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Analysis of poly(A)$^+$RNA patterns in human tissues

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A novel method for analysing and comparing the relative amounts of the most abundant (higher than 0.1% abundance) individual mRNAs present in different poly(A)$^+$RNA preparations has been developed. This method is based on the synthesis of short (10–30 nucleotide) cDNA termination products, by reverse transcription of poly(A)$^+$RNA primed with a 5′-labeled oligonucleotide. A set of 30 different oligonucleotides are used as primers in separate reactions, their length and sequences having been chosen to provide more than a 90% probability of initiating synthesis from any individual RNA present in the poly(A)$^+$RNA Each primer produces about 10–60 bands per track, following polyacrylamide gel electrophoresis under denaturing conditions Data presented reveals poly(A)$^+$RNA pattern differences for a number of human tissues and identifies changes in RNA patterns between normal tissues and neoplastic human tumors (myoma of the uterus) from several individuals

Poly(A)$^+$RNA pattern; Reverse transcriptase; Differentially expressed genes; Arbitrary primer; Human tissue; Myoma of the uterus

1. INTRODUCTION

Current estimates of the total number of expressing genes in a typical mammalian cell range from 10,000 to 20,000 [1]. High resolution 2-dimensional protein gel electrophoresis is commonly used for monitoring 1000–2000 proteins expressed simultaneously in different cell types [2]. Unfortunately, to date, an efficient technique for monitoring gene activity by the simultaneous quantitative and qualitative analysis of large numbers of individual mRNAs has not been available. In this report we describe a new method for determination of the poly(A)$^+$RNA composition (pattern) of a given cell or tissue type based on the analysis of short cDNA termination products, synthesized by reverse transcription of poly(A)$^+$RNA, primed with a short oligonucleotide. Recently, we have described limitations of the method, reproducibility of the results, linearity range of the quantitative data analysis, interpretation of the position of the bands on electrophoregram and criteria used for selection of primary structure of primers [3]. In this paper we show the field of application of the developed method, by analysing the differences in the pattern of poly(A)$^+$RNAs from different normal and transformed human tissues. A similar approach for analysing differences between mRNA populations, involving PCR amplification of cDNA templates using a pair of short primers, has been published recently [4,5].

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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 370A Applied Biosystems DNA synthesizer and labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase [6]

2.2 *cDNA synthesis*

0.2 pmol of 5′-^{32}P-labeled primer was annealed to 0.4 μg of the poly(A)$^+$RNA template, in a volume of 1 μl of deionized water, by heating the mixture for 2 min at 95°C, followed by incubation for 10 to 20 min at 25°C. cDNA synthesis was then initiated by mixing the annealed primer-RNA with 20 units of M-MLV reverse transcriptase (Gibco-BRL) in a final volume of 2 μl, containing 50 mM Tris-HCl (pH 8.3 at 22°C), 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 50 μg/ml of BSA, 20 μM each of dATP, dGTP, dCTP, 50 μM ddUTP(5AA-Fam) (modified by fluorescein (Fam) residue at the 5-position of the uridine base [7]) and incubated for 12 h at 42°C. For selective hydrolysis of non-terminated oligonucleotides 50 μg/ml of snake venom phosphodiesterase (Pharmacia) and 100 mM glycine-NaOH, pH 10.1, were added and then incubated for 45 min at 56°C [3]. The phosphodiesterase reaction was stopped by addition of 2 μl of 95% formamide, 10 mM EDTA, pH 8.0, then heated for 2 min at 95°C. One-half of the sample was then loaded into a single well of a 20% polyacrylamide-urea gel and electrophoresed as described in [3]. Oligo d(T)$_n$ was used as the size marker.

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

All normal tissues were obtained within 1–2 h of sudden death. Myometrium (without endometrium impurities) and myoma nodules

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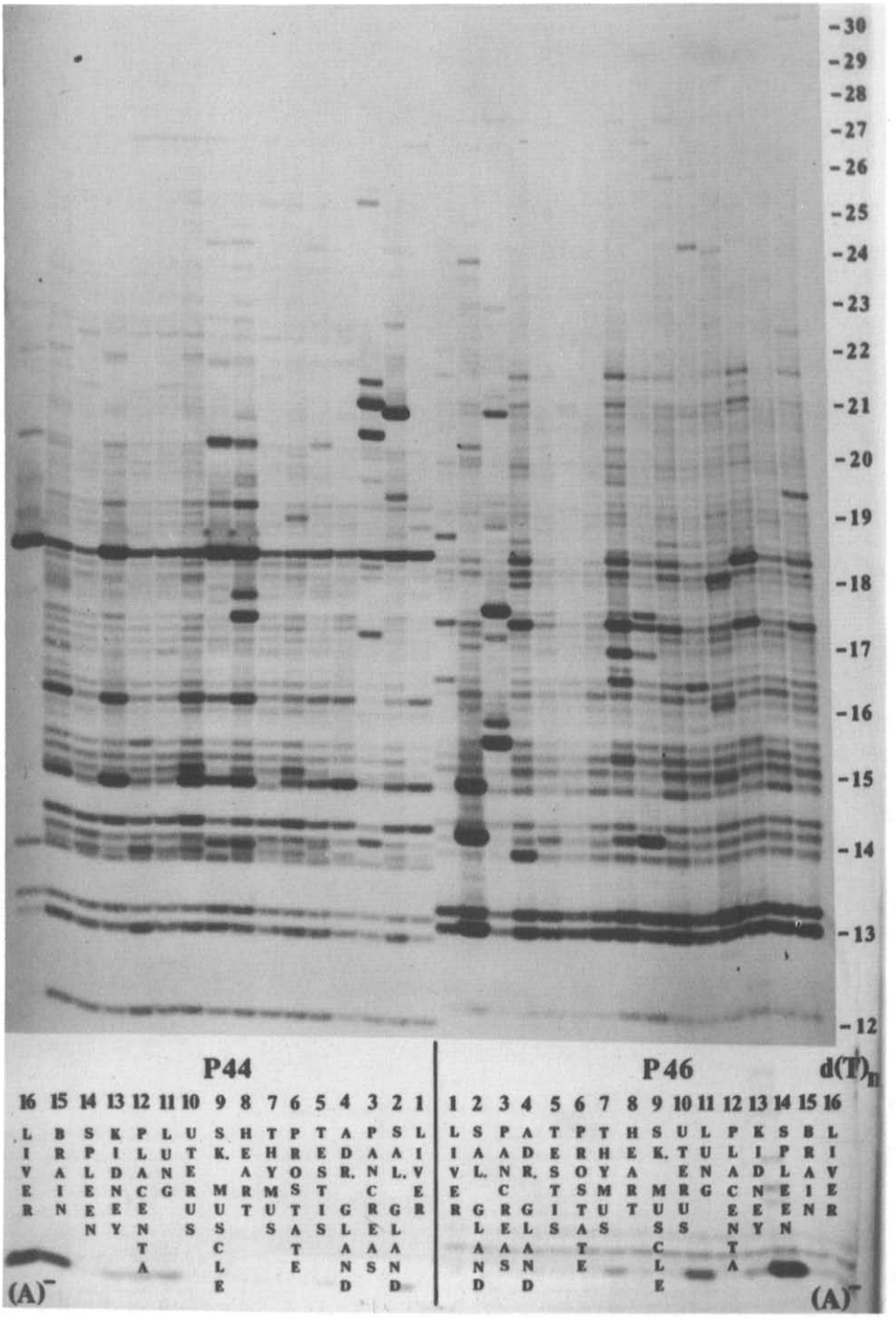

Fig. 1. Poly(A)⁺RNA pattern of terminated cDNA synthesis products catalyzed by M-MLV reverse transcriptase on human poly(A)⁺RNAs (1–15) or poly(A)⁻RNA (16) using primers P44 or P46.

were obtained at the time of uterus ablation from three patients. Leomyoma of the uterus was diagnosed histologically for all three cases in accordance with classification [8].

Total RNAs were purified from frozen tissue or cells using homogenization guanidine thiocyanate and phenol/chloroform extraction [6]. The 28S:18S RNA ratio was at least 2:1 for all preparations of total RNAs, as determined by denaturing agarose-glyoxal gel electrophoresis. Poly(A)⁺RNAs were purified from total RNAs by two cycles of oligo d(T)-cellulose chromatography [6].

3. RESULTS AND DISCUSSION

3.1. *Comparison of the poly(A)⁺RNA patterns for different normal human tissues*

Fig. 1 shows the differences in electrophoretic pattern of cDNA products synthesized by M-MLV reverse transcriptase from poly(A)⁺RNAs purified from 15 different human tissues (lanes 1–15) or poly(A)⁻RNA

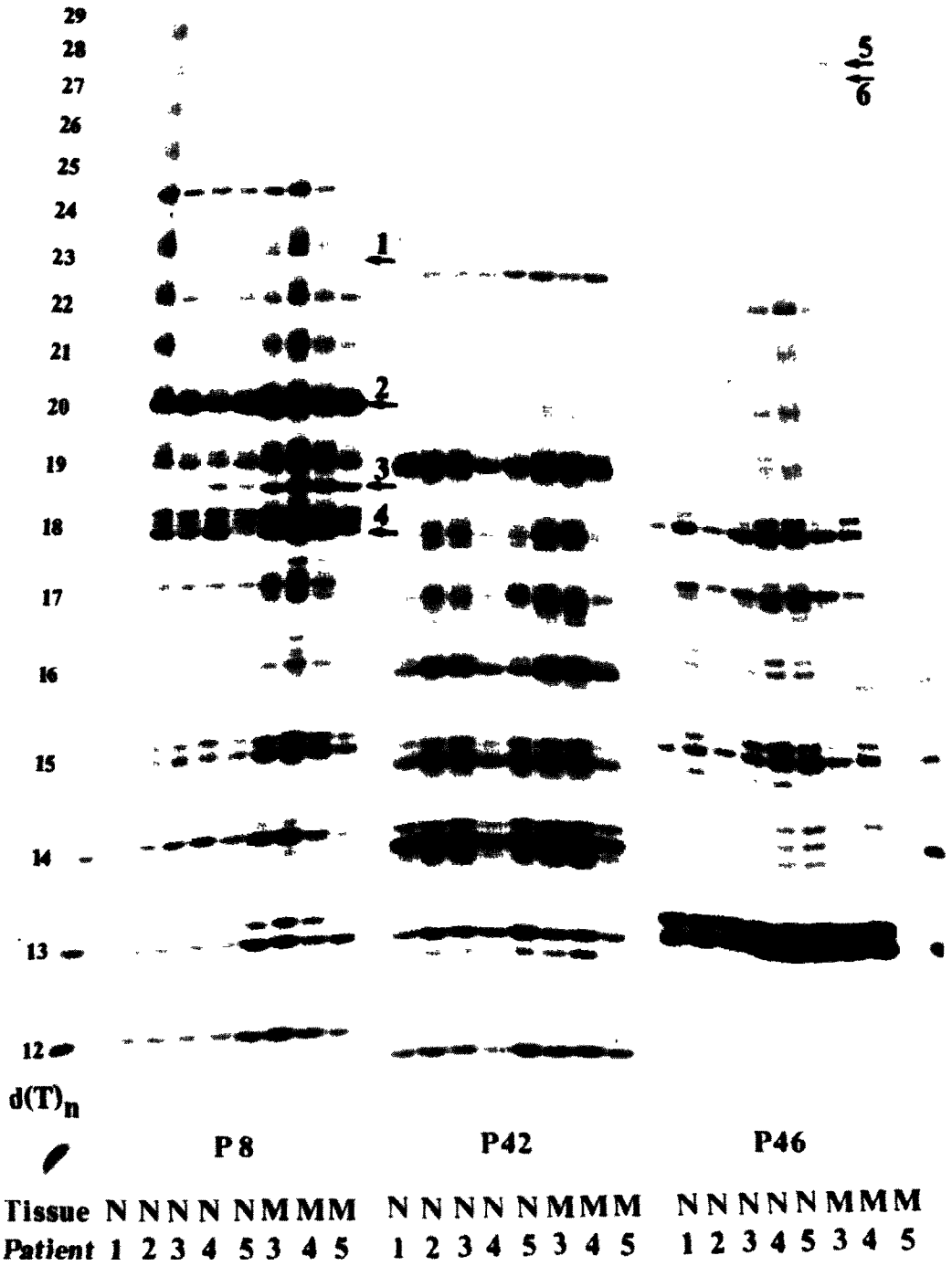

Fig. 2. Comparison of patterns for poly(A)$^+$RNAs purified from normal myometrium (N, patients 1, 2), myometrium, surrounding myomas (N, patients 3, 4, 5) and myomas (M, patients 3, 4, 5) from human uteri using primers, P8, P42, P46.

(lane 16) used as templates, and with 5′-labeled oligo P44 or P46 used as primers. The electrophoretic patterns obtained are very reproducible and unique for each poly(A)$^+$RNA when either of the oligonucleotide listed in section 2 is used as the primer. Intensity of the bands is generally proportional to the concentrations of template RNAs responsible for their synthesis when these are in the 0.1–30% abundance range (data not shown) [3]. Quantitative comparison between different poly(A)$^+$RNAs for a given mRNA/primer product will therefore be valid providing the gel band contains only one specific product. Based on this data we estimate that the most intense signals observed in Fig. 1 commonly correspond to individual mRNAs with abun-

dance in the range 5–20%. These intensive bands are characterized for poly(A)$^+$RNAs purified from tissues overproducing specific proteins (for example glands: pancreas (lane 3), salivary (lane 2), adrenal (lane 4), liver (lane 1), and muscle tissues: heart (lane 8), skeletal muscle (lane 9), and uterus (lane 10)). Poly(A)$^+$RNA patterns from histologically different tissues (for example, from liver (lane 1) and brain (lane 15)) possess more differences than poly(A)$^+$RNAs from similar tissues (for example, skeletal muscle (lane 9), heart (lane 8) and uterus (lane 10)). The simplest patterns of cDNA synthesis products are typical for RNAs with low complexity (e.g. poly(A)$^-$RNA (lane 16) and poly(A)$^+$RNA from some glands: pancreas (lane 9) and liver (lane 1)). Increasing the complexity of the poly(A)$^+$RNA leads to the appearance of a greater number of bands per lane – see brain (lane 15), spleen (lane 14). All the other primers (see section 2) produce of similar complexity reproducible patterns for all used poly(A)$^+$RNAs [3].

3.2. *Myoma of the uterus*

It is essential to demonstrate the significance of individual-specific differences in poly(A)$^+$RNA spectra and possibility to reveal differences in poly(A)$^+$RNA patterns for closely related tissues. The model of the uterus myoma formation from normal myometrium seems to be ideal for such studies for the following reasons.

(1) Myoma of the uterus is a very widespread oncological disease, so it is possible to obtain statistically reliable data using multiple samples.

(2) Normal myometrium consists of an homogeneous population of smooth-muscle cells (98%) and myoma is characterized by the appearance of only one type of cell [8].

(3) Normal myometrium surrounding the myoma could serve as an internal control to myoma, without any individual differences.

We have tried to observe individual-specific and myoma-specific differences in the poly(A)$^+$RNA spectra from two normal (without pathology) uteri and three pathological uterine (myometrium and myoma) samples. For this purpose we used the set of 30 primers (see section 2) providing the analysis of approximately 90% of all the RNAs in poly(A)$^+$RNA within the detection limit (> 0.1% abundance) [3]. Only six primers out of 30 primers tested showed individual-specific differences (about 0.2% of total number of bands in poly(A)$^+$RNA pattern) and only 2 primers could detect myoma-specific differences (about 0.1% of bands). The data for three primers P8, P42 and P48 are shown in Fig. 2. The P8 primer identifies individual-specific changes such as the appearance of a new RNA (band 1, patient 4) or changes in the content of individual RNAs (band 2, patient 5; band 4, patient 3). Primer P8 also reveals an increased content (about 3–5-fold) of individual RNA (band 3) both in myometrium and in myoma in all 3 cases (patients 3, 4, 5) compared to the normal uterine samples (patients 1, 2). Also primer P46 reveals the increasing intensity of band 6 and decreasing intensity of band 5 for all three myomas (patients 3, 4, 5) compared to normal myometrium (patients 1, 2) and myometrium surrounding myomas (patients 3, 4, 5).

The presented examples show that the poly(A)$^+$RNA pattern method could reveal the genes equally expressed in distantly related tissues and differentially expressed genes in the more related tissues. Each pattern is very stable and reproducible. It can be used to identify individual-specific genes or genes differentially expressed due to pathological processes. The same approach may be successfully applied for searching for differentially expressed genes during the processes of differentiation, transformation, cell cycle regulation and mutagenesis in cells cultivated in vitro as well as changes appear due to action of drugs or media condition changes.

Changes identified on the level of poly(A)$^+$RNA pattern (bands on electrophoresis) can be fluently transformed into sequence information just by slicing the band of interest and sequencing the oligonucleotide. The sequence of 14–30 nucleotide long oligonucleotide is sufficient to search for target template mRNAs in the GeneBank database, for direct sequencing of mRNA templates in poly(A)$^+$RNA, for PCR amplification/cloning [4] and for the cloning of differentially expressed genes from a cDNA library using a hybridization approach. Results of this type of analysis for the model of normal, fetal and cirrhotic human liver and differentiation of F9 mouse embryonal carcinoma cells will be published in the next communication [9].

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