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Identification of rapid turnover transcripts overexpressed in thyroid tumors and thyroid cancer cell lines: use of a targeted differential RNA display method to select for mRNA subsets

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ABSTRACT

The mRNAs of transiently expressed proteins such as cytokines and proto-oncogenes are commonly subject to rapid transcriptional activation and degradation. Transcript turnover is determined in part by association of certain proteins with consensus AU-rich motifs (AUUUA) in the 3′-untranslated region of the transcripts. Here we report a modification of differential RNA display (DRD) to detect differentially expressed rapid turnover mRNAs containing AU-rich motifs from thyroid cancer tissues and cell lines. RNA of normal and thyroid cancer tissues was differentially displayed using a 3′ anchor primer to the poly(A) tail and an arbitrary 5′ primer incorporating an AUUUA sequence. The appropriateness of the strategy was established by its ability to display known early response genes, such as c-*fos*, using partially degenerate primers. To test whether the novel cDNAs isolated coded for transcripts subject to rapid turnover, they were used as probes for Northern blots of RNA from clonal human thyroid carcinoma cell lines treated for varying periods with either cycloheximide or actinomycin D. A number of novel differentially expressed cDNA fragments were isolated from human papillary thyroid carcinoma tissues, among them a cDNA with zinc finger motifs and homology to other zinc finger proteins. Using this fragment to probe a cDNA library, a full-length cDNA (*ZnF20*) was isolated that was 4333 bp in length and contained an open reading frame of 1029 amino acids. The *ZnF20* cDNA hybridized to multiple transcripts in a thyroid cancer cell line (8.0, 4.5 and 2 kb) that increased after cycloheximide treatment and decayed <2 h after addition of actinomycin D. The *ZnF20* mRNA was overexpressed in three of six thyroid papillary carcinomas as compared with paired normal thyroid tissue controls. The data presented here support the use of a targeted DRD approach for the isolation of rapid turnover mRNAs, many of which may be interesting candidate oncogenes.

INTRODUCTION

A number of mRNA transcripts that code for proteins involved in control of DNA synthesis are expressed transiently. Several studies indicate that *trans*-acting factors play a role in mediating the destabilization of mRNA, in part through their binding to discrete sequence elements present in the message. The cap structure and the 5′-untranslated region (5′-UTR), as well as the open reading frame, of mRNAs are reported to contain destabilizing sequences involved in RNA–protein interactions that culminate in the degradation of specific transcripts (1). However, binding of proteins to the 3′-untranslated region (3′-UTR) of the mRNA and metabolism of the poly(A) tail are generally believed to play a pivotal role in mRNA decay. AU-rich elements within the 3′-UTR of certain classes of rapid turnover mRNAs can stimulate transcript degradation, in part through mechanisms which require association of specific proteins with AU-rich consensus motifs (AUUUA) (2–4), which are often reiterated several times within an AU-rich region of the 3′-UTR sequence. Of the mRNAs reported to contain such motifs, many are nuclear proto-oncogenes and cytokines (4). Indeed, several proto-oncogene mRNAs undergo rapid degradation, presumably because of a requirement to act at discrete points in the cell cycle. Some of these rapid turnover genes have the potential to become oncogenic when mutated or inappropriately expressed (e.g. *fos*, N-*myc* and *jun*). Overexpression of oncogenes of this class (i.e. through amplification, translocation or other mechanisms) is associated with several human neoplasms. N-*myc* amplification and overexpression has been reported in up to 53% of patients with end stage neuroblastoma (5). In addition, amplification of c-*myc* has been reported in breast cancer (6), gastric adenocarcinoma (7), colon carcinoma (8) and acute myelogenous leukemia (9). Increased expression of c-*myb* was found in colon carcinoma cell lines (10). The *PRAD1*/*cyclin D1* gene is rearranged in parathyroid tumors leading to its overexpression as an oncogene (11). Overexpression of this gene has also been linked to certain forms of leukemia and breast cancer (12,13). Truncation of the AU-rich region of *cyclin D1* stabilizes its mRNA and leads to its accumulation in a breast cancer cell line (14). An increased abundance of c-*myc* and c-*fos* mRNA has been reported in thyroid

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neoplasms, primarily in thyroid carcinomas (15). Overexpression of the high mobility group protein HMG1 mRNA, which also has an AUUUA motif in its 3′-UTR, is also found in thyroid cancers (16). HMG1 is believed to be involved in condensation of centromeres, telomeres and GQ bands during mitosis (16).

Based on the assumption that among the mRNA species undergoing rapid turnover many may be involved in the regulation of cell division, we have designed a strategy to isolate such transcripts that are overexpressed in thyroid neoplasms. The recent description of differential RNA display (DRD) allows the application of strategies to rapidly identify and clone differentially expressed transcripts using primers bracketing sequences within 150–1000 bases of the poly(A) tail (17). Downstream primers are composed of 5′(dT)11VN or (dT)12VN) (where V = A, C or G; N = T, A, C or G). In the original method upstream primers were arbitrary 9mers or 10mers. Statistical calculations show that ~200–250 different pairs of randomly chosen primers are needed to represent all messages being expressed in an individual cell (50–100 products are obtained with each reaction), with some combinations identifying the same product by virtue of the fact that more than one upstream primer may bind to different regions of the same cDNA. This calculation does not consider that the resolution of certain differentially expressed products is only possible under certain amplification conditions (i.e. template concentrations, annealing temperature, etc.). This technology is likely to be more powerful if it is targeted to mRNAs that share structural and functional features. Our approach requires the upstream primer to be composed of an AT-rich sequence complementary to the motif(s) determining rapid turnover. We have taken advantage of these motifs in the 3′-UTR of mRNAs (i.e. AUUUA) as a means to identify overexpressed and/or potentially mutated candidate early response genes in human thyroid neoplasms by differential mRNA display. A bioassay utilizing Northern blots of thyroid cell lines treated with cycloheximide or actinomycin D was set up to verify that the mRNA fragments isolated by this method are subject to protein-synthesis dependent rapid turnover. With this technique we report the isolation of a number of cDNAs with rapid turnover properties from human thyroid carcinoma cell lines as well as from thyroid carcinoma tumor tissues. In this manner, fragments of the glutaminase transcript, a novel cDNA with a zinc finger binding motif, and a number of unknown cDNAs have been identified.

MATERIALS AND METHODS

Cell lines and tissue sample collection

The human thyroid carcinoma cell lines NPA, ARO, FRO and WRO were propagated in RPMI-1640 medium containing 10% fetal calf serum, glutamine (286 mg/l) and Fungi-bact (Gibco BRL, Gaithersburg, MD), as described (29). Some flasks of cells were treated with 1 µg/ml actinomycin D or 10 µg/ml cycloheximide for the indicated periods of time. Human thyroid tissue was collected at time of surgery and immediately frozen in liquid N_2 until assayed. Whenever possible samples were taken from both tumor and normal tissue from the same individual.

RNA extraction

RNA was isolated from thyroid carcinoma cell lines using the method of Chomczynski and Sacchi (19). RNA from thyroid tissue, snap frozen in liquid N_2 at time of surgery, was prepared using the guanadinium/CsCl procedure (16). All RNA samples used for DRD were treated with DNase I (GenHunter, MA) as recommended by the manufacturer.

Differential RNA Display

Aliquots of 100–200 ng total RNA were reverse transcribed with 200 U Superscript reverse transcriptase (Gibco-BRL) in the presence of 2.5 µM downstream primer (T)11GA or (T)12GA, 20 µM dNTP for 60 min at 35°C, followed by 5 min heat inactivation at 95°C. Two microliters of the cDNA reaction mixture was amplified in the presence of 1 µM each primer, 20 µM dNTP, 1.25 mM $MgCl_2$ and 0.5 µM [^{35}S]dATP (1200 Ci/mmol). Other components in the PCR reaction were as suggested by the manufacturer. PCR parameters were 94°C 30 s, 40°C 2 min, 72°C 30 s for 40 cycles, followed by a 5 min extension at 72°C. From each reaction 4 µl was loaded on a 6% polyacrylamide sequencing gel or a non-denaturing MDE gel (AT Biochem, Malvern, PA) for analysis.

PCR primers

Primers used for reverse transcription were either (T)11AG, (T)12AC or (T)12AG. The 10mer oligo used as a second primer for the PCR reaction containing the 3′-UTR rapid turnover motifs was one of the following as indicated: GGCATTTACG, GGCAGCATTTA, GGCATTTACG or GCTTATTTAT. In an attempt to isolate sequences further upstream from the 3′-UTR, a separate set of experiments were performed using primer pairs consisting of the 10mer encompassing the rapid turnover AUUUA sequence, GGCATTTACG, as well as the random 10mer CTTGATTGCC.

Cloning of differential RNA display products

DRD products were cut from the gel and rehydrated in 100 µl 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA for 15 min at 25°C followed by elution at 95°C for 15 min. NaOAc was added to a final concentration of 0.3 M and the DNA precipitated in 100% ethanol overnight at –20°C. The product was centrifuged at 4°C for 30 min, dried and resuspended in 10 µl water. A 5 µl aliquot was then used for re-amplification. The resulting PCR product was cloned into either the TA cloning vector (Invitrogen, San Diego, CA) or a Prime PCR Cloner system (5 prime-3 prime, Boulder, CO). Cloned cDNA products of interest were sequenced with a Sequenase 2.0 kit (US Biochemical Co., Cleveland, OH) according to the manufacturer's instructions.

Northern blot analysis

To determine whether the cDNA fragments isolated using targeted DRD represent mRNAs that are subject to protein synthesis-dependent rapid turnover, 20 µg total RNA of cells treated for the indicated periods of time with either cycloheximide or actinomycin D were electrophoresed through a 1% agarose–formaldehyde gel (4-MOPS buffer) and transferred to nylon membranes (Micron Separation Inc., Westborough, MA). Probes were synthesized from PCR cloned DRD products using the M13-40 sequencing primer (GTTTTCCCAGTCACGAC) and T7 promoter oligonucleotide (TAATCGACTCTATAGGGGAA) primer set, followed by random priming in the presence of [^{32}P]dCTP.

Preparation and screening of the NPA cell cDNA library

Poly(A)$^+$ mRNA was isolated from NPA thyroid cells using a PolyATtract mRNA system (Promega, Madison, WI). cDNA was generated from poly(A)$^+$ mRNA and then cloned into a λ expression vector using a ZAP Express Vector Kit (Stratagene, San Diego, CA). The λ cDNA library was then screened as directed by the manufacturer (Stratagene, San Diego, CA), using a probe labeled by random priming in the presence of [^{32}P]dCTP. The probe used was a gel-purified fragment isolated from the clone whose sequence contains the zinc finger domains. Positive plaques were isolated and the ExAssist helper phage (Stratagene, San Diego, CA) used to generate a recircularized pBK-CMV (Stratagene, San Diego, CA) phagemid containing the positive cDNA.

Sequencing of the full-length *ZnF20* cDNA

Sequencing of the zinc finger cDNA was performed by the nested deletion method as described by manufacturer (Pharmacia Biotech Inc., Piscataway, NJ). Briefly, the PBK-CMV *ZnF20* phagemid was cut with *Sac*I and *Sal*I (5′-end) or *Kpn*I and *Xba*I (3′-end). The linearized DNA was incubated with exonuclease III and aliquots of the reaction taken every 2 min for 40 min. The reaction was stopped by addition of S1 nuclease, the reaction mixture incubated at room temperature for 30 min and the size of the remaining insert determined by agarose gel electrophoresis. Aliquots containing the appropriate size inserts were recircularized by ligation with T4 DNA ligase and used to transform DH5α cells. DNA prepared from the resulting clones was confirmed to have the desired size insert and sequenced using an ABI sequencing machine. Initially 20 clones generated by 3′ and 5′ nested deletion were sequenced. Sequences generated from the different nested deletion clones were aligned to give a full-length sequence, using the Lasergene Seqman program (DNASTAR Inc., Madison, WI). Regions not represented by at least three independent clones were sequenced using primers 3′ and 5′ of the region.

RESULTS

Optimization of differential RNA display using AT-rich primers

To identify novel mRNA transcripts that may be subject to rapid turnover, we modified the DRD technique to target the consensus sequence AUUUA present in the 3′-UTR of mRNAs with short half-lives. The AUUUA motif in the 3′-UTR of this class of mRNAs is often repeated multiple times (2,4). A significant concern in the experimental design was that the preferential A+T composition of the primers mapping to overlapping AUUUA sequences could hinder the PCR amplification. Initial experiments were set up to analyze conditions needed for amplification utilizing an arbitrary (T)12NN primer, designed to anchor to the origin of the poly(A) tail of the mRNA, and a targeted AT-rich 10mer, to selectively amplify mRNAs containing an A-rich 3′-UTR. As a model to address this issue, a clonal human follicular thyroid carcinoma cell line was treated for varying periods of time with either the protein synthesis inhibitor cycloheximide, which 'superinduces' or stabilizes rapid turnover mRNAs such as c-*fos* (20,21) through impairment of protein-mediated RNA degradation, or with the transcriptional inhibitor actinomycin D, which should result in a faster reduction of rapid turnover transcripts. As shown in Figure 1A, treatment of FRO thyroid

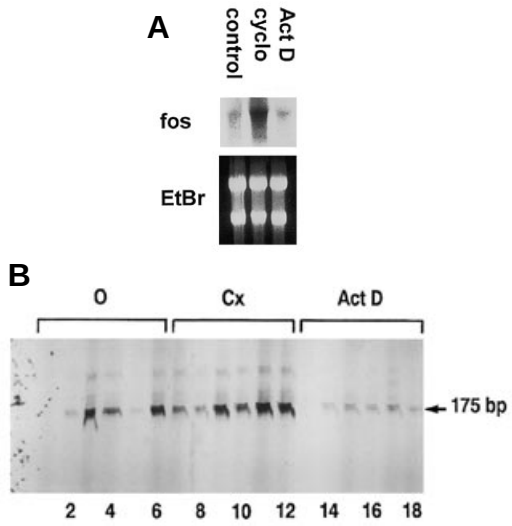

Figure 1. (**A**) Northern blot of 20 µg RNA from FRO cells grown in 10% fetal calf serum (FCS) with or without treatment with 10 µg/ml cycloheximide for 4 h or 5 µg/ml actinomycin D for 2 h. (Top) Hybridization with c-*fos* cDNA. (Bottom) Ethidium bromide staining of 28S and 18S ribosomal subunit bands. (**B**) Autoradiogram of a 6% denaturing DNA sequencing gel showing differential display of cDNAs from: lanes 1–6, FRO cells in 10% FCS; lanes 7–12, + cycloheximide for 4 h; lanes 13–18, + actinomycin D for 2 h. Primer pair 1 [(T)12AC and ATTTATTTGT], lanes 1, 2, 7, 8, 13 and 14; primer pair 2 [(T)12AC and ATTTGTTTAT], lanes 3, 4, 9, 10, 15 and 16; primer pair 3 [(T)12AC and ATTTATTTAT], lanes 5, 6, 11, 12, 17 and 18. The arrow points to 175 bp amplified fragment of the 3′-UTR of c-*fos* cDNA. PCR conditions were as follows: primer concentration 1 mM, 1.5 mM $MgCl_2$, 1.5 U Taq polymerase; amplification, 94°C 30 s, 40°C 2 min, 72°C 30 s, 40 cycles, 72°C 5 min. Not shown, no bands were displayed when MMLV reverse transcriptase was omitted from the reaction. Size marker, single track #(ddG) sequencing of pBR322 DNA.

carcinoma cells with 10 µg/ml cycloheximide for 4 h evoked a marked accumulation of c-*fos* mRNA transcripts and c-*fos* mRNA levels were markedly reduced in cells exposed to 5 µg/ml actinomycin D for 2 h. To determine the impact of the AT content of the upstream primer on the pattern of RNA display, cDNA was generated from untreated cells or from cells exposed to either cycloheximide for 4 h or actinomycin D. The cDNA was then subjected to amplification with sets of primers designed to be either perfectly complementary to an AUUUA motif in the 3′-UTR of c-*fos* (Fig. 1B, lanes 5, 6, 11, 12, 17 and 18) or divergent by 1 nt either outside the AUUUA consensus sequence (lanes 3, 4, 9, 10, 15 and 16) or within it (lanes 1, 2, 7, 8, 13 and 14). As shown in Figure 1B, the three sets of primers amplified primarily a 175 bp fragment, the precise predicted size of c-*fos* cDNA. The abundance of this PCR fragment was maximal in cycloheximide-treated cells and low or absent in cells treated with actinomycin D. The three pairs of primers amplified primarily the same product. However, few other bands were displayed with these primers (even after varying $MgCl_2$ concentrations, annealing temperature, primer or template concentrations). Thus, conditions of amplification with upstream primers with an AT content of 100% (primer with perfect homology to the c-*fos* 3′-UTR) or 90% (primers which had a 1 nt substitution) are not adequate to allow a broad display of cDNA products.

RNA display was found to be much improved when the AT content of the upstream primer was decreased to 80% (Fig. 2). Using two different downstream primers, the upstream primer

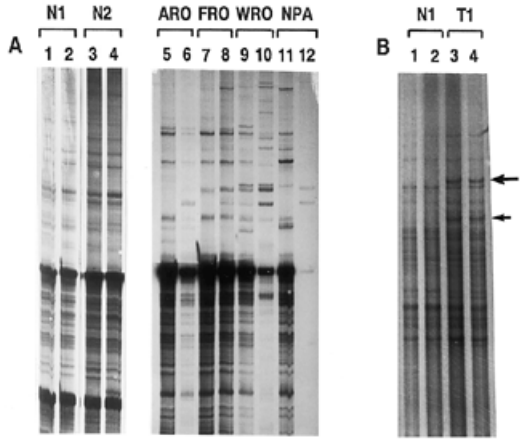

Figure 2. (**A**) Differential display of RNA from normal thyroid tissues from two patients or from clonal human thyroid carcinoma cell lines. Downstream primer, 5′(dT)11GC; upstream primer, 5′-GCTTATTTAT-3′. Normal thyroid 1, lanes 1 and 2; normal thyroid 2, lanes 3 and 4; anaplastic carcinoma cell line ARO, lanes 5 and 6; follicular carcinoma cell line FRO, lanes 7 and 8; WRO, lanes 9 and 10; papillary carcinoma cell line NPA, lanes 11 and 12. Lanes 1, 3, 5, 7, 9 and 11, 2 µg RNA; lanes 2, 4, 6, 8, 10 and 12, 4 µg RNA. (**B**) Differential display of RNA from paired samples of normal thyroid tissue (lanes 1 and 2) and a follicular adenoma (lanes 3 and 4). Downstream primer, 5′(dT)11CA; upstream primer, 5′-GCTTATTTAT-3′. Lanes 1 and 3, 2 µg RNA; lanes 2 and 4, 4 µg RNA. The arrow indicates differentially expressed product. Conditions of amplification and negative controls were as described in the legend to Figure 1.

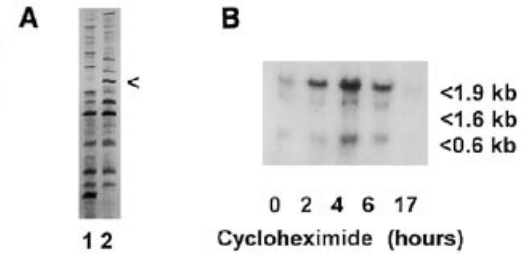

Figure 3. (**A**) Differential display of RNA from NPA cells treated without (lane 1) or with 10 µg/ml cycloheximide (lane 2) for 4 h. Upstream primer, 5′-GCTTATTTAT-3′; downstream primer, 5′(T)12NC3′. PCR reaction was as described in the legend to Figure 1, except that dXTP concentrations were 20 mM. (**B**) Northern blot of 20 µg RNA of NPA cells treated with cycloheximide for the indicated time and hybridized with random prime-labeled PCR product obtained from re-amplification of the gel-purified band indicated in (A) (arrow).

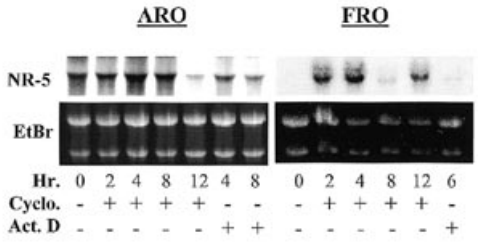

Figure 4. Northern blot of 20 µg RNA from ARO or FRO cells treated with either cycloheximide or 1 µg/ml actinomycin D for the indicated time and hybridized with random prime-labeled PCR product obtained from re-amplification of a gel-purified band (NR5) differentially displayed in a thyroid cancer cell line treated with cycloheximide as described in the legend to Figure 3. Upstream primer 5′-GCAGCATTTA-3′; downstream primer, 5′(T)12NG3′. PCR conditions were as described in the legend to Figure 1.

GCTTATTTAT (which retains the rapid turnover consensus motif ATTTA) resolved 20–50 products from cDNA of normal thyroid tissue and four different clonal thyroid carcinoma cell lines (Fig. 2A). The pattern of bands was consistent within the same cDNA sample, but varied somewhat with template concentration and revealed a similar banding pattern between the various normal samples tested. Not unexpectedly, the thyroid carcinoma cell lines displayed many bands not present in normal thyroid tissue. When cDNA from paired normal and tumor tissue from a patient with a follicular adenoma were examined (Fig. 2B), almost identical patterns were observed between the normal and adenoma RNA with the exception of two bands. This is consistent with a much more discrete set of abnormally expressed gene products between normal and tumor tissue *in vivo*.

Isolation of rapid turnover transcripts from cycloheximide-treated human thyroid carcinoma cell lines

Human thyroid carcinoma cell lines were treated with cycloheximide to superinduce short-lived transcripts and the cDNA products analyzed by targeted differential display using a primer containing the ATTTA motif. Only fragments which appeared to be superinduced following cycloheximide treatment and that were decreased or absent following actinomycin D treatment were chosen for further investigation. Bands of interest were cut out of the gel and DNA was eluted, precipitated and re-amplified. Following isolation and re-amplification, these fragments were used as probes for Northern blot analysis of human thyroid carcinoma cell lines that had been treated for varying times with cycloheximide or actinomycin D. Those PCR fragments hybridizing to mRNA bands that appeared to have been stabilized or induced by cycloheximide and that decreased after actinomycin D treatment were considered to be compatible with a model of protein synthesis-dependent rapid turnover and were cloned and sequenced. Figure 3A depicts a typical band that was isolated from the FRO thyroid carcinoma cell line treated with cycloheximide. As seen in Figure 3B, there is rapid induction of this mRNA, followed by its turnover. This fragment was cloned and sequenced and found to be identical with an expressed sequence tag (EST) isolated from a HepG2 cell line (22). Figure 4 shows a Northern analysis of another cDNA fragment isolated from a thyroid carcinoma cell line treated with cycloheximide. In ARO cells this mRNA increased by ~2-fold with cycloheximide and rapidly disappeared with actinomycin D (half-life ~4 h). A similar observation was seen in FRO cells, except that the mRNA exhibited a biphasic increase. The short half-life of 4 h in ARO cells and the increase seen with cycloheximide suggests that this mRNA is indeed a rapid turnover message. Sequence analysis of this mRNA revealed significant partial homology with a number of mRNAs, among them prostatic antigen, the transcriptional activator DNA binding protein HNF-1 and a zinc finger protein. Table 1 summarizes the results obtained from targeted differential display with two different thyroid carcinoma cell lines treated with cycloheximide. Eight differentially displayed fragments were isolated, of which four were found to have a rapid turnover pattern in the cycloheximide/actinomycin D assay. All isolated fragments were indeed AT rich and all contained the AUUUA consensus motif. These data supported the use of a targeted differential display strategy to isolate rapid turnover cDNA fragments from surgical material of thyroid cancers.

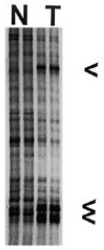

Figure 5. Differential display of RNA paired samples of normal and papillary thyroid carcinoma tissue from the same patient. Duplicate lanes were run for each sample. Upstream primer, 5′-CTTGATTGCC-3′; downstream primer, 5′-GGCATTTACG-3′. The PCR reaction was as described in the legend to Figure 1, except that dXTP concentrations were 20 mM. Arrows indicate differentially displayed bands isolated and cloned for further investigation.

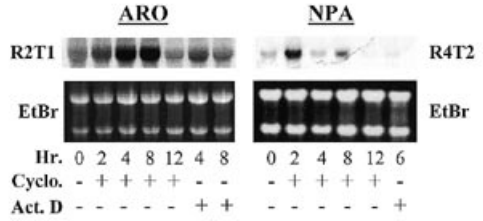

Figure 6. Northern blot of 20 µg RNA from NPA or ARO cells treated for the indicated periods of time with either 10 µg/ml cycloheximide or 1 µg/ml actinomycin D. Northern blots were hybridized with two separate random prime-labeled PCR products, R4T2 or R2T1, obtained from differentially displayed gel-purified bands amplified from two papillary thyroid carcinoma tumors. Primer pairs used for differential display are outlined in Table 2.

Table 1. Isolation of differentially displayed products after treatment of thyroid carcinoma cell lines with cycloheximide

Cell type	Clone no.	Primers	Up-regulation
FRO	FA193	(T)12GC and GCTTATTTAT	Yes
FRO	FA293	(T)12AG and GCAGCATTTA	Yes
FRO	FA893	(T)12AG and GCAGCATTTA	No
NPA	NR393	(T)12AG and GCAGCATTTA	Biphasic
NPA	NR493	(T)12AG and GCAGCATTTA	ND
NPA	NR593	(T)12AG and GCAGCATTTA	Yes
NPA	NR693	(T)12AG and GCAGCATTTA	ND
NPA	NR793	(T)12AG and GCAGCATTTA	ND

ND, not determined.

Isolation of rapid turnover transcripts from human papillary thyroid carcinoma tissue

RNA was isolated from human papillary thyroid carcinomas as well as normal adjacent thyroid tissue. Targeted differential display was carried out with a primer set anchoring at the poly(A) tail and a 10mer targeted to the 3′-UTR sequence ATTTA. In order to isolate coding sequences >500 bases upstream of the poly(A) tract, a primer set was chosen with the 10mer containing the ATTTA sequence now being used for reverse transcription and an upstream arbitrary 10mer used in conjunction with the PCR reaction. Figure 5 illustrates a typical RNA display pattern seen with targeted primers. Bands that were differentially displayed were isolated, cloned and then used as probes to a Northern panel

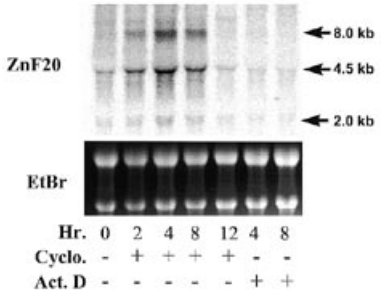

Figure 7. Northern blot of 20 µg RNA from ARO cells treated for the indicated periods of time with either 10 µg/ml cycloheximide or 1 µg/ml actinomycin D. The Northern blot hybridized with random-prime labeled 2750 bp cDNA generated by *Eco*RI/*Xho*I restriction digestion of the *ZnF20* cDNA clone (see Fig. 8).

of mRNA from thyroid carcinoma cell lines treated with cycloheximide or actinomycin D to verify that they were subject to rapid turnover. Figure 6 demonstrates Northern analysis of two of these fragments. Both fragments hybridized to RNA species that accumulated following cycloheximide treatment. Of these, R4T2 was of particular interest, because it accumulated rapidly after cycloheximide and decayed with a half-life of ~6 h. Table 2 summarizes the data obtained from targeted differential display of RNA from papillary thyroid carcinoma tissues. A number of new cDNAs have been identified, among them a cDNA with zinc finger motifs and homology to a recently reported partial sequence of a zinc finger cDNA (HUMZIFI) that maps to the 3p21 locus (23). This mRNA was induced 2.7-fold by cycloheximide and had a half-life of 2 h (Fig. 7). We considered this message to be a good example of a rapid turnover mRNA isolated by this approach and it was therefore selected for further analysis. To obtain the full-length cDNA we generated a cDNA library from the NPA thyroid carcinoma cell line. The cDNA library was screened with the isolated differentially displayed cDNA fragment containing the zinc finger motif. The three phage plaques that revealed a positive signal were isolated and determined to be identical by restriction digestion. Sequencing of a representative clone (*ZnF20*) established that the message has a length of 4333 bp and an open reading frame of 1029 amino acids that codes for a protein with a predicted size of 118.9 kDa. Sequence analysis revealed the presence of 20 C2H2-type zinc finger motifs (Fig. 8) as well as PKC, ck2 and cAMP phosphorylation sites. Two other previously reported partial cDNA sequences [*HUMZIFI* (23) and *HSP18MR* (24)] have almost 100% homology to the 3′- and 5′-ends of *ZnF20* respectively. These zinc finger genes and thus the *ZnF20* gene map to chromosome 3p21. Results from Northern blots of thyroid carcinoma cell lines treated with cycloheximide and actinomycin D (Fig. 7), as well as identification of three AUUUA motifs in the 3′-UTR of the *ZnF20* RNA (Fig. 8), suggests that this is indeed a mRNA subject to rapid decay. Probing Northern blots of thyroid papillary carcinomas and adjacent normal tissue (Fig. 9) with the *ZnF20* cDNA reveals transcripts of ~8.0, 4.4, 3.0 and 2 kb. The gel has been overexposed to better visualize the 8 kb transcript, since it is overexpressed in three of the six thyroid papillary carcinomas. The identification of a 5′ ATG and multiple polyadenylation signals (AATAAA) just prior to the poly(A) tract indicates that the cDNA clone sequenced is likely to be a full-length transcript that corresponds to the 4.4 kb band found in Northern blots probed with the *ZnF20* cDNA.

Table 2. Isolation of differentially displayed products from human thyroid carcinoma tissues

Name	FastA homology	Primers	Size (bp)	AT %	Up-regulation
R1T3	Line element	CTTGATTGCC and GGCATTTACG	145	59	ND
R1T4	COXIII	CTTGATTGCC and GGCATTTACG	146	57	ND
R1T5	Actin capping protein	CTTGATTGCC and GGCATTTACG	162	57	ND
R1T6	No match	CTTGATTGCC and GGCATTTACG	192	57	ND
R2T1	No match	(T)11AG and GCAGCATTTA	378	57	No
R2T2	Glutaminase	(T)11AG and GCAGCATTTA	379	61	Yes
R2T4	No match	(T)11AG and GCAGCATTTA	456	62	ND
R4T1	No match	(T)11AG and GCAGCATTTA	145	54	Yes
R4T2	No match	(T)11AG and GCAGCATTTA	359	64	Yes
R4T5	Zinc finger motifs	CTTGATTGCC and GGCATTTACG	207	59	Yes

ND, not determined.

The cDNA for human glutaminase was likewise found to be up-regulated in the cycloheximide-treated thyroid carcinoma cell lines. The human glutaminase gene has not been cloned. However, the rat glutaminase mRNA is known to be up-regulated in tumors and does indeed possess a number of 3′-UTR AUUUA rapid turnover sequences (25–27).

DISCUSSION

Disruption of growth control involves in part inappropriate expression of mRNAs which are usually present at discrete time intervals in the cell cycle. There are few unbiased assays to identify transiently expressed mRNAs that may be involved in the pathogenesis of neoplasia. In this paper we have described a modification of the DRD technique to target mRNAs with rapid turnover properties. DRD and RNA fingerprinting by arbitrary primed PCR (RAP) have been used to detect differentially expressed genes based on arbitrary priming of short length cDNA products (8,28). In the original DRD technique two primers were used, one to anchor the poly(A) tail of the mRNA and the other, an arbitrary 10mer of random sequence, designed to amplify a discreet array of cDNA fragments possessing a sequence complementary to it (17). RAP utilizes one random 20mer for PCR amplification as well as cDNA synthesis (28). Both methods have been utilized to isolate differentially expressed mRNA transcripts. A disadvantage of these strategies is that they are unable to target a specific subset of gene products. A modification of RAP utilizing nested primers has been used to enrich for gene families of rare transcripts (29). Stone and Wharton developed an additional modification to try and detect changes in expression of a specific gene family (30). They altered the RAP technique so that cDNA was generated by arbitrary PCR followed by amplification with an arbitrary primer as well as a primer homologous to a conserved region within the coding sequence of a gene family. Technical difficulties encountered utilizing the differential RNA technique are related in part to the relatively low stringency of the PCR amplification, which gives rise to false positive results, and problems of reproducibility. In our studies duplicate samples were run for all the targeted differential display samples. Bands were considered of interest only when displayed in duplicate lanes. In addition, we focused our attention on those bands that were displayed in more than one tumor specimen from different patients. An additional consideration is that the band of interest, although apparently discrete, often contains several superimposed cDNA fragments. This results in the isolation of a mixture of cDNAs, not all of which represent differentially expressed products. In order to avoid this problem we separated the cDNA fragments on a non-denaturing gel matrix, as has been suggested (31). Additionally, all differentially displayed cDNA bands were cloned and sequenced. To determine whether the cDNA fragments were of interest, they were hybridized to Northern blots of mRNA isolated from clonal thyroid cancer cell lines treated with either cycloheximide, to superinduce rapid turnover transcripts, or actinomycin D, to permit rapid mRNA decay. The use of this bioassay is not without some drawbacks. Although the human thyroid cell lines were originally isolated from tumors, they are no longer subject to normal growth controls and there is the possibility that some rapid turnover transcripts could be permanently up-regulated in these cells. However, the use of four cell lines for each hybridization reaction minimizes this caveat.

The 3′-UTR sequences of several proto-oncogene, cytokine and lymphokine mRNAs contain a number of repeats of the AUUUA pentamer (2–4). Addition of these AU-rich sequences to rabbit globin mRNA reduces its stability, while removal of these sequences from unstable mRNAs increases their half-lives. A number of proteins have been isolated that bind to these AU-rich sequences and may play a role in determining mRNA turnover (32–34). These factors bind not only to the AU-rich elements, but to other U-rich elements in the 3′-UTR. However, the presence of AUUUA repeats in the 3′-UTR is not an invariable hallmark of rapid turnover. The c-*sis* proto-oncogene contains AU-rich elements, yet possesses a long half-life (35). While deletion of the 3′ AU-rich elements in rapid turnover mRNAs generally leads to stabilization, removal of the AU-rich element of TFIIIA mRNA had a minimal effect on its stability (36). Thus, it is not clear that all AUUUA-containing mRNAs have short lives or that all such RNAs are indeed relevant to abnormal growth. Using a combination of targeted DRD and a simple bioassay, our approach is designed to select only those mRNAs that are rapidly degraded. However, the presence of the AUUUA pentamer in the 3′-UTR and the rapid mRNA decay may not always be causally related.

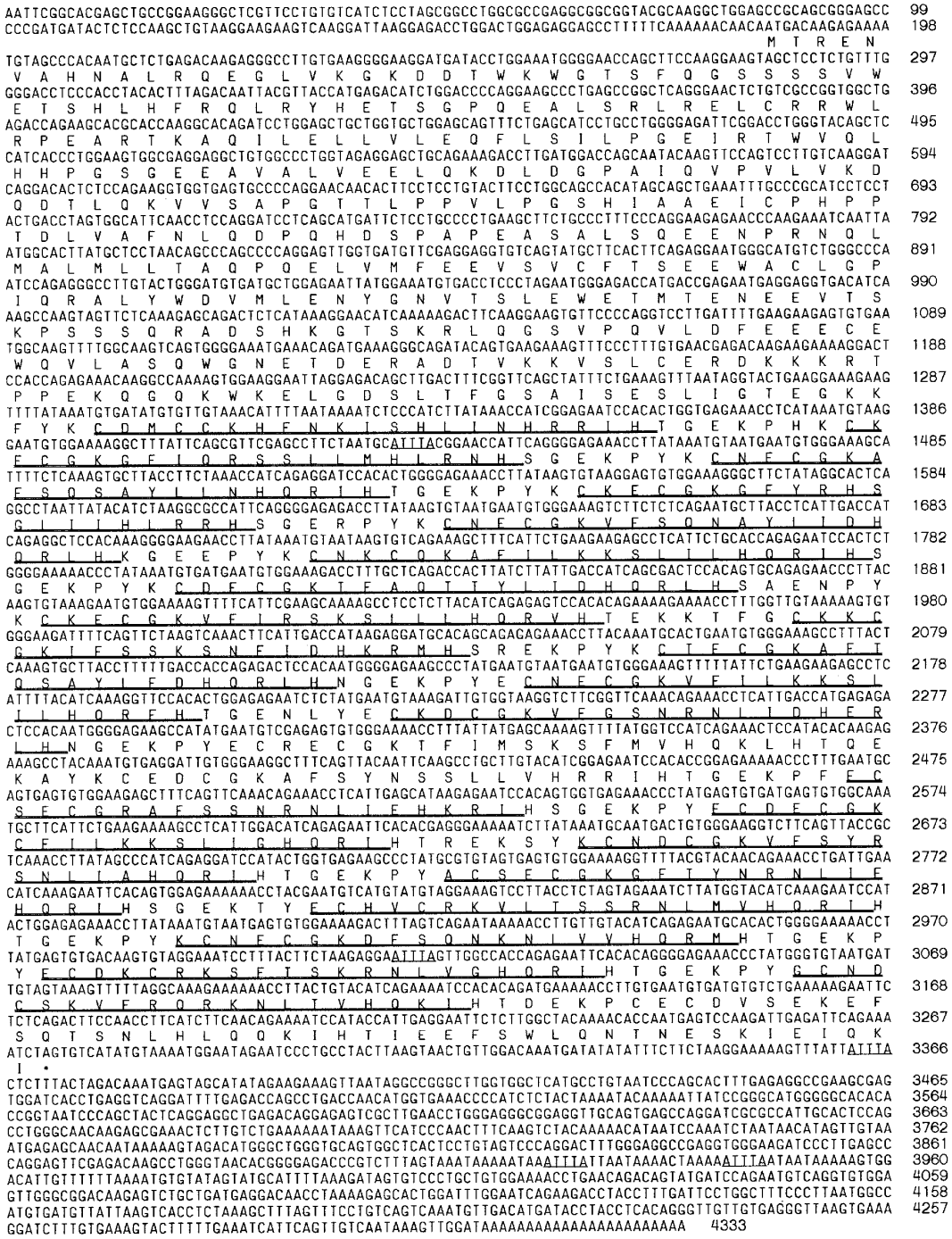

Figure 8. DNA and amino acid sequence of the *ZnF20* cDNA. The 4333 bases of the *ZnF20* cDNA were sequenced as described in Material and Methods. Underlined nucleotides indicate the position of the rapid turnover motifs (AUUUA) and double underlined amino acids demonstrate the position of the 20 C2H2 zinc finger domains identified using the Lasergene, Protean program (DNASTAR Inc. Madison, WI).

One of the cDNA fragments identified by the targeted DRD contains zinc finger domains and was used to screen a NPA cDNA library. The resulting cDNA product (*ZnF20*) has an open reading frame of 1029 amino acids and almost 100% homology with two partial mRNA sequences of two previously reported zinc finger proteins, HUMZIFI (23) and HSP18MR (24). *HUMZIFI* and *HSP18MR* match *ZnF20* at the 3′- and 5′-ends respectively, but miss overlapping each other by 25 bases. Thus it seems *ZnF20* unites the two partial messages giving a full-length cDNA of 4.4 kb. This is supported by the presence of a 5′ ATG and multiple polyadenylation signals just 5′ of the poly(A) tract of the *ZnF20* cDNA as well as the 4.4 kb band seen in Northern blots probed

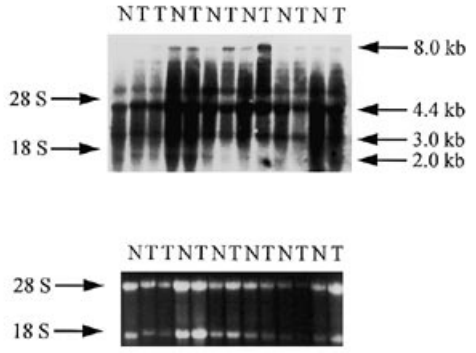

Figure 9. Northern blot of 3–10 µg RNA from thyroid papillary carcinomas and normal thyroid tissue from the same patient which were loaded to ensure a similar quantity of RNA between the tumor/normal pairs. Lanes 2 and 3 show two different tumor fragments from the same patient, with corresponding normal tissue in lane 1. In all other normal/tumor pairs the tumor is preceded by its corresponding normal tissue sample. (Upper) Northern blots were hybridized with random prime-labeled 2750 bp cDNA generated by *Eco*RI/*Xho*I restriction digestion of the *ZnF20* cDNA clone. (Lower) Ethidium bromide staining of the gel to control for loading.

with the *ZnF20* cDNA (Fig. 7). Alternatively, the three different zinc finger cDNAs represent alternatively spliced products, which is supported by multiple bands seen in Northern blots (Fig. 7; 23,24) and the observation that the *HSP18MR* cDNA was cloned with a polyadenylation signal and a poly(A) tail (24). Both *HUMZIFI* and *HSP18MR*, and thus the *ZnF20* gene, map to chromosome 3p21, a region known to be frequently rearranged in malignancies. *ZnF20* is of particular interest since rearrangements and/or loss of heterozygosity of this 3p21 region has been implicated in thyroid cancer (37). This region has also been implicated in cancers of the uterine cervix (38), ovary (39), testis (40), breast (41) and lung (42–46), as well as mesoteliomas (47) and renal cell carcinomas (48–50). Positional cloning of genes from this region reveals the presence of multiple genes coding for zinc finger proteins arrayed in a cluster (23). Zinc finger proteins are known to bind zinc ions and thereby interact with DNA in a sequence-specific manner to regulate DNA transcription. Since many of them control transcription of genes regulating cell proliferation as well as differentiation, they may well be potential candidate oncogenes. Further work is being undertaken to identify the function of this protein and its possible role in thyroid tumorigenesis.

Another cDNA fragment isolated from a human papillary carcinoma exhibited 80% homology with the rat glutaminase gene (cloning of human glutaminase has not been reported). This enzyme may be important in the abnormal metabolism of tumors, which often use glutamine as an energy substrate (25). Indeed, there is evidence that many malignant tumors have increased glutaminase activity (26,27).

A number of isolated fragments contained homology to Alu or LINE sequences. Alu sequences are generally believed to act as mobile elements and have been implicated in homologous recombination. These sequences are AT rich, which probably accounts for the fact that they were isolated in our targeted differential display technique. However, there have been intriguing reports that repetitive sequence elements accumulate in RNA populations during tumorigenesis and in transformed cells (51–53). An increase in the expression of Alu elements and genomic instability has been observed in colonic mucosa at risk for tumor development (54). Although most Alu sequences are present in intergenic regions or in intronic sequences, the LDL receptor possesses an Alu sequence within the 3′-UTR of the last exon of its mRNA (55). Since most of the differentially displayed cDNA sequences with Alu homology were <400 bp in length, it would be difficult for us to identify the transcript from which they originated.

A fragment with autonomously replicating activity was likewise isolated by targeted DRD. This fragment contains homology to the *coxIII* mitochondrial sequence that not only has self-replicating properties, but has been reported to have undergone an insertion that interrupts the c-*myc* proto-oncogene in the HeLaTG cell line leading to a chimeric transcript (56).

In conclusion, the modification of DRD presented here allows targeting and identification of mRNAs that have rapid turnover properties. This targeted differential RNA display may be a useful approach to isolate new candidate oncogenes that may play a role in the development or phenotypic progression of neoplasia.

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