR products. As some RADES–PCR products in these

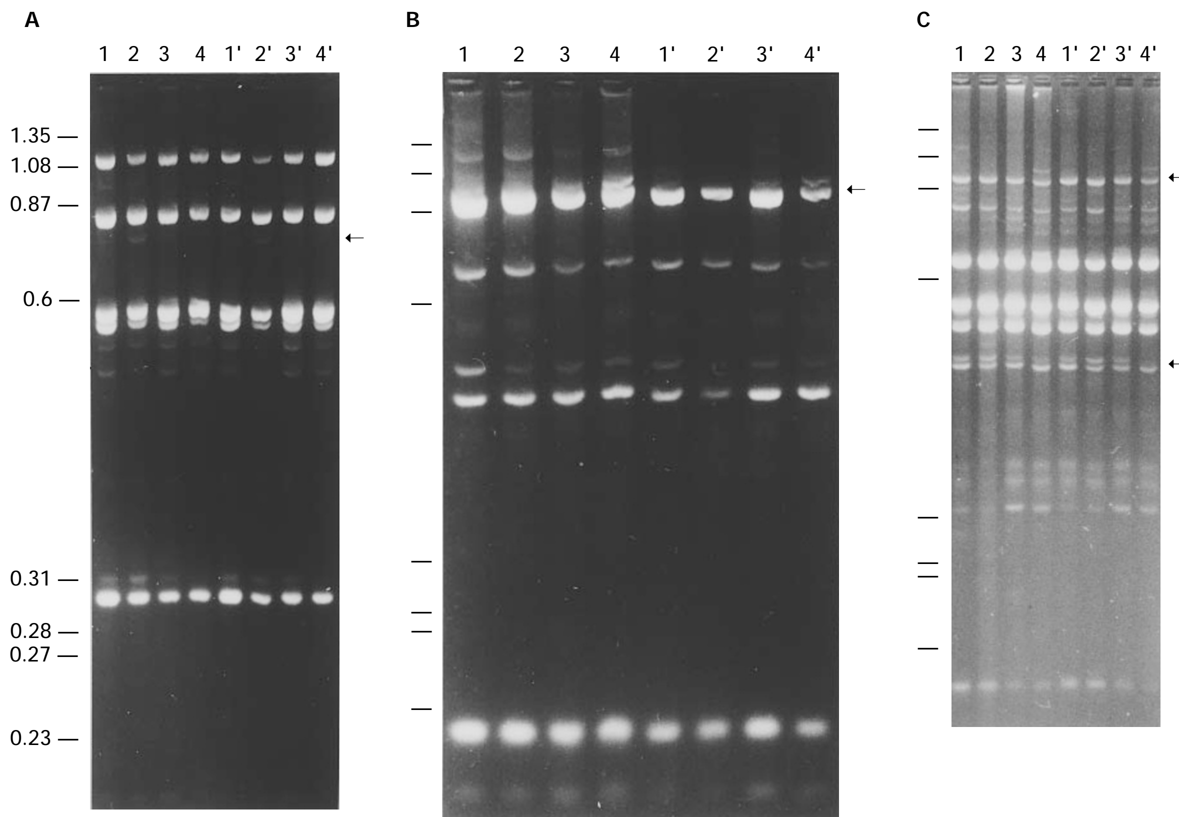

Figure 2 Fingerprinting of cDNA generated from procyclic form *T.b. rhodesiense* treated for 0 (control), 7, 24 and 48 h with ConA using the RADES–PCR technique. Examples of reproducible fingerprints for two different template concentrations are displayed (lanes 1–4 1 ng/μl, lanes 1′-4′ 0.2 ng/μl template). An EtdBr-stained 3% MetaPhor agarose gel of RADES–PCR fingerprints from control untreated and ConA treated for 7 h; 24 h and 48 h procyclic cDNA(s), left to right respectively. The lanes which carry a prime suffix were PCR reactions performed on the same DNA but using a 5 × lower template concentration. Primers used in the examples shown above were: (A) miniexon primer ILO 2452 (GTACTATATTGNG) with arbitrary primer ILO 1499 (AAGCGAGCCG), (B) miniexon primer ILO 2453 (GTACTATATTGNT) with arbitrary primer ILO 1501 (CGGCCGGTCA) and (C) miniexon primer ILO 2451 (GTACTATATTGNC) with arbitrary primer ILO 1501 (CGGCCGGTCA). Arrows point to products differentially amplified (either up regulated or down regulated) for both template concentrations and which are discussed in the text

reactions will be generated by only one of the two primers it is likely that the 850 bp product which shows increased amplification in cells treated for 48 h with ConA in both sets of reactions is amplified by ILO 1501 alone and is an example of single primer annealing (Figure 2B and 2C lanes 4 and 4′).

As well as products that showed either transient or a gradual increased amplification during the death process, others showed decreased amplification. A product representing a transcript which appeared to be down regulated during the death process is evident in Figure 2C (arrow) at 500 bp amplified using minexon primer ILO 2451 and arbitrary primer ILO 1501. This transcript is shown in Figure 2C lanes 1, 2, 3 and 1′, 2′ and 3′ corresponding to control, 7 h ConA treatment and 24 h ConA treatment respectively but is absent in cells treated with ConA for 48 h (Figure 2C lanes 4 and 4′).

Discussion

The present study has demonstrated that ConA can induce apoptosis in procyclic form *T.b. rhodesiense* parasites at a concentration of 1 μg/ml and that parasite death was recoverable up to 7 h following ConA treatment but after 24 h treatment the parasites appeared to be irreversibly committed to death. In a similar experimental system, when murine macrophages were treated with various concentrations of ConA in the presence of FBS to induce apoptosis, a dose dependent decrease in mitochondrial activity was observed when the incubation time was more than 8 h and DNA fragmentation was only observed in cells treated for more than 8 h with ConA (Kong *et al*, 1996). The ability of both trypanosomes and macrophages to recover from short exposure to ConA may reflect the passage of these cells through the condemned and execution stages of apoptosis characteristic of other cell types. The condemned stage may be highly variable with a duration of hours to days even within synchronised cell populations; this is followed by the execution phase which includes the stages responsible for the morphological characteristics of apoptosis (Earnshaw, 1995).

Signals which induce apoptosis in metazoa are varied and often the same signals which may induce death in one cell type may also induce differentiation and proliferation under other circumstances. The concentrations of ConA used to induce death in procyclic *T.b. rhodesiense* and which induce apoptosis in murine peritoneal macrophages are similar to doses which illicit a mitogenic response in T lymphocytes (Kong *et al*, 1996). Similarly, a lectin present in tsetse fly midguts stimulates cell death in the insect form trypanosome but has also been shown to be required for the subsequent maturation and successful cyclical transmission of the parasite through the insect vector (Maudlin and Welburn, 1994), suggesting close links between death and differentiation in the insect form of this parasite.

Apoptosis in metazoa is considered to be the result of an active cellular response involving initiation of *de novo* synthesis of mRNAs permitting the cell to follow an orderly and specific cascade of molecular events into death (Arends *et al*, 1990; Williams and Smith, 1993). The present study demonstrates that this is also the case for ConA-induced apoptosis in *T.b. rhodesiense*. ConA-induced DNA fragmentation of procyclic parasites was found to be reduced in the presence of a protein synthesis inhibitor, suggesting that activation of the apoptotic process in trypanosomes may be dependent on synthesis of new proteins during the cell death program (Welburn *et al*, 1996). In some but not all metazoan cell types apoptosis can be suppressed by inhibitors of RNA or protein synthesis (Martin *et al*, 1988, McConkey *et al*, 1990). However, treatment of trypanosomes with cycloheximide alone was also found to induce DNA cleavage (Welburn *et al*, 1996). In metazoa while *de novo* RNA and protein synthesis are required to carry out the apoptotic programme, other evidence suggests that continuous signalling is required to maintain homeostasis in certain cell types and that the inhibition of RNA or protein synthesis can also induce PCD (Martin *et al*, 1990, Waring, 1990). The observation that two disparate receptor pathways that by themselves cause PCD may mutually antagonise one another has previously been shown in T cells (Zacharchuk *et al*, 1990).

For ConA induced apoptosis in *T.b. rhodesiense* the RADES–PCR differential display method has been shown to be a highly sensitive system for the examination of differentially expressed genes during parasite cell death. On average, one product displaying altered amplification at both template concentrations for the different time points and representing the product of a differentially expressed gene was observed for each set of primers tested. The observation of only single band alterations in these profiles indicates the integrity of the RNA from which the template was prepared during the treatment periods sampled. The molecular mechanisms of lectin-induced apoptosis in trypanosomes and the identification of genes involved in the regulation of cell death in these parasites can be rapidly screened using this method. The observed up regulation of two transcripts late in the death process (at 48 h ConA treatment) strongly suggests that the parasites are undergoing a form of PCD in which they are actively participating in their own destruction in a regulated manner. These products are currently being cloned and sequenced.

In conclusion, we have demonstrated that ConA-induced apoptosis in procyclic form *T.b. rhodesiense* parasites is an active process involving differential gene expression and that the RADES–PCR method can be effectively applied to these parasites, and kinetoplastids in general, to rapidly identify genes which are differentially regulated during the process. These studies should allow the dissection of PCD in kinetoplastids and the determination of whether the mechanisms are similar to those of metazoan organisms. Such studies may not only reveal the molecular mechanisms of PCD in these organisms, but will allow a comparative analysis with metazoan organisms to determine whether PCD in these evolutionary divergent primitive organisms resembles that of metazoan organisms and whether the process has become established more than once in the evolutionary process. It is clear that it is possible to identify transiently expressed messages visible during the ‘execution’ phase of the death process and

because ConA-induced apoptosis in *T.b. rhodesiense* is reversible up to 7 h following ConA treatment, it should be possible to identify anti-apoptotic genes as well as those intimately involved in the cell death process.

Materials and Methods

Trypanosomes

T.b. rhodesiense (stock Obwang, isolated from a patient during a sleeping sickness epidemic in S.E. Uganda, 1990) were transformed to procyclic form by transmission through tsetse flies (Welburn and Maudlin, 1987).

Trypanosomes were dissected from tsetse fly guts and cultured at 27°C in Cunningham's medium supplemented with 17% FBS (Cunningham, 1977).

Lectin treatment

To assess the concentration of ConA required to induce death in the parasites, *T.b. rhodesiense* log phase procyclic trypanosomes (10^6 trypanosomes/ml) were treated with 0.1, 1, 10 and 50 μg/ml ConA. The parasites were harvested after 72 h ConA treatment and DNA prepared (Welburn *et al*, 1996). After electrophoresis through 1% agarose the resulting DNA was examined for fragmentation (oligonucleosomal ladder). Results for trypanosomes treated with 50, 10 and 1 μg ConA were identical and so the lowest dose which served to induce DNA fragmentation was selected for subsequent experiments. To examine at which point in time after treatment with ConA the parasites were irreversibly committed to die log phase trypanosomes were treated with 1 μg/ml ConA in Cunninghams culture medium supplemented with 17% FBS. At each of 2, 7, 24, 48 and 72 h of ConA treatment parasites were harvested washed in medium without ConA and allowed to recover. Only parasites treated for 7 h with ConA were able to recover from the treatment and survive to form persistent populations. Parasites treated for 24 h were irreversibly committed to death.

For RNA analysis the different procyclic *T.b. rhodesiense* samples were generated by treatment of log phase trypanosomes (10^6 trypanosomes/ml) with 1 μg/ml ConA type IV (Sigma, U.K) in procyclic culture media (Cunningham's supplemented with 17% FBS) and harvested 2, 7, 24 and 48 h after treatment.

RNA purification

Total RNA was isolated by the acid guanidinium-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) with improvements by Puissant and Houdebine (1991), from untreated control parasites and parasites treated with ConA at each time point (2, 7, 24 and 48 h after treatment) and analysed by electrophoresis (Pelle and Murphy, 1993). For *T. brucei* s.l RNA, three distinct ribosomal RNA bands (approximately 2.2, 1.7 and 1.5 kb) and smaller RNA transcripts, including tRNA molecules were clearly visible at all time points. RNA conversion to cDNA was effected using Moloney murine leukaemia virus (M-MLV) reverse transcriptase and an oligo (dT) primer containing an *Asc*I restriction site, ILO 1487 (TAGGCGCGCC(T)18).

Generation of double-stranded cDNA by PCR amplification

Following first strand cDNA synthesis, double-stranded cDNA was generated using specific primers for the fixed 5′ and 3′ ends of mRNAs of trypanosomes. Since trypanosome mRNAs contain a common 5′ miniexon sequence (De Lange *et al*, 1984; Parsons *et al*, 1984), and the different species of African trypanosomes in which this gene has been characterised have complete homology for 25 nt at the 3 end of this sequence (Cook and Donelson, 1987; De Lange *et al*, 1984; Parsons *et al*, 1984), a primer, ILO1488 (TAGGCGCGCCTAGAACAGTTTCTG-TACTATATTG), also containing an *Asc*I restriction site, was used which contains nt 16 to 39 of the miniexon sequence. PCRs contained single-stranded cDNA/10 μl 10 × Taq buffer [10 mM Tris HCl (pH8.3)/2 mM $MgCl_2$/ml] 4 μl of 5 mM dNTPs/2.5 units Taq (Thermus aquaticus) DNA polymerase (Promega Inc.)/1 μl (100 ng/μl stock) each of the oligo (dT) primer, ILO 1487, and miniexon primer, ILO 1488, in 100 μl total volume. Reactions were performed on a programmable thermal cycler (MJ Research Inc.). Cycling conditions were 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for 40 cycles followed by a 5 min extension at 72°C. Amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gels and detected by UV illumination following staining with EtdBr. The products appeared as a smear with the majority of products migrating close to 1 kbp. The total amount of product generated in the 100 μl PCR was approximately 10 μg of double-stranded cDNA, as estimated from the samples loaded on the gels. Buffer, primers and dNTPs were removed by ultrafiltration in a Centricon 30 column (Amicon, Inc.) and cDNAs were recovered and diluted to a final concentration of 20 ng/μl in TE buffer.

RADES – PCR differential display analysis of cDNA

RADES – PCR was carried out essentially as previously described (Murphy and Pelle, 1994). Reactions were conducted at two template concentrations to distinguish artefactual products from products that are genuinely derived from differentially expressed genes. PCR reactions were carried out using different miniexon specific primers together with three different arbitrary primers as follows: (A) miniexon specfic primer ILO 2452 GTACTATATTGNG, where N is a mixture of all four bases, together with arbitrary primer ILO 1499 AAGC-GAGCCG; (B) miniexon primer ILO 2453 GTACTATATTGNT with arbirary primer ILO 1501 CGGCCGGTCA; and (C) miniexon specific primer ILO 2451 GTACTATATTGNC with arbitrary primer ILO 1501. PCRs contained 1 μl (either 20 ng or 2 ng) of the target cDNAs and the volume brought to 20 μl with water/10 mM Tris HCl(pH8.3)/ 50 mM KCl/3 mM $MgCl_2$/0.05% (v/v) NP40/ 0.05% (v/v) Tween 20/200 μM of the four dNTPs/0.6 μM of each primer/0.5 units of Taq DNA polymerase. Cycling conditions were 94°C for 45 s, 40°C for 1 min and 72°C for 1 min for 40 cycles followed by a 5 min extension at 72°C.

Amplification products were analysed by electrophoresis in 2% (w/v) agarose gels and detected by UV illumination following staining with EtdBr. For fine separation of the PCR products, 10 μl of each sample set generating clear and reproducible fingerprints was electrophoresed in a 3% (w/v) MetaPhor™ agarose gel.

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