Research Report

Differential Display Probes for cDNA Arrays

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ABSTRACT

PCR with a combination of one arbitrary and one oligo(dT) anchor primer can be used to generate an effective probe for cDNA arrays. The method uses less than 1/200 of the amount of RNA used in some other array hybridization methods. Each fingerprint detects approximately 5% of the transcribed mRNAs, sampled almost independent of abundance, using inexpensive E. coli *colony arrays of expressed sequence tag (EST) clones. It proved necessary to alter the differential display (DD) protocol to generate a sufficient mass of PCR products for use as a probe. The use of different oligo(dT) anchor primers with the same arbitrary primer resulted in considerable overlap among the genes sampled by each probe. This can be avoided by using different arbitrary primers with each oligo(dT) anchor primer. Four genes not previously known to be regulated by epidermal growth factor (EGF) and three genes known to be regulated by EGF in other cell types were characterized using DD fingerprints as probes for arrays. It should be possible to convert archived DD fingerprints into effective probes for arrays, allowing thousands of experiments that have already been performed to yield further information. The use of DD fingerprints as probes should increase the rate of identification of differentially regulated genes several fold while obviating the need for cloning and sequencing.*

INTRODUCTION

We have recently shown that RNA arbitrarily primed polymerase chain reaction (RAP-PCR) fingerprints (15) can be used to produce reduced complexity, non-stoichiometric probes for cDNA arrays (14). These probes have reduced complexity relative to total cDNA probes because only small portions of some of the mRNAs in the population are efficiently amplified by RAP-PCR. The probe is non-stoichiometric because the primers select only certain RNAs for efficient amplification, including rare RNAs.

Using a collection of 18 000 partly normalized expressed sequence tags (ESTs) on a nylon membrane array, a radiolabeled "RAP-array" generated using total RNA from a cell line will hybridize to about 2000 cDNAs, as detected by a phosphor imager. The clones detected in this manner primarily correspond to transcripts that are too rare to observe on these membranes using a labeled total cDNA probe. Thus, the method is useful for surveying the relative levels of expression of rare mRNAs in total RNA samples.

Here, we describe the adaptation of fingerprints generated by the 3′ anchor method of Liang and Pardee (10) so that the differential display (DD) protocol can also be used to generate probes for arrays. In principle, the use of probes derived from oligo(dT) anchoring has some potential advantages for certain types of arrays. Some arrays are generated by oligo(dT) primed reverse transcription (RT), and these clones are 3′ biased. A probe generated by an oligo(dT) anchored primer and an arbitrary primer should also be 3′ biased, so that each PCR product should hybridize to the corresponding 3′ biased clone. In contrast, a probe generated using arbitrary priming alone may sample regions internal to mRNAs. Such an internal fragment of a gene might not hybridize to a 3′ truncated clone if the arbitrary product is located further 5′ in the mRNA. Previously, we used oligo(dT) priming followed by RAP-PCR sampling with two arbitrary primers, which should also be biased towards sampling 3′ ends (14).

HaCaT keratinocytes respond to epidermal growth factor (EGF) by undergoing a dramatic change in morphology. We had previously identified 13 genes regulated during this process. Here, we add an additional seven genes to this list using probes developed in this manuscript.

MATERIALS AND METHODS

RNA Preparation

RNA from the human keratinocyte cell line HaCaT was prepared as described previously (14). Briefly, cells were grown to confluence and maintained at confluence for 2 days. The medium was changed 1 day before the experiment. EGF (Life Technologies, Gaithersburg, MD, USA) was added at 20 ng/mL. Treated and untreated cells were harvested after 4 h, and total RNA was prepared with the RNeasy® Total RNA Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' protocol. To remove remaining genomic DNA, the extracted total RNA was treated with RNase-free DNase (Boehringer Mannheim, Indi-

anapolis, IN, USA) and cleaned again using the RNeasy kit. The purified RNA was adjusted to 400 ng/μL in water and checked for quality by agarose gel electrophoresis.

Differential Display

Standard DD was performed using the materials supplied in the RNAimage® Kit (Genhunter, Nashville, TN, USA) (**www.nashville.net/~genhunt**), AmpliTaq® DNA Polymerase (PE Biosystems, Foster City, CA, USA) and [α-^{32}P]dCTP according to the kits protocol, except that each RNA template was used at four different concentrations (800, 400, 200 and 100 ng per reaction of 20 μL) with each anchored oligo(dT) primer (0.2 μM). The PCR contained only 2 μM dNTPs (for a total of 4 μM including the carryover from the cDNA mixture), 0.2 μM each primer and 1/10 of the newly synthesized cDNA (corresponding to 80, 40, 20 and 10 ng RNA). The anchored oligo(dT) primers (H-T_{11}G, H-T_{11}A and H-T_{11}C) were used in all possible combinations with four different arbitrary primers (H-AP1, H-AP2, H-AP3 and H-AP4).

Modified DD

RT was performed using four different concentrations of each RNA template (1000, 500, 250 and 125 ng per reaction of 10 μL). The reaction mixture containing 1.5 μM oligo(dT) anchored primer [AT_{15}A, GT_{15}G, T_{13}V (V = A, G or C)], 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 0.2 mM each dNTP, 8 U RNase inhibitor (Boehringer Mannheim) and 20 U MuLV Reverse Transcriptase (Promega, Madison, WI, USA) were ramped for 5 min from 25°–37°C, held at 37°C for 1 h, and finally the enzyme was inactivated at 94°C for 5 min. The newly synthesized cDNA was diluted 4-fold in water.

The PCR was performed after adding 10 μL of reaction mixture to 10 μL of the diluted cDNAs (corresponding to 250, 125, 62.5 and 31.25 ng of RNA) to yield a 20-μL final reaction volume containing (not including the carryovers from the RT reaction) 2 μM anchored oligo(dT) primer, 0.4 μM arbitrary primer (KA2: GGTGCCTTTGG or OPN28: GCACCAGGGG) 2.5 U AmpliTaq DNA Polymerase Stoffel Fragment (PE Biosystems), 2 μCi [α-^{32}P]dCTP, 175 μM each dNTP, 10 mM Tris, pH 8.3, 10 mM KCl, 3.125 mM $MgCl_2$. The reactions were thermal cycled for 35 cycles of 94°C for 40 s, 40°C for 1 min 40 s and 72°C for 40 s.

An aliquot of the PCR products resulting from the four different concentrations of the same RNA template were displayed side by side on a 5% polyacrylamide gel and visualized as described previously (Figure 1) (14).

Labeling of DD Products for Use as Probes Against cDNA Arrays

Random primed labeling of DD products was performed as described previously (14). The DD PCRs (14 μL) were purified using a QIAquick™ PCR Purification Kit (Qiagen), and the DNA was recovered in 50 μL 10 mM Tris, pH 8.3. Random primed synthesis was performed using a standard protocol. Briefly, the recovered DD products (5 μL) were combined with 3 μg random hexamers, boiled for 3 min and placed on ice. The hexamer/DNA mixture was combined with the reaction mixture to yield a 25-μL reaction containing 0.05 mM three dNTPs (minus dCTP), 50 μCi of 3000 Ci/mmol [α-^{32}P]dCTP, 1× Klenow fragment buffer and 4 U Klenow fragment (both from Life Technologies). The reaction was performed at room temperature for 4 h, chased for 15 min at room temperature by adding 1 μL of 1.25 mM dCTP and incubated for an additional 15 min at 37°C. The unincorporated nucleotides and hexamers were removed with the QIAquick Nucleotide Removal Kit (Qiagen) and the purified products were eluted using two aliquots of 140 μL 10 mM Tris, pH 8.3.

Hybridization to the Array

Hybridization to the array was performed according to our previously described protocol.

Prewash. The cDNA membranes (Genome Systems, St. Louis, MO, USA) were prewashed in three changes of 2× standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) in a horizontally shaking flat bottom container to reduce the residual bacterial debris. The first wash was carried out in 500 mL for 10 min at room temperature. The second and third washes were each carried out in 1 L of prewarmed (55°C) prewash solution for 10 min. Membranes are no longer available from this source. cDNA arrays can be obtained from **www.RZPD.de** and **www.resgen.com**.

Prehybridization. The membranes were transferred to large roller bottles and prehybridized in 60 mL prewarmed (42°C) prehybridization solution containing 6× SSC, 5× Denhardt's reagent, 0.5% SDS, 100 μg/mL fragmented, denatured salmon sperm and 50% formamide for 1–2 h at 42°C.

Hybridization. The prehybridization solution was exchanged with 10 mL prewarmed (42°C) hybridization solution containing 6× SSC, 0.5% SDS, 100 μg/mL fragmented, denatured salmon sperm and 50% formamide. To decrease the background hybridization due to repeats (e.g., Alu and Line elements), sheared human genomic DNA was denatured in a boiling water bath for 10 min and immediately added to the hybridization solution to a final concentration of 10 μg/mL. An aliquot of 10 ng/mL poly(dA) can be added to block oligo(dT) stretches in the radiolabeled probe. Simultaneously, the labeled probe was denatured in a boiling water bath for 4 min and immediately added to the hybridization solution. The hybridizations were carried out at 42°C for 18–20 h.

Wash. The hybridization solution was poured off, and the membranes are thoroughly washed in six changes of wash solution (including a transfer of the membranes from the roller bottles to a horizontally shaking flat bottom container and back to the roller bottles) over 2–3 h. The stringency of the washes was increased stepwise from 2× SSC, 0.1% SDS at room temperature to 0.1× SSC, 0.1% SDS at 64°C. As the membranes are washed individually/separately in the roller bottles, it is important to ensure that the temperatures in the washes are exactly the same for all the membranes. Therefore, the last high-stringency wash should be at least 40 min to ensure exactly equilibrated

temperatures in all bottles. The final wash solution was removed, and the membranes were briefly rinsed in 2× SSC at room temperature, blotted with 3 MM paper, wrapped in Saran® Wrap while moist and placed against Kodak Biomax film (Eastman Kodak, Rochester, NY, USA).

Confirmation of Differential Expression Using Low-Stringency RT-PCR

Normally, the first level of confirmation is the use of two RNA concentrations per sample. Only those hybridization events that seem to indicate differential expression at both RNA concentrations in both RNA samples can be relied upon.

Most arrays now commercially available are associated with partial sequences in the GenBank® database. In cases where there is no sequence, the clones can be ordered from Genome Systems and sequenced. Sequences

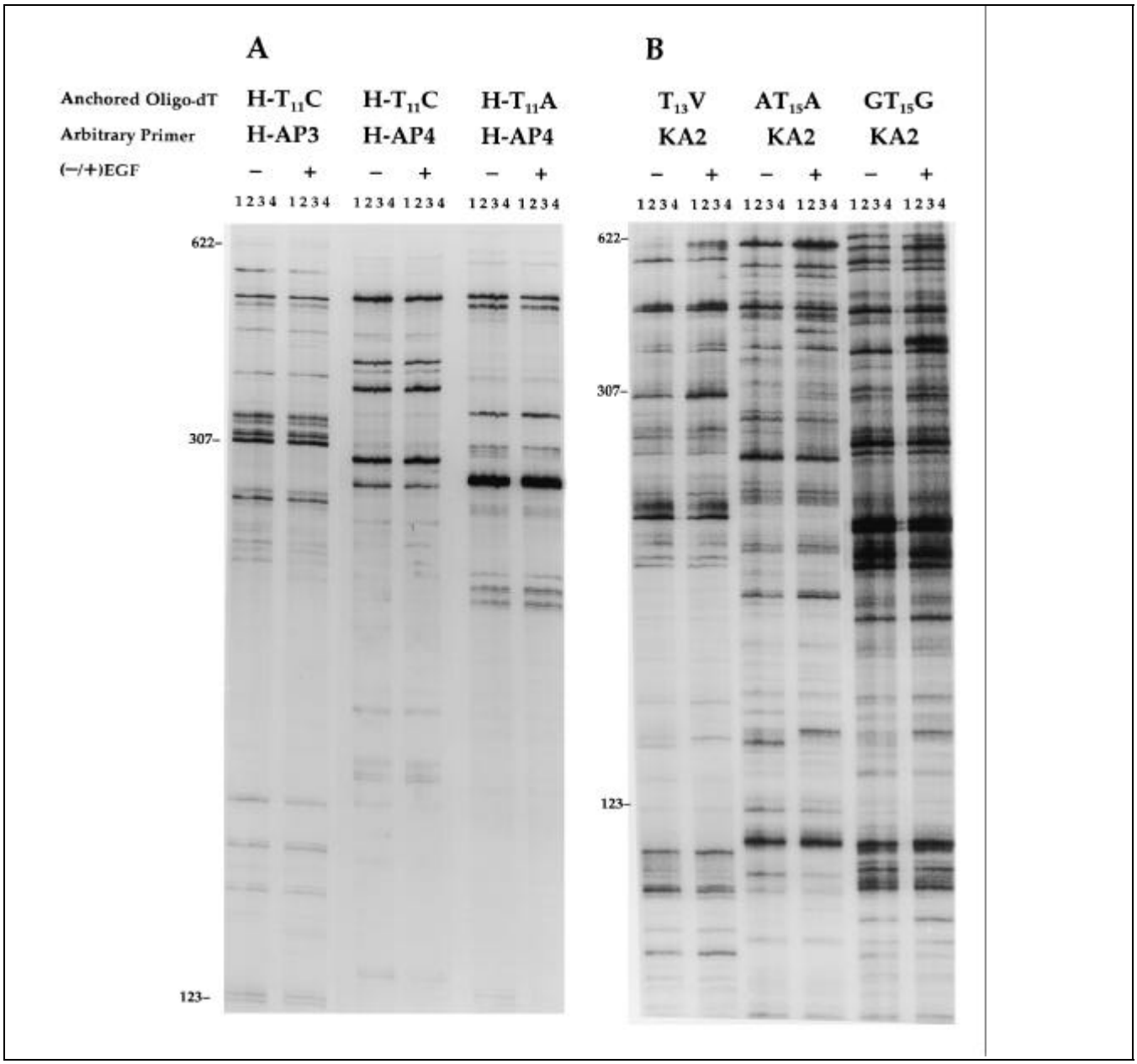

Figure 1. DD of untreated and EGF-treated HaCaT cells. (A) DD reactions were performed according to the RNAimage kit protocol except that four different starting concentrations (1–4) of total RNA were used: 1, 800 ng; 2, 400 ng; 3, 200 ng; 4, 100 ng. One tenth of this material was then used for PCR. The anchored oligo(dT) primer H-T11C was used with two different arbitrary primers (H-AP3 and H-AP4); the arbitrary primer H-AP4 was used with two different anchored oligo(dT) primers (H-T_{11}C and H-T_{11}A). The reactions that share either the arbitrary primer or the anchored oligo(dT) primer show almost no visible overlap in the visible bands. (B) DD using an arbitrary primer (KA2: GGTGCCTTTGG) with three different anchored oligo(dT) primers (T_{13}V, AT_{15}A and GT_{15}G). The DD protocol was adjusted to yield more mass and a higher complexity of the generated products. The starting concentrations of RNA were: 1, 1000 ng; 2, 500 ng; 3, 250 ng; 4, 125 ng. One fourth of this material was then used for PCR. Again, using different oligo(dT) anchored primers changes the pattern of the displayed bands almost entirely.

were used to derive PCR primers of 18–25 bases in length using MacVector® 6.0 (Oxford Molecular Group, Oxford, England, UK). Generally, primers were chosen that generate PCR products of 100–250 bp, have melting temperatures of at least 60°C and are preferably located close to the polyadenylation site of the mRNA so as to reduce the chance of sampling family members.

RT was performed on total RNA using two RNA concentrations per sample and an oligo(dT_{15}) primer (Genosys Biotechnologies, The Woodlands, TX, USA). The reactions containing 100 or 50 ng/µL total RNA, 0.5 µM oligo-(dT_{15}) primer, 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 20 mM DTT, 0.2 mM of each dNTP, 0.8 U/µL RNase inhibitor and 2 U/µL of MuLV-reverse transcriptase were ramped for 5 min from 25°–37°C and held at 37°C for 1 h. The enzyme was inactivated by heating the reactions at 94°C for 5 min, and the newly synthesized cDNA was diluted 4-fold in water.

Diluted cDNAs (10 µL) were mixed with 2× PCR mixture containing 20 mM Tris, pH 8.3, 20 mM KCl, 6.25 mM $MgCl_2$, 0.35 mM of each dNTP, 3 µM of each specific primer, 2 µCi [α-^{32}P]dCTP (ICN Biomedicals, Costa Mesa, CA, USA) and 2 U AmpliTaq DNA Polymerase Stoffel fragment, for a 20-µL final reaction. The following low-stringency thermal profile was used: 94°C for 40 s, 40°C for 40 s and 72°C for 1 min, for 17 and 19 cycles. The reaction is carried out in two sets of tubes at different cycle numbers because the abundance of the transcripts, the performance of the primer pairs and the amplifiability of the PCR products can vary. PCR products were run under the same conditions as above on a 5% polyacrylamide, 43% urea gel. The gel is dried and placed for 18–72 h on a phosphor imager screen and read with a Storm Phosphor Imager® (Molecular Dynamics, Sunnyvale, CA, USA). Invariance among the other arbitrary products in the fingerprint was used as an internal control to indicate the reliability of the relative quantitation. The gene-specific products from four sets of reactions per differentially regulated gene were quantitated using ImageQuant™ Software (Molecular Dynamics).

Primer pairs used for confirmation of differential expression: R72714 (Egr-1) (155-nucleotide [nt] product), (A) 5′-CACGTCTTGGTGCCTTTTG-TGTG-3′, (B) 5′-GAAGCTCAGCTC-AGCCCTCTTCC-3′. H14529 (ACTB, β-actin) (174-nt product), (A) 5′-

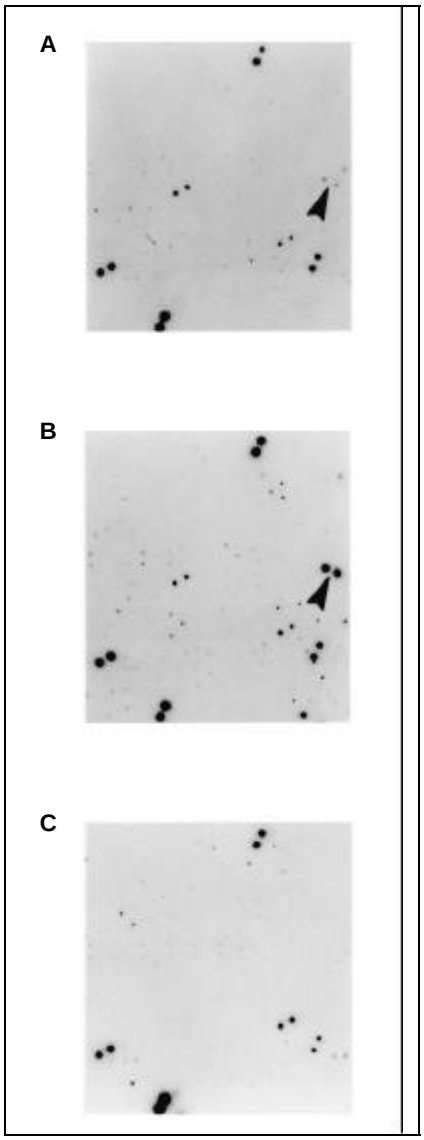

Figure 2. Hybridization of DD reactions to cDNA arrays. (A and B) The DD products generated with the primers $GT_{15}G$ and KA2 from untreated (A) and EGF-treated (B) HaCaT cells in Figure 1B, were labeled by random priming and hybridized to cDNA arrays. A section representing <5% of a membrane is shown with a differentially hybridized clone H14529, encoding β-actin, indicated by an arrow. (C) Hybridization of DD products generated with the primers $AT_{15}A$ and KA2 from untreated HaCaT cells. Note the overlap of the hybridization signals with panel A and C that were not obvious from the polyacrylamide display in Figure 1B.

CCAGGGAGACCAAAAGCCTTCA-TAC-3′, (B) 5′-CACAGGGGAGGT-GATAGCATTGC-3′. H27389 (A+U-rich element RNA binding factor) (144-nt product), (A) 5′-GTGCTTTTCA-AAGATGCTGCTAGTG-3′, (B) 5′-G-CTCAATCCACCCACAAAAACC-3′. H05545 (Protein phosphatase 2A catalytic subunit) (141-nt product), (A) 5′-TCCTCTCACTGCCTTGGTGGA-TG-3′, (B) 5′-CACAGCAAGTCAC-ACATTGGACCC-3′. H27969 (103-nt product), (A) 5′-CCAAAGACATTCA-GAGGCATGG-3′, (B) 5′-GAGGTGG-GGAAGGATACAGCAG-3′. R73247 (Inositol tris phosphate kinase) (168-nt product), (A) 5′-GAAAAGGGTTGG-GGAGAAGCCTC-3′, (B) 5′-TCTC-TAGCGTCCTCCATCTCACTGG-3′. H21777 (α-tubulin isoform 1) (155-nt product), (A) 5′-ACAACTGCATCCT-CACCACCCAC-3′, (B) 5′-GGACAC-AATCTGGCTAATAAGGCGG-3′.

Figure 3 was assembled using Adobe® Photoshop® (Adobe Systems, San Jose, CA, USA).

RESULTS AND DISCUSSION

The experiments discussed here used total RNA from immortalized keratinocytes (HaCaT) (2), treated and untreated with EGF, as previously described (14). The first DD protocol tried was RNAimage. The anchor primers oligo(dT)-G, oligo(dT)-C or oligo(dT)-A were used for RT, and then each cDNA was used in combination with four different arbitrary primers for PCR. The fingerprints were resolved on a denaturing acrylamide gel for quality control purposes. The fingerprints generate approximately 30–50 clearly visible products. Fingerprints were generally reproducible in the range from 100–800 ng of total mRNA used in these experiments, with very few RNA concentration dependent products. Three of the most reproducible fingerprints that shared either an oligo(dT) anchored primer or an arbitrary primer (Figure 1A) were radiolabeled by random priming in the presence of three unlabeled dNTPs and [α-^{32}P]dCTP, and each was used to probe identical arrays of 18 000 double spotted *Escherichia coli* colonies carrying ESTs from the IMAGE consortium (**http://www-bio.llnl.gov/bbrp/image/image.html**). The arrays were hybridized and washed, as previously described (14).

The kit protocol used 0.2 μM of the arbitrary primer and 4 μM dNTPs compared to 1 μM primers and 200 μM dNTPs used in our RAP-PCR protocol (14). The fingerprint reaction contained <40 ng of product in 20 μL, presumably because of limiting components. This was approximately five times less DNA than is recommended for the probe in our published protocol (14). For this reason, it took about ten days with an intensifying screen to obtain an adequate exposure of X-ray film, rather than the two days or less we reported previously for RAP-PCR fingerprints. Approximately 500 products were easily discernible with each probe after a sufficient exposure. The number of reliably observable genes is usually increased by at least twofold or more when using a phosphor imager screen (data not shown). Furthermore, pooling of separate labeled fingerprints into the same probe can increase throughput even further.

To reduce the exposure time for probe hybridization to arrays, we performed experiments at the higher concentration of primer and dNTPs that are used in our RAP-PCR protocols (Figure 1B) (14). These experiments yielded the expected increase in product mass and a corresponding reduction in exposure times for arrays.

We explored the selectivity of oligo(dT) primers with different anchor bases. When the arbitrary primer was changed, while keeping the same anchor primer, then the pattern of clones hybridized changed almost entirely, with typically <5% overlap between any two fingerprints. In contrast, probes containing the same arbitrary primer and different anchored primers shared approximately 30% of the clones to which they hybridized. Figure 2, A and C show examples of such shared products from a small portion of an array. Similar observations were made using fingerprints generated under a wide variety of conditions, including the protocols and primers from the RNAimage kit, our modified protocols and using primers of our own design. The possibility of this overlap being due to repeats was excluded by the use of genomic and total mRNA probes against the same membranes (Reference 14 and data not shown).

There are at least two possible reasons for these observations. One is that the anchored primers are not as discriminating as one would hope. The other possibility is that products that contain the arbitrary primer at both ends are common in the mixture. Surprisingly, the overlap among probes that had different anchored primers, but shared the same arbitrary primer, was not reflected in any noticeable similarity in the fingerprint products when resolved on a denaturing polyacrylamide gel. For example, the probes used in Figure 2, A and C are shown in Figure 1B where there are no easily discerned similarities, at least not to the extent of 30% of the products being in common. It is hard to draw the conclusion that the products that are shared in the

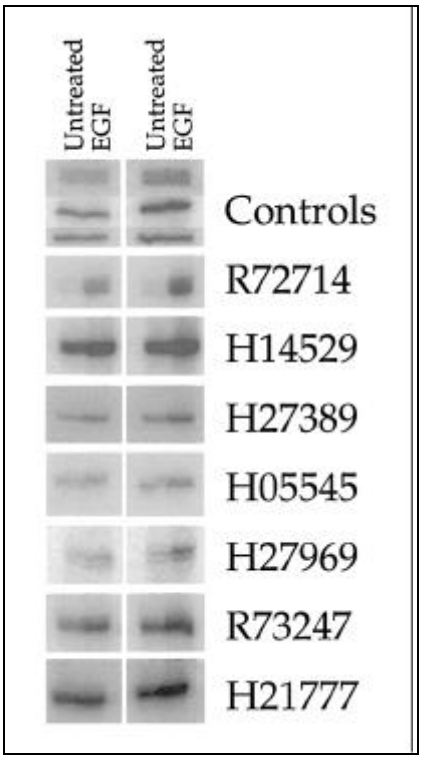

Figure 3. Confirmation of differentially regulated genes using low-stringency RT-PCR. RT was performed at two different RNA concentrations for each gene, 125 ng in the left column and 250 ng in the right column. In the data shown, PCR was performed for 19 cycles. Unregulated internal control bands are labeled as well as regulated genes corresponding to GenBank Accession Nos.: R72714, H14529, H27389, H05545, H27969, R73247 and H21777. The extent of regulation of each gene is listed in Table 1.

probes are not generally visible on the gel, because many of the shared products were among the most intensely hybridizing clones on the array. Perhaps some of the products visible on the gel share the arbitrary primer at one end, but during PCR, the products are preferentially primed at multiple different locations in the opposite direction by the different anchored primers. This would result in fingerprints that had little or no similarity in a polyacrylamide display, while being compatible with the observation that probes with the same arbitrary primer, but different anchored primers, overlap by 30% in the clones to which they hybridize.

Shared products seem to be a general phenomenon for anchored fingerprints that share an arbitrary primer under a fairly wide range of conditions. Fortunately, for practical purposes, the problem of overlap among fingerprints can simply be avoided by not using the same arbitrary primer with different anchored primers. This goes against the recommendations in the kits, but is, nevertheless, easy for the user to implement.

Comparison of the pattern of hybridizing clones with that generated by total genomic DNA, showed that the clones hybridizing to a probe generated by the Genhunter fingerprint did not generally contain the Alu repetitive element that occurs in a few percent of mRNA 3′ untranslated regions (UTRs). The clones hybridized by the probe did not overlap significantly with clones hybridized by a total cDNA probe derived from RT of poly(A)$^+$ mRNA, indicating that the genes sampled were not heavily biased towards the most abundant RNAs. The overall overlap with the total cDNA probe and with the genomic DNA probe was <5% as determined by alignment of all the detectable hybridizing clones. These two encouraging findings are consistent with results we obtained when using only arbitrary primers for fingerprinting (14) and indicate that arbitrary priming combined with anchored oligo(dT) priming can be used to monitor rare genes in cDNA arrays. It also reaffirms the idea that RAP-PCR and DD are not heavily biased toward abundant transcripts.

Among over 2000 clones surveyed for differential gene expression between untreated and EGF-treated HaCaT cells, there were 29 different clones that appeared to clearly reflect differential expression at one RNA concentration. The 12 most promising were chosen, and specific primers were designed for RT-PCR. An example of one of these putative differentially expressed genes is indicated by an arrow in Figure 2, A vs. B. Differential expression of at least 1.5-fold was confirmed for seven genes as determined by quantitative phosphor imager analysis. Differential regulation was determined by radioactive incorporation and quantitation on a phosphor imager. The values presented are the mean ± the standard deviation (SD), which were obtained by analysis of eight RT-PCRs which were generated from two separate RNA preparations at two different RNA concentrations and

Table 1. Genes Regulated by EGF After a 4-h Treatment

Gene	Accession No.	Up-Regulation ± SD
EGR1	R72714, X52541	8.3 ± 3.4
ACTB, β-actin	H14529, M10277	2.0 ± 0.3
A+U-rich element RNA binding factor	H27389, D89092, D89678	1.9 ± 0.3
Protein phosphatase 2A catalytic subunit	H05545, J03804	1.6 ± 0.4
Unknown	KIAA0061, H27969	1.6 ± 0.4
Inositol tris phosphate kinase	R73247, U51336	1.6 ± 0.3
α-tubulin isoform 1	H21777, K00558	1.6 ± 0.3

two different cycle numbers each, except in the cases of EGR1 and α-tubulin, which were tested on one RNA prep. The results are shown in Figure 3 and summarized in Table 1.

Egr-1 was already known to be differentially regulated by EGF in other cell types (3,7–9). The observations of changes in β-actin and α-tubulin expression are perhaps associated with the change in morphology that we observe these cells to undergo after EGF treatment. Regulation of these genes by EGF has been observed in other cell types (1,6,12,13). These observations independently validate the treatments and the method used to detect differential expression. The regulation of protein phosphatase 2A mRNA has not previously been observed, but is consistent with the role of this protein in transduction of the EGF signal (4). Similarly, the gene associated with the metabolism of inositol phosphates had not previously been shown to be regulated by EGF, but such regulation is consistent with previous observations of increases in the compounds generated by this enzyme after EGF treatment in another ectodermal cell type (5). Regulation of two other genes by EGF, an unknown gene, H27969 and an RNA binding protein, D89692, was not previously reported in any cell type.

Five other genes were not confirmed to be regulated when RT-PCR was used. The number of false positives can vary from experiment to experiment and depends on both the quality of the fingerprints and on the quality of the commercially available membranes. To identify false positives before the confirmation stage, we generally recommend that differential expression be observed at two RNA concentrations on arrays before confirmation by RT-PCR (14). The experiments presented here involved only a single concentration because they were primarily designed to determine the efficiency of coverage and overlap among probes made by the oligo(dT)-X anchored priming method. Nevertheless, based on previous work, which is confirmed here, we have found that over half of the differentially hybridizing clones observed at one concentration are real, and when two array hybridizations are performed for each treatment at two different input template concentrations, the error rate is well below 10%.

It is important to note that 3′ anchored probes, while suitable for probing 3′ biased libraries, will not be suitable for certain applications; for example, sampling RNAs that do not have poly(A) tails, such as most bacterial RNAs. Also, these probes would not be suitable for PCR arrays based on internal products, a current example being the Atlas™ Array (CLONTECH Laboratories, Palo Alto, CA, USA) (**www.clontech.com**), which contain coding sequence regions. The lack of 3′ non-coding region in these arrayed products would make a 3′ anchored probe a liability. Similarly, for random primed libraries, there is no reason to

use a 3′ biased probe. Nevertheless, in the case of arrays of 3′ biased ESTs, anchored-arbitrary probes will generally prove eminently suitable.

We began these experiments with the idea that it was probable that the number of clones identified by each arbitrarily primed probe could be increased without adversely affecting the sensitivity and selectivity of the probe. We had the reasonable expectation that fingerprints generated using anchored oligo(dT) and an arbitrary primer, when converted to a radiolabeled probe, would improve the frequency of sampling of genes in a 3′ biased cDNA array. So far, this has not proved to be true, and if anything, the throughput is somewhat less than when two arbitrary primers are used, for reasons we do not understand. Nevertheless, we have demonstrated that changes to the current DD protocols allow the use of such fingerprints as probes for arrays.

The very large number of fingerprints that various laboratories throughout the world have accumulated can be converted to effective probes if the mass is increased by performing PCR on an aliquot of each fingerprint in the presence of sufficient dNTPs (100 μM) and primers (ca. 1 μM). We have previously shown that fingerprints can be re-amplified (11). This will allow thousands of archived DD experiments to yield several fold more information than they yielded when originally resolved as fingerprints on gels. It is expected that throughput will be increased and the detection of rare transcripts enhanced even further if fluorescently-labeled DD probes are used against cDNA arrays on glass slides.

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REFERENCES

1. **Ball, R.L., T. Albrecht, W.C. Thompson, O. James and D.H. Carney.** 1992. Thrombin, epidermal growth factor, and phorbol myristate acetate stimulate tubulin polymerization in quiescent cells: a potential link to mitogenesis. Cell Motil. Cytoskeleton *23*:265-278.
2. **Boukamp, P., S. Popp, S. Altmeyer, A. Hulsen, C. Fasching, T. Cremer and N.E. Fusenig.** 1997. Sustained nontumorigenic phenotype correlates with a largely stable chromosome content during long-term culture of the human keratinocyte line HaCaT. Genes Chromosom. Cancer *19*:201-214.
3. **Cao, X.M., G.R. Guy, V.P. Sukhatme and Y.H. Tan.** 1992. Regulation of the Egr-1 gene by tumor necrosis factor and interferons in primary human fibroblasts. J. Biol. Chem. *267*:1345-1349.
4. **Chajry, N., P.M. Martin, C. Cochet and Y. Berthois.** 1996. Regulation of p42 mitogen-activated-protein kinase activity by protein phosphatase 2A under conditions of growth inhibition by epidermal growth factor in A431 cells. Eur. J. Biochem. *235*:97-102.
5. **Contreras, M.L.** 1993. Nerve growth factor stimulates the production of inositol 1,3,4- and 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate in PC12 cells. J. Neurochem. *61*:1035-1042.
6. **Hazan, R.B. and L. Norton.** 1998. The epidermal growth factor receptor modulates the interaction of E-cadherin with the actin cytoskeleton. J. Biol. Chem. *273*:9078-9084.
7. **Ito, E., L.A. Sweterlitsch, P.B. Tran, F.J. Rauscher and R. Narayanan.** 1990. Inhibition of PC-12 cell differentiation by the immediate early gene fra-1. Oncogene *5*:1755-1760.
8. **Iwami, K., N. Ashizawa, Y.S. Do, K. Graf and W.A. Hsueh.** 1996. Comparison of ANG II with other growth factors on Egr-1 and matrix gene expression in cardiac fibroblasts. Am. J. Physiol. *270*:H2100-H2107.
9. **Kujubu, D.A., J.B. Stimmel, R.E. Law, H.R. Herschman and S. Clarke.** 1993. Early responses of PC-12 cells to NGF and EGF: effect of K252a and 5′-methylthioadenosine on gene expression and membrane protein methylation. J. Neurosci. Res. *36*:58-65.
10. **Liang, P. and A.B. Pardee.** 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*:967-971.
11. **Ralph, D., M. McClelland and J. Welsh.** 1993. RNA fingerprinting using arbitrarily primed PCR identifies differentially regulated RNAs in mink lung (Mv1Lu) cells growth arrested by transforming growth factor beta 1. Proc. Natl. Acad. Sci. USA *90*:10710-10714.
12. **Shinji, T., N. Koide, H. Hada, S. Sasaki, T. Oka, N. Takayama, S. Shiraha, K. Ujike et al.** 1997. Reciprocal gene expression of rat fibroglycan and beta-actin during the course of regeneration after D-galactosamine liver injury. Hepatogastroenterology *44*:239-244.
13. **Torok, N., R. Urrutia, T. Nakamura and M.A. McNiven.** 1996. Upregulation of molecular motor-encoding genes during hepatocyte growth factor- and epidermal growth factor-induced cell motility. J. Cell Physiol. *167*:422-433.
14. **Trenkle, T., J. Welsh, B. Jung, F. Mathieu-Daude and M. McClelland.** 1998. Non-stoichiometric reduced complexity probes for cDNA arrays. Nucleic Acids Res. *26*:3883-3891.
15. **Welsh, J., K. Chada, S.S. Dalal, R. Cheng, D. Ralph and M. McClelland.** 1992. Arbitrarily primed PCR fingerprinting of RNA. Nucleic Acids Res. *20*:4965-4970.

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