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Fluorescent differential display: arbitrarily primed RT-PCR fingerprinting on an automated DNA sequencer

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Abstract We established robust, reliable protocols for 'Differential Display (DD),' an RNA fingerprinting method originally developed by Liang and Pardee [(1992) Science 257, 967–971] using RT-PCR with arbitrary primers. Our protocols are optimized so that reliable DD analysis can be performed on a fluorescent DNA sequencer to ensure high throughput as well as improved operational safety, compared with the original one using radioactive compounds. Such 'Fluorescent Differential Display (FDD)' techniques will accelerate the identification of differentially expressed as well as polymorphic transcripts to address various biological questions.

Key words: Differential display; Arbitrary primer; RT-PCR; Fingerprinting; Fluorescent DNA sequencer

1. Introduction

The use of arbitrary primers in PCR was a breakthrough providing novel and promising approaches for DNA fingerprinting. Such techniques can be readily applied to any species without prior information on target sequences. A unique protocol termed 'Arbitrarily Primed PCR' (AP-PCR) [1] allows one to use any primer, that had been originally synthesized for other individual purposes, for successful DNA fingerprinting of organisms with various genome sizes ranging from bacteria to human. Another fingerprinting approach called 'Random Amplified Polymorphic DNA' (RAPD) [2] was devised using decamers designed to permit priming from multiple sites in complex genomes. Since the introduction of these techniques, numerous reports have appeared to prove their utility in various fields of biology, including genetic mapping, taxonomy, cancer research, epidemiology, population biology, phylogenetics and so on.

Recently, the concept of PCR fingerprinting using arbitrary primers was extended to the analysis of complex RNA populations, such as total cellular or tissue transcripts. A protocol termed 'RAP' (RNA fingerprinting by Arbitrarily primed PCR) [3–5] was developed as an extension of AP-PCR. In RAP, a single arbitrarily chosen primer is used for the first strand cDNA synthesis by reverse transcriptase as well as the second strand synthesis by *Taq* DNA polymerase under low stringency conditions. Subsequent stringent PCR specifically amplifies the fragments both ends of which were tagged with the arbitrary primers during the cDNA synthesis steps. Another procedure called 'Differential Display' (DD) was also described [6–8]. In DD of mRNAs, the first strand of cDNA was synthesized by reverse transcription using a 3′-anchored oligo-dT primer, while the second strand synthesis and subsequent amplification of 3′-end portions of selected cDNAs were conducted by PCR using an arbitrary decamer, just like RAPD, and the anchored oligo-dT primer.

Both methods generate fingerprints on polyacrylamide gels, each of which is composed of a subpopulation of cDNAs sampled from the original complex RNA source in a primer-dependent manner. Parallel comparison of such fingerprints (i.e. individual lanes) allows one to identify differentially expressed species with ease. It should be noted that more than two samples can be readily compared by such methods, unlike so-called subtractive techniques that are essentially for the comparison between two samples. Furthermore, it gives access to low abundunce transcripts that are too rare to detect with conventional differential hybridization techniques. In addition, since the products of these methods were tagged at their both ends with the primers, they can be easily recovered from the gel, re-amplified and used for various further analyses.

These features make such fingerprinting approaches quite attractive to researchers from various fields where the identification of differentially expressed transcripts is of particular interest and importance. However, to scan most transcripts in complex organisms, hundreds of primers or primer combinations have to be used. It is thus quite critical for such a large scale analysis to increase the throughput by accelerating each experiment, in particular, time-consuming steps such as electrophoresis, gel processing and signal detection. In addition, considering the number of reactions to be handled, elimination of radioactive compounds is desirable to ensure operational safety.

To meet these ends and to extend the utility of these techniques, we tried to run these protocols on a fluorescent DNA sequencer. While RAP can be performed with minimum modification (to be published elsewhere), DD was found refractory to generating signals strong enough for fluorescence detection. Since DD generally amplifies more bands and is thus suitable for scanning larger numbers of transcripts than RAP, we tried to develop novel protocols for 'Fluorescent Differential Display' (FDD). Two simple but robust protocols were developed for FDD. They will lay the foundation for a unique high throughput system for expressed sequence scanning, that would be of general use in various biological studies.

2. Materials and methods

2.1. RNA isolation

Total RNAs were isolated by a modified acid-guanidine thiocyanate-phenol-chloroform method [9] using TRIzol (BRL, MD) or ISOGEN (Nippon Gene, Japan) reagents, according to the manufacturers' instructions with minor modifications. RNAs from *Xenopus* embryos were further purified by LiCl precipitation. Some samples were treated

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with RNase-free DNase (BRL or Promega, WI) to remove residual contaminating DNAs. RNAs isolated by other methods were also successfully used. Rabbit β-globin mRNA was obtained from BRL.

2.2. First strand cDNA synthesis

Total RNAs (2.5 μg) were mixed with 50 pmol of the Fluorescein isothiocyanate (FITC)-labeled 3′-anchored oligo-dT primer (see below) in 10 μl of DEPC-treated water, heated at 70°C for 10 min and chilled by immersing the tube in ice-water. To this solution, 10 μl of 2 × RT solution (1 × RT solution = 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 0.1 mg/ml BSA and 0.5 mM each dNTP) containing 200 units of SuperScript II reverse transcriptase (BRL) was added, mixed well and incubated at 25°C for 10 min and at 42°C for 50 min. Following the incubation at 90°C for 5 min, the reaction mixture was diluted 5-fold by addition of 80 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at −20°C until use.

2.3. Polymerase chain reaction

The reaction mixture for the second strand synthesis and subsequent amplification was prepared in each well of a 96-well microtiterplate (Techne, UK) so that it contains 1 nmol of each dNTP (50 μM each at final concentration), 1 unit of *Taq* DNA polymerase, 10 pmol of arbitrary primer, 5 pmol of FITC-labeled 3′-anchored oligo-dT primer and 2 μl of cDNA solution described above in 20 μl of 1 × PCR buffer supplemented with 1.5 mM $MgCl_2$. (Enzymes and buffers from various suppliers gave comparable results.) The 96-well plate was subjected to thermal cycling using a Techne PHC-3 apparatus. Thermal cycling parameter for Protocol I, that uses longer arbitrary and 3′-anchored oligo-dT primers (see below), was as follows; 94°C for 3 min, 37°C for 5 min and 72°C for 5 min for the second strand synthesis, followed by 20–25 cycles of 95°C for 15 s, 55°C for 1 min and 72°C for 2 min for amplification. For protocol II using shorter primers, a thermal cycling profile composed of 94°C for 3 min, 40°C for 5 min, 72°C for 5 min followed by 20–25 cycles of 95°C for 15 s, 40°C for 2 min and 72°C for 1 min was employed. Also, an additional final extension step at 72°C for 5 min was employed in both protocols.

The FITC-labeled 3′-anchored oligo-dT primers used in this study were synthesized on a Model 392 DNA synthesizer (Applied Biosystems Inc., CA) using FluorePrime (Pharmacia, Sweden). Various primers of 16–24 nt long synthesized in our laboratory for other individual purposes were recruited as arbitrary primers for the protocol I. Arbitrary decamers for protocol II were obtained from Operon Technologies (CA).

2.4. Gel electrophoresis

Each PCR product was mixed with the same amount of dye solution (98% formamide, 10 mM EDTA and 0.01% Methyl violet), denatured at 90°C for 2 min and chilled on ice. Two μl of each sample was applied to each well of a 6 (or 4.5)% polyacrylamide/7 M urea gel in 1 × TBE buffer cast between the half-length gel plates (20 cm × 17 cm × 0.3 mm). The gel was run on a HITACHI SQ-3000 DNA sequencer (Hitachi, Japan) at 800 V for 2.5–3 h. The second set of samples can be applied to the same gel immediately after the first run. Gel images appeared on the display of the NEWS workstation (NWS-3470, SONY, Japan) operating the sequencer were visually inspected. The images were also printed by a SONY UP-930 multiscan videoprinter connected to the workstation.

For the detection on a FluorImager 575 (Molecular Dynamics, CA), 5–6 μl of samples containing the dye solution was applied to each well of a 0.35 mm thick polyacrylamide gel (20 cm × 33 cm). Following the removal of upper glass plate, the gel was put on the sample tray and scanned at the high sensitivity mode. We also confirmed that FDD can be successfully performed with XRITC (Rhodamine X isothiocyanate)-labeled primers and FMBIO-100 (Takara Shuzo, Japan), the other commercially available fluorescent image analyzer (not shown).

2.5. Cloning of the bands of interest

Since the initial analysis on the DNA sequencer revealed the size of the band of interest, one can choose such gel concentration that optimizes the separation of the target for preparative electrophoresis. The fingerprinting pattern is visualized by the FluorImager 575 and printed on a transparency film by its actual size, onto which the gel with lower glass plate was overlaid and the gel piece containing the band of interest was excised using a blade. The gel can be scanned again to confirm the precise excision.

An alternative method requiring no special equipment is the use of non-denaturing gel: the reaction products (~10 μl) can be separated by native PAGE, and detected by staining with SYBR Green I (Molecular Probes, OR), a sensitive fluorescent dye excited by conventional ultraviolet illuminator.

For the re-amplification of the excised band, we found that the direct addition of the gel piece to a 50 (or 100) μl PCR reaction gave satisfactory results. Neither tedious electroelution procedures nor even boiling steps were found necessary. Usually, 30 cycles of PCR yielded enough amount of the fragment for subsequent subcloning into a 'T-vector' (pT7Blue, Novagen, WI) as well as direct sequencing.

3. Results and discussion

3.1. Rapid fingerprinting on a modified DNA sequencer

Our goal is to establish a system for the scanning of expression profiles of most, if not all, transcripts in complex organisms based on the arbitrarily primed RT-PCR fingerprinting technique. Toward this end, we attempted to introduce an automated fluorescent DNA sequencer into the system, since it ensures high throughput analysis with minimum operator interaction, automates the data acquisition and storage, and improves operational safty.

We routinely use a HITACHI SQ-3000 DNA sequencer for such analyses (see below). It can display gel images just like a fluorescent gel scanner. For the identification of differences among a number of samples (i.e. lanes), visual inspection of gel images rather than electropherograms was found more sensitive and rapid. The signal detection system of this particular machine allows one to use sample combs of any shape. We usually use a 42-well shark-tooth comb and load 40 samples. Another important modification of the apparatus is the shortening of the gel length (17 cm) to the half of the default one. Such a short gel drastically saves the running time. For instance, bands within the range of DD (up to ~600 nt) can be separated within 2.5–3 h with satisfactory resolution. It should be noted that short running time minimizes the damage of gel

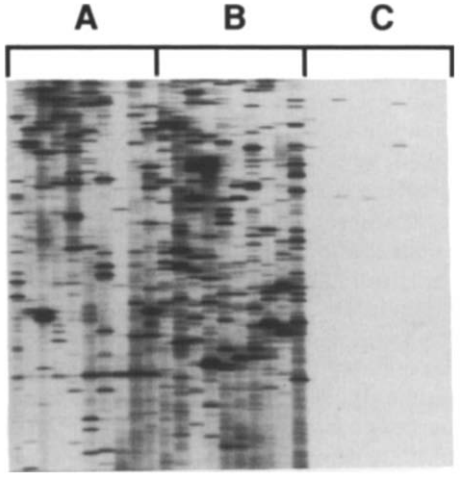

Fig. 1. Comparison of protocols. Rat liver RNAs were subjected to differential display analysis using three different protocols. (A) Protocol I using FITC-MluTG primer and ten longer arbitrary primers (24 cycles including the second strand synthesis step). (B) Protocol II using FITC-GT_{15}MG and ten arbitrary decamers. (24 cycles). (C) A protocol similar to the original one using FITC-T_{12}MG and the same decamers as B (40 cycles). The image was taken with the modified HITACHI SQ-3000 DNA sequencer.

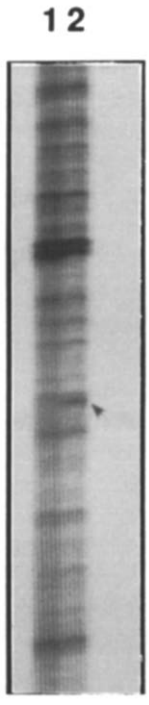

Fig. 2. Sensitivity of FDD. Total RNAs (2.5 μg) from glioma U251 cells mixed without or with 0.5 pg of rabbit β-globin mRNA (BRL) were prepared and subjected to FDD protocol I as described in section 2. An oligonucleotide derived from the globin sequence was used as the upstream arbitrary primer. A portion of FDD obtained from the samples without and with the β-globin mRNA was shown in lanes 1 and 2, respectively. Arrow indicated the band derived from the added β-globin mRNA, that showed the expected size and restriction patterns (not shown).

matrix to enable the re-use of the same gel for the separation of further samples. Consequently, consecutive use of two gels allows three or four sample loadings a day, thereby considerably increasing the throughput.

3.2. Protocols for FDD

We had tried to run DD on the modified fluorescent DNA sequencer, but found that DD using FITC-T_{12}MN (where M = mixture of A, C and G; N = one of A, C, G and T) and decamers, similar to the original protocol, generated weaker and fewer signals than expected even with various modifications in reaction conditions. We concluded that the protocols using the original primer design are not suitable for fluorescent detection, and thus tried to develop novel protocols for fluorescent DD (FDD).

Since signals of enough strength were readily obtained with RAP using fluorescent primers, we adopted the idea of RAP to develop a novel FDD protocol. This protocol, termed protocol I, uses arbitrary primers of ~20 nt long and a 3′-anchored oligo-dT primer (BamTN: FITC-CCCGGATCCT$_{15}$N, or MluTN: FITC-CGTACGCGT$_{15}$N, where N = one of A, C or G). Following the first strand synthesis with one of the 3′-anchored oligo-dT primers and reverse transcriptase, the second strand is synthesized during the first cycle of PCR, where a low temperature (37°C) and longer duration for annealing are employed so that even a long arbitrary primer can anneal with multiple targets and initiate the second strand synthesis. In the following PCR cycles, higher annealing temperature (55°C) is used to ensure specific amplification of the primer-tagged cDNA fragments generated during the first cycle. (The restriction enzyme sites at the 5′-sides of the oligo-dT primers are added not only to make their melting temperature comparable to those of long arbitrary primers but to allow uni-directional cloning of the PCR products.) As shown in Fig. 1, a number of bands with enough intensity were readily obtained with much fewer cycles by this method than the protocol close to the original one.

Another protocol was also established using arbitrary decamers and termed protocol II. Although DD protocols with such short primers were not suitable for the fluorescent detection, modification of the 3′-anchored oligo-dT primer was found to drastically improve the intensity of signals obtained. The modified primer currently used in our laboratory is

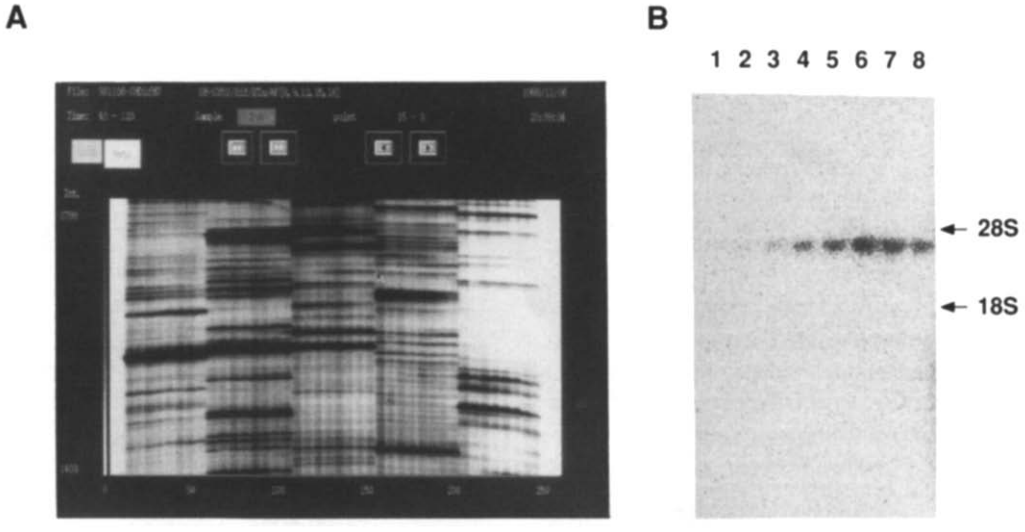

Fig. 3. Identification of transcripts differentially expressed during the differentiation of a neuroblastoma cell line. (A) Total RNAs were isolated from a human neuroblastoma cell SH-SY5Y at 0, 2, 6, 12, 24, 48, 120 and 240 h after the addition of retinoic acid (10 μM) to the culture medium (from left to right), subjected to FDD protocol I with FITC-BamTG and five arbitrary primers, and resolved on the modified HITACHI SQ-3000 DNA sequencer. This figure is a screen dump from the NEWS workstation operating the sequencer. The arrow indicated an induced species. (B) The band indicated by the arrow in (A) was cloned and used as a probe for Northern blot hybridization, according to the standard procedure [10].

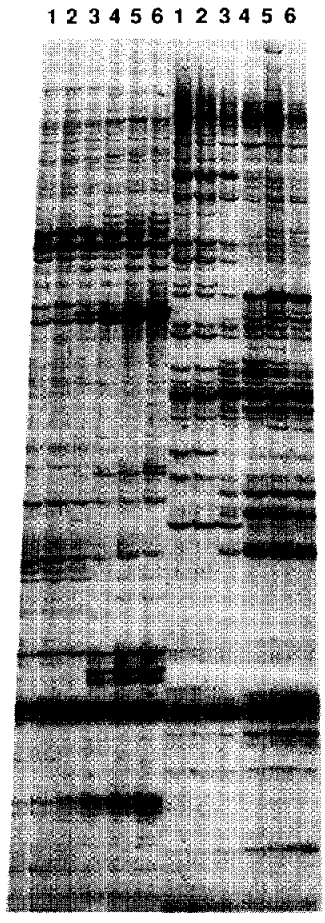

Fig. 4. Gene expression profile during the early embryonic development of *Xenopus laevis*. Total RNAs isolated from cleavage, blastula, gastrula, neurula, tail bud and tadpole stages were subjected to FDD using protocol II and resolved on a denaturing polyacrylamide gel electrophoresis in lanes 1–6, respectively. Primers used were FITC-GT_{15}MG and two arbitrary decamers (Operon). Note that this particular image was obtained by FluorImager 575.

5′-FITC-GT_{15}MN-3′ (M = the mixture of A, C and G; N = one of A, C, G or T. Since the second most 3′ nucleotide was found to have bare effects on the discrimination ability, coincident with the findings of others [8], the position is occupied by the mixture of A, C and G.) Elimination of the G at the 5′-end considerably dropped the intensity of the signals, whereas further addition of nucleotide to the 5′-end did not improve the signal intensity so much. This protocol generates much superior signals than those using shorter 3′-anchored oligo-dT primers (Fig. 1). It should be noted that the protocol II is more sensitive to the presence of contaminating DNA than the protocol I, since it uses lower annealing temperature throughout the PCR.

Both protocols generate fingerprints composed of 50–100 (or more) bands in a primer-dependent manner. Since we usually load 40 samples per each run, and make three or four runs a day as described above, data on ~10,000 transcript species can be obtained a day. The reproducibility of fingerprint pattern is of satisfaction: most (~95%), if not all, of the bands seem to be reproducibly amplified. However, faint bands fluctuating from run to run are occasionally observed. Such irreproducible species should not be chosen for further analysis.

3.3. Sensitivity of FDD

One of the major advantages of DD is its potential to detect low abundance messages. To evaluate such ability of FDD, a model experiment was designed, where total RNA from a human glioma cell line U251 'doped' with a known amount of rabbit β-globin mRNA was subjected to the modified differential display protocol using a primer derived from the globin sequence as the upstream primer. As shown in Fig. 2, the expected band originated from the added globin mRNA was detected even in a sample where the added RNA occupied only 0.00002% of total amounts. Considering the average content and length of mRNAs [10], this result indicated that the sensitivity of FDD is comparable to that of the original DD protocol using radioactive compounds [6]. The amount of RNA required for FDD (10–200 ng/reaction) is also in the same range with that for radioactive DD. We routinely use 50 ng of total RNA for each reaction.

3.4. Molecular cloning of the bands of interest

The use of fluorescent DNA sequencer allows the rapid identification of the primer combination displaying the band of interesting behavior. Once such a primer pair is identified, the rest of the reaction (~19 μl) can be used for the preparative isolation of the band. As described in section 2.5, following the visualization of fingerprint pattern with fluorescent image ana-

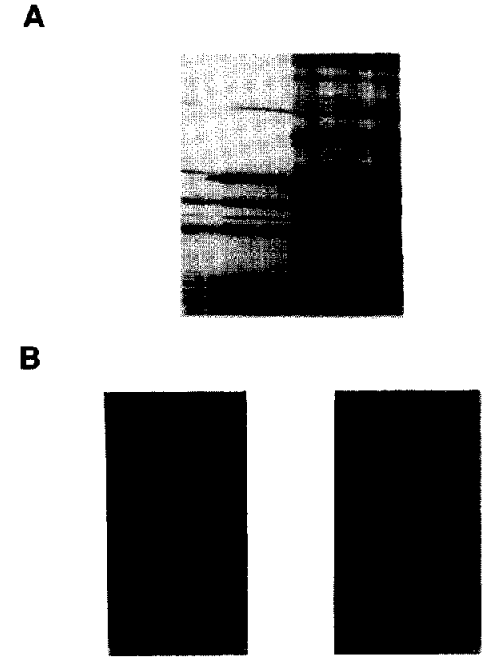

Fig. 5. Detection of polymorphic transcripts. (A) Total liver RNAs from *Mus musculus domesticus* (B6), *Mus musculus molossinus* (MSM) and reciprocal F1 hybrids (MSM/B6 and B6/MSM) were subjected to FDD protocol I with FITC-BamTG and two different arbitrary primers. Two individuals from each species were used (From left to right, B6/B6, MSM/MSM, MSM/B6 and B6/MSM). A portion of FDD is shown. (B) Polymorphism confirmed by RT-PCR using specific primers. The polymorphic bands indicated by the arrow and the arrow head in (A) were further analyzed to synthesize specific primers. Two-step RT-PCR assay, where FDD-PCR products after 10 cycles were diluted 100-fold and used as templates for PCR using specific primers, revealed the polymorphic natures of these bands. While the band indicated by the arrow showed the presence/absence polymorphism (left), the band pointed by the arrow head showed length polymorphism (right).

lyzers or sensitive fluorescent staining, the gel piece was excised and directly used for the re-amplification of the band of interest.

Although the re-amplified products are occasionally pure enough to be used as templates for direct cycle sequencing, they are, in most cases, composed of more than a single species, particularly when a non-denaturing gel is used for excision. Thus, we usually clone the re-amplified products first and, then, identify the correct ones corresponding to the bands of interest as follows.

As the first step for such identification, we usually perform 'colony PCR' to amplify the insert with the arbitrary and the 3′-anchored oligo-dT primers and run the products in parallel with the original FDD reaction. Clones bearing inserts comigrating precisely with the band of interest are subjected to DNA sequencing. If, unfortunately, two (or more) different species of the same length are revealed, further tests are necessary. A simple diagnostic test is the digestion of original FDD reaction by appropriate restriction enzymes chosen based on the nucleotide sequences of candidate clones. Such simple experiments often help one identify the correct clone among the candidates. Also, various hybridization-based tests would be plausible for the purpose. For instance, one can probe the Southern blot of FDD fingerprints with candidate clones, as recently reported with RAP [5]. Alternatively, candidate clones can be screened by differential hybridization using FDD products as probes.

Following such tests, an oligonucleotide derived from the sequence of the identified clone is synthesized. This specific primer can be used to perform a nested PCR test to prove the identity of the target band and the clone, in which test diluted DD products are used as templates for PCR with the specific and the arbitrary primers. Such PCR would generate the product of expected size and behavior, thereby providing a solid confirmation of the validity of clone selection. It should be noted that the primer can be used in so-called 5′-RACE (Rapid Amplification of cDNA Ends) [11] to clone 5′-portion of the transcript. Since DD bands are derived essentially from the 3′-untranslated regions of mRNAs, such efforts are often required to obtain the information on the products encoded by the transcripts identified with DD.

3.5. FDD analysis of differentially expressed transcripts

Both FDD protocols described above are currently applied to various projects in our laboratory. For example, Fig. 3A shows the FDD pattern obtained by protocol I during retinoic acid-induced in vitro differentiation of a human neuroblastoma cell line SH-SY5Y. An induced band indicated by the arrow was cloned and used as a probe for Northern blot hybridization. It detected a ~4 kb RNA induced during the differentiation, proving the validity of the expression pattern on FDD (Fig. 3B). While the nucleotide sequence of this particular band failed to find any homologs in the sequences deposited in the databases, known genes were also found among the induced, reduced and transiently-induced bands we had cloned. For example, one of the induced bands was identical to neurofilament-M, a typical marker of neural differentiation (data not shown). The FDD pattern obtained by protocol II from various stages of *Xenopus laevis* early embryo is shown in Fig. 4. This fingerprint was visualized by the fluorescent image analyzer. Bands of various behaviors, including induced, reduced, stage-specific and unchanged ones, were readily observed, reflecting the dynamic change in gene expression programs during early embryonic development. Further analyses on these and other differentially expressed clones will be published elsewhere.

3.6. FDD analysis of polymorphic transcripts

Besides the transcripts showing quantitative changes, DD can occasionally detect qualitative differences (polymorphisms) among transcripts, as was shown with RAP previously [3]. This unique but underrecognized feature of these techniques may provide a novel way for genetic mapping. As demonstrated previously [12], polymorphisms between different mouse (sub)species are frequently found in the 3′-untranslated regions, from which portions most DD bands are derived. Accordingly, high incidence of polymorphic DD pattern is expected on inter(sub)specific comparison. In fact, FDD readily revealed polymorphic bands between liver RNAs from *Mus musculus domesticus* and *Mus musculus molossinus* (Fig. 5A). Each primer pair was found to reveal ~10 (or more) bands showing polymorphic pattern. Two types of polymorphism should be detected: presence/absence and length polymorphisms, the former and latter of which are presumably due to the polymorphism at and between the priming sites, respectively. Although it is hard to tell which category each polymorphic band belongs to, subsequent cloning reveals its nature, as shown in Fig. 5B. More efficient polymorphism detection would be possible by SSCP (Single Strand Conformation Polymorphism) electrophoresis [13] as recently demonstrated with AP-PCR [14].

Considering the average length of the 3′-terminal exons, it is quite conceivable that STSs (Sequence-Tagged Sites) generated from polymorphic DD bands can be amplified from genomic DNA as well as cDNA. In our preliminary results, five out of five such STS primers tested successfully amplified their target sequences directly from mouse genomic DNA (data not shown). Thus, these STSs can be genetically localized onto the mouse genome map using an appropriate panel of DNAs from an intersubspecific backcross. Since this strategy uses STSs derived from transcribed regions, it provides a rapid way to genetically put 'genes' rather than anonymous DNA markers onto the map as proposed previously [12], in which strategy FDD serves as an efficient way for the enrichment of polymorphic cDNAs to be used.

Furthermore, if a high quality panel of RNAs from an appropriate mapping population is available, it is, in principle, possible to genetically map cDNAs (= genes) in a multiplexed manner through simply analyzing DD patterns without any cloning and sequencing efforts. Obviously, FDD will further accelerate the throughput of this high speed mapping strategy. The feasibility of such approach is under pursuit.

3.7. Conclusions

Robust, reliable protocols for FDD were developed for high throughput analysis of gene expression profile on a fluorescent DNA sequencer. A similar attempt to run DD on a fluorescent DNA sequencer was reported by others [15]. While their protocol is based on a two-step PCR using the same 3′-anchored oligo-dT primers as the original ones and fluorescently-labeled arbitrary decamers, ours use modified 3′-anchored oligo-dT primers labeled with fluorescent dye in a simple single-step amplification procedure. We believe our protocols can be adapted to any other non-radioactive as well as radioactive detection systems with minimum modifications.

In our initial attempts, we cloned 22 bands showing differentially expressed or polymorphic FDD patterns from various sources. Subsequent Northern blot hybridization and/or RT-PCR analysis using specific primers derived from the identified clones proved the validity of FDD pattern in all the cases tested (Fig. 3 and 5, and unpublished data). Compared with the high incidence (70–80%) of false positives reported in the literature using the original DD protocol [16–18], the robustness and reliability of our procedures are obvious. We attribute the high success rate not only to the careful clone examination step but also to the novel primer design that allows the use of more standard and well-established RT-PCR conditions compared with the unusual ones in the original DD. The protocols described here will facilitate the identification of differentially expressed as well as polymorphic transcripts to address various biological questions.

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