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Application of poly(A)$^+$RNA patterns method for searching of differentially expressed genes

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Poly(A)$^+$RNA composition differences for normal, fetal and cirrhotic human liver before and after retinoic acid-induced differentiation of the F9 embryonal carcinoma cell line were analyzed by a novel poly(A)$^+$RNA patterns method. The method is based on the polyacrylamide gel electrophoretic analysis of short cDNA termination products, synthesized by reverse transcriptase using poly(A)$^+$RNA as a template, a set of short 5′-end labeled primers, three natural and one terminator deoxyribonucleotide. A number of known differentially expressed genes and some unknown ones were then identified by direct sequencing of the differentially represented bands excised from a gel and searching a complementary mRNA target sites in GenBank database

Poly(A)$^+$RNA pattern; Reverse transcriptase; Differentially expressed genes; Fetal liver, Cirrhosis; F9 embryonal carcinoma

1. INTRODUCTION

The development of new methods that allow the detection and isolation of differentially expressed genes is important for studying gene regulation and differentiation. The approaches, using high resolution 2-dimensional gel protein analysis [1], differential screening of cDNA libraries [2,3] and differential analysis of mRNA composition by arbitrary primed PCR [4,5] are used. Recently, we have reported the description of a novel method for determination of the poly(A)$^+$RNA composition of a given cell or tissue type [6,7]. The composition of poly(A)$^+$RNA is revealed as electrophoretic patterns of short cDNA termination products, synthesized by reverse transcription of poly(A)$^+$RNA, primed with a short oligonucleotide. In this report we attempted to make a link from electrophoretic pattern data to the level of primary structure data for a number of differentially represented bands. To this end we analyzed the differences in the patterns of poly(A)$^+$RNAs from three different but still closely related tissues: normal, cirrhotic and fetal livers and the changes of poly(A)$^+$RNA patterns during F9 cell differentiation. The direct chemical sequencing of specific bands excised from a gel then provide enough information to identify the differentially expressed genes, direct primer-extension sequencing of poly(A)$^+$RNA [8], PCR amplification/cloning [4] and cloning of differentially expressed genes from a cDNA library using a hybridization approach.

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2. MATERIALS AND METHODS

2.1. *Oligonucleotides*

F1(TGCAGGCTG), F2(TGCAGGTGG), F4(TGGAGCTGG), F5(TGTGGCTGG), F6(TGTGGCAGG), P8(TGCTGGGGA), P10(TGCTGGAGG), P32(TGCTGGAGT), P33(TGCTGGACG), P34(TGCTGGATG), P35(TGCTGGCGG), P36(TGCTGGTGG), P37(TGCTGCAGG), P38(TGCTGTAGG), P39(TGCTGGCGT), P40(TGCTGGCGC), P41(TGCTGGCAG), P42(TGCTGGCCG), P43(TGCTGACGG), P44(TGCTGTCGG), P46(TGCTGGTGT), P47(TGCTGGTGC), P48(TGCTGGTAG), P49(TGCTGGTCG), P50(TGCTGGCGA), P51(TGCTGGTGA), P55(TGCTGGGTG), P56(TGCTGGTTG), P57(TGCTGGCTG), P59(TGCTGCATG) were synthesized on a 370A Applied Biosystems DNA synthesizer and labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase [9].

2.2. *cDNA synthesis*

Conditions for cDNA synthesis by M-MLV reverse transcriptase in the presence of three natural nucleotides and one terminator nucleotide (ddUTP(5AA-Fam)), subsequent treatment with snake venom phoshodiesterase and polyacrylamide-urea gel electrophoresis were previously described [4,5]. Oligo d(T)$_n$ was used as the size marker.

2.3. *Poly(A)$^+$RNA purification*

All normal tissues, fetal liver (24-weeks foetus) and cirrhotic liver were obtained within 1–2 h of sudden death and characterized in accordance with [4]. The F9 embryonal carcinoma cells were grown and induced to differentiate by treatment with retinoic acid and dibutyryl cAMP as previously described [10]. Poly(A)$^+$RNAs were purified from frozen tissue or cells and characterized as previously described [4,5].

2.4 *Chemical sequencing of oligonucleotides*

Bands were excised from the gel and oligonucleotides were eluted by incubating each gel slice in 1 ml of deionized water containing 5 μg/ml of total yeast RNA for 3 h at 55°C. Each solution was then loaded onto a DE52 cellulose column (5 μl), and the oligonucleotide was eluted in 20 μl of 1 M NaCl, desalted on a G15 sephadex column (200 μl) equilibrated with deionized water, and dried under vacuum Cleavage at G residues with dimethylsulphate, G+A residues with piperidinium formate and T residues with potassium permanganate

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were carried out as described [11]. The reaction on C residues was performed with hydrogen peroxide [12]. Piperidine strand cleavage was carried out for all reactions [11]. Electrophoresis of the cleavage products was performed using 20% polyacrylamide-urea gels.

2.5. *Searching for mRNA target sites*

We have used Wisconsin University GCG computer program[13] and the GenBank (release 70.0) database to identify sequences complementary to the sequenced oligonucleotides.

3. RESULTS AND DISCUSSION

3.1. *Comparison of normal, cirrhotic and fetal livers*

Previously, we showed that a set of 30 primers provides 90–95% probability of initiating synthesis from any individual RNA present in the poly(A)$^+$RNA. This provides a comprehensive analysis of all RNAs having abundances higher than 0.1% [4,5]. Of the 30 primers used, at least 28 showed clear reproducible quantitative or qualitative differences in the individual RNA patterns for normal and fetal liver poly(A)$^+$RNAs, but only 5 primers produced different poly(A)$^+$RNA spectra for normal and cirrhotic livers. The data shown in Fig. 1 demonstrate such differences in the poly(A)$^+$RNA spectra for normal and fetal livers (primer F1, F6, P55, P41, P10, P35, P36, P57), and for normal and cirrhotic livers (F6, bands 1, 2; P55, band 3; P35, band 4; P36, bands 5, 6; P57, band 7) with no such differences observed for primer P32. All differentially represented bands (Fig. 1) did not show individual-specific differences although six primers out of 30 primers tested revealed individual-specific differences (about 0.2% of the total number of bands in the poly(A)$^+$RNA pattern) (data not shown). To prove that the observed differences in the poly(A)$^+$RNA spectra reflect differences in the poly(A)$^+$RNA composition we isolated and sequenced several arbitrarily chosen bands 8–18. Table IA shows the sequences of these bands and the results of searching for possible complementary sequences in the GenBank database. Out of 11 sequences analyzed, 2 sequences (bands 14 and 15) did not correspond to any sequence deposited in the GenBank database. For the remaining 7 sequences (bands 9, 10, 11, 12, 13, 17, 18), a unique complementary sequence was found. One sequence (band 16) corresponded to two target individual mRNAs and there were a number of mRNAs (6 human genes) which possessed a complementary target site for the sequence of band 8. In accordance with data shown in Table IA and Fig. 1, α1-globin, α2-globin, A_γ-globin, G_γ-globin and insulin-like growth factor mRNAs apparently exhibit enhanced expression in fetal liver compared with normal liver. This is in accordance with numerous data concerning enhanced expression of these genes during fetal development [14,15]. Mismatch analysis shows that the allowed mismatches are located within the first four nucleotides at the 5′-end of the primer (Table 1).

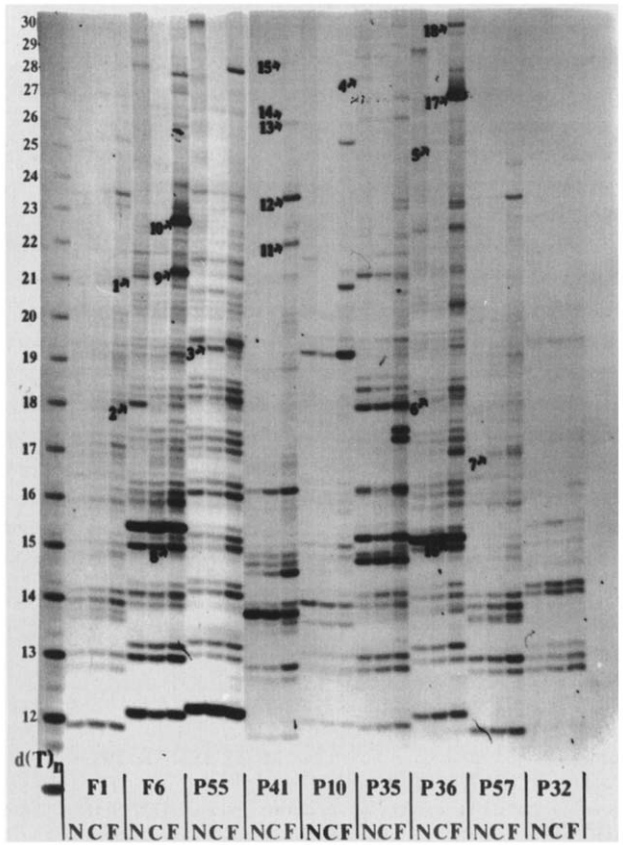

Fig. 1. Comparison of poly(A)$^+$RNA patterns for normal (N), cirrhotic (C) and fetal (F) human livers using primers, F1, F6, P55, P41, P10, P35, P36, P57, P32.

3.2. *Differentiation of F9 embryonal carcinoma cells*

F9 embryonal carcinoma cells (derived from a mouse testicular teratocarcinoma) can be induced to differentiate to parietal endodermic cells by treatment with retinoic acid and dibutyryl cAMP [10]. Changes in the cellular phenotype, which occur in vitro after 2–3 days of such treatment, are well defined by coordinate changes in the expression of specific marker genes [16].

Analizing differences in the poly(A)$^+$RNA patterns from nondifferentiated and differentiated F9 cells we found that out of 30 primers used, 8 primers identify at least 10 separate differentially expressed mRNAs. Primers P35, P37, P43 and P55 revealed 4 individual bands (Fig. 2, bands 1, 3, 4, 5) induced at least 3-fold after 6 days of differentiation. Primer F6 (band 2, 6 days of differentiation) provides an example of a small (less than 2-fold) change in mRNA content. Primers P36 and P48 did not reveal any differences. Table IB shows the results of sequencing the differentially represented bands 1, 3, 4, 5 and the bands 6, 7, 8 which did not change their level of expression. As in the case of fetal liver (Table IA, bands 9, 11, 12, 13) we found several examples of unclear sequencing (Table 1B, bands 4, 6, 7) and misincorporation (bands 1, 3). Searching for complementary mRNAs target sites in the GenBank database revealed that band 1 corresponds to mouse α1-collagen type IV mRNA, band 3 to mouse T-com-

Table I

Sequence of the individual bands and identification of RNAs in GenBank database

Band No	Sequence[a] of Primer-synth. cDNA (5′→3′) RNA(s) target site (3′←5′)	Name of primer/RNA(s)	GenBank database references (5′-end RNA target site position)
A. Normal, cirrhotic and fetal livers (Fig. 1)			
8	TGTGGCAGGgggt Numerous	F6 6 mRNAs	
9	*TGT*GGCAGG$^{T}_{C}$ggaacggct *CGC*CCGTCCTCCTTGCCGA	F6 α2-globin	HUMAGL1 (837)
10	TGT-GGCAGGagacagcaccat ACAGCCGTCCTCTGTCGTGGTA	F6 α1-globin α2-globin	HUMAGL1 (137)
11	*TGCT*GGCAGg$^{T}_{C}$ggaacggct *T*CG*C*CGTCCTCCTTGCCGA	P41 α2-globin	HUMAGL1 (837)
12	TGCTGGCAGg$^{G}_{A}$gacagcaccat AC*AG*CCGTCCTCTGTCGTGGTA	P41 α1-globin	HUMAGL1 (137)
13	TGCTGGCAG$^{T}_{C}$ggcagaggacaggt AC*T*ACCGTCTCCGTCTCCTGTCCA	P41 G_γ-globin A_γ-globin	HUMBGG (162) HUMHBGAB (444)
14	TGCTGGCAGgggacagcaccacggt None	P41 None	
15	TGCTGGCAGgggggggcagcgcaggggct None	P41 None	
16	*T*GCTGGTGGggat *C*CGACCACCCCTA	P36 Cytochrome P450 Cystein proteinase inhibitor	HUMCYPC219 (1088) HUMINCP (1280)
17	TGCTGGTGGggaaggacaggaacat AC*C*ACCACCCCTTCCTGTCCTTGTA	P36 α1-globin α2-globin	HUMGL1 (350)
18	*T*GCTGGTGGgcagagcgcgggcaggcgt *G*CGACCACCCGTCTCGCGCCCGTCCGCA	P36 Insulin-like growth factor II	HUMIGF2FP (269)
B F9 Cell differentiation (Fig. 2)			
1	*T*GCTGGGTGgggagcgcagagccccggcgt *T*CGACCCAC*G*CCTCGCGTCTCGGGGCCGCA	P36 α1 (α2)-collagen type IV	MUSCOL4A (403)
3	*T*GCTGGCGGacagcct *G*CGACCGCC*C*GTCGGA	P35 T-complex testis expressed peptide	MUSTCTEI (1465)
4	TGCTGACGGg$^{T}_{C}$ggaacggct None	P37 None	
5	*T*GCTGACGGcat(agt)[b] *G*CGACTGCCGTA(TCA)	P43 Laminin A	MUSLASMAR1 (552)
6	*TGCT*GGCGGG$^{A}_{G}$agacgggccggt *CGCG*CCGCCC*G*CTCTGCCCGGCCA	P35 28S rRNA	MUSRRM (1277)

Table I (continued)

Band No	Sequence[a] of Primer-synth. cDNA (5′→3′) RNA(s) target site (3′←5′)	Name of primer/RNA(s)	GenBank database references (5′-end RNA target site position)
7	TGCTGGCGGg[a,b]cggcgagacggcgt	P35	
	None	None	
8	*TGCTGACGGgcgagagggcggccccct*	P43	
	*TGC*ACTGCCCGCTCTCCCGCCGGGGGA	28S rRNA	MUSRRM (4554)

[a]Upper case letters in the upper row show the seguence of the primer used, mismatched bases are printed in italics and underlined. The synthesized cDNA sequence is printed in lower case letters. Uncertainties in sequencing are marked by two bases at one position and underlined.
[b]Brackets mark the sequence of band 5 after extension in the presence of 0.2 μM dTTP.

plex associated testis-expressed peptide (Tctc 1) mRNA, band 5 to mouse laminin A mRNA, and bands 6 and 8 to mouse 28S rRNA. The sequences of bands 4 and 7 did not correspond to any complementary sites among genes/mRNAs accumulated in GenBank database.

As the length of band 5 was too short (12 nucleotides) for searching complementary sequences in the GenBank database, we 'extended' this band by deliberately adding 0.2 μM of dTTP, in addition to ddUTP(5AA-Fam) and sequenced the band terminated at the second T, which was 3 nucleotides longer. This resulted in the finding of the laminin A mRNA sequence (Table 1B, band 5). Among the detected differentially expressed mRNAs, mouse α1-collagen type IV mRNA and laminin A mRNA are well known markers of differentiated F9 cells [16] and Tctc 1 mRNA [17] is a new marker for F9 cell differentiation.

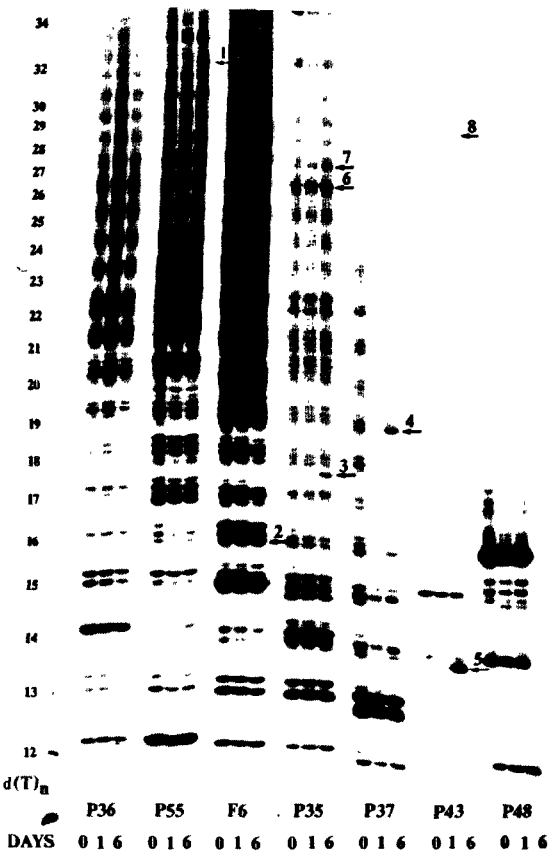

Fig. 2. Changes in cDNA patterns synthesized by reverse transcriptase using poly(A)$^+$RNA from non-differentiated (0) or F9 cells, treated with retinoic acid and dibutyryl cAMP for 1 day (1) or 6 days (6) using primers, P36, P55, F6, P35, P37, P43, P48.

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