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Alternatively spliced p53 RNA in transformed and normal cells of different tissue types

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ABSTRACT

The alternatively spliced RNA species of tumor suppressor gene p53, containing an additional 96 bases derived from intron 10, is present at approximately 25 to 30% the level of regularly spliced p53 RNA in both normal epidermal and carcinoma cells. The presence of this alternatively spliced RNA in 10T1/2 fibroblast cells, mouse liver and testis suggests that this alternative splicing may be universal. The level of alternatively spliced p53 RNA was increased coordinately with that of regularly spliced p53 in 10T1/2 cells in response to epidermal growth factor. Immunoprecipitation analysis of epidermal cells using monoclonal antibodies which recognize different epitopes of p53 suggested that distinct p53 proteins may be translated from both RNA species. Considering previous observations on the potential importance of carboxyl terminal sequences in p53 function, knowledge of the ubiquitous presence of alternatively spliced p53 is important for future studies of p53 function in normal cells and in oncogenesis.

INTRODUCTION

Wild-type p53 is known to have a negative regulatory function on cell growth while mutant p53 promotes cell transformation *in vivo* and *in vitro* (1). The p53 found to be elevated in many tumors and transformed cells is reported to be associated either with mutations at the coding sequences or complex formation with viral proteins such as SV40 large T antigen and adenovirus type 5 E1B proteins. Using a murine epidermal cell transformation model system, we have demonstrated that p53 expression is altered at the RNA and protein levels in chemically induced squamous cell carcinomas in the absence of mutations at the coding sequences or 5′ and 3′ untranslated regions examined, implying a novel mechanism of p53 inactivation (2).

Alternatively spliced p53 RNA, containing an additional 96 bases derived from intron 10, has been found previously in a chemically transformed fibrosarcoma cell line, but was not detected in a nontransformed helper T cell cDNA library (3). Thus it has been considered to be transformation-specific, possibly due to mutation in intron 10 to generate a new splice acceptor site (3). In this report, we present the first experimental evidence that the alternatively spliced p53 is present also in normal cells of different tissue types.

MATERIALS AND METHODS

Cell culture

The normal epidermal cells of strain 291 were grown in conditioned 'low-calcium' Eagle's minimum essential medium, i.e. modified to contain 0.02–0.04 mM Ca^{2+}, supplemented with 10% fibroblast conditioned medium and 10 ng/ml epidermal growth factor (EGF) to enrich for basal proliferating epidermal cells, or in 'high-calcium' Eagle's medium (1.4 mM Ca^{2+}) with nonessential amino acids, 5% fetal bovine serum and 1% antibiotic-antimycotic (HCEM) for 48 h to enrich for spinous cells committed to terminal differentiation (4, 5). Tumor cell derivatives were grown in HCEM (6). 10T1/2 cells were cultured in Eagle's basal medium supplemented with 10% fetal bovine serum (7). Quiescent 10T1/2 cells (obtained by serum starvation of post-confluent cultures for 4 days) were treated with EGF (20 ng/ml) for 30 min, at which time maximum induction of c-*fos* and c-*myc* expression was achieved (7), and harvested for RNA extraction.

Polymerase chain reaction (PCR) and sequencing

RNA was isolated from cultured cells or fresh tissue samples by guanidinium/cesium chloride extraction and dissolved in diethylpyrocarbonate-treated water as described previously (5, 8). cDNA was made from RNA isolated from 291 normal, 05RAT or 03RAT tumor cells using AMV Reverse Transcriptase (Promega, Madison, WI) as described (9). 3′ p53 cDNA segment [RS4 (nt 1042 to 1539, +1 being the first nucleotide of the initiation codon ATG] (10) was amplified by PCR with the sense (AGTCGAATTCAAGGATGCCCATGCTACAGA) and the antisense (AGTCGGATCCAGGGTGGGGGGTGGATAAA) primers, as described (9). As denoted by underlining, the primers contained either an EcoRI restriction site (sense primer) or a

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BamHI site (antisense primer) at the 5′ end to increase cloning efficiency. PCR was performed for 35 cycles of denaturation (95°, 30 sec), annealing (60°, 1 min) and extension (72°, 3 min) in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). Amplified fragments were desalted by centricon 100 ultrafiltration (Amicon, Beverly, MA), digested with EcoRI and BamHI, isolated from 3% low melting temperature agarose (FMC, Rockland, ME) using Geneclean II (BIO 101, La Jolla, CA) and cloned into pGEM3zf(+) (Promega) for sequencing of the sense strand of p53 cDNA or pBluescript KS(+) (Stratagene, La Jolla, CA) for the antisense strand, and transfected into *E.coli* strain JM109 (11). Single-stranded DNA isolated after transfection with helper phage R408 (Promega) was used as a template for dideoxynucleotide sequencing with M13(−40) forward (for the sense strand) or T7 (for the antisense strand) primers and [α-^{35}S]dATP (NEN), using Sequenase Version 2.0 (United States Biochemical Co, Cleveland, OH) according to manufacturer's instructions. Three or more clones containing p53 cDNA segments isolated from 291 normal, 05RAT or 03RAT tumor cells were sequenced. The reaction products were loaded on 6% polyacrylamide/8M urea gels and electrophoresis was carried out at 70 watts. Gels were immersed in 15% methanol/5% acetic acid for 45 min, dried at 80°C and exposed to Kodak XAR film.

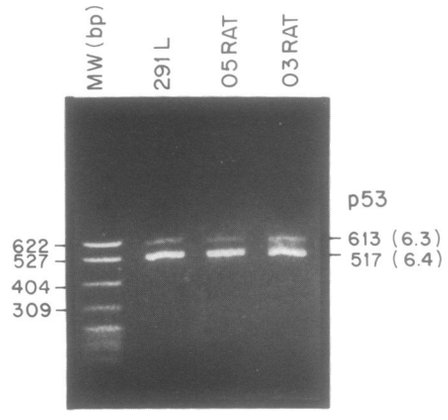

Fig.1. PCR of 3′ segment of cDNA (RS4). p53 cDNAs from each cell line were amplified by PCR as described in materials and methods. Amplified segments were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The molecular weight markers (MspI-digested pBR322) are shown at the left side of the gel (MW). Sequencing analysis revealed that the minor band (AS-p53) was 613 bp and the major band (R) 517 bp. The higher molecular weights of the bands shown on the gel compared to molecular weight markers are due to non-specifically added nucleotides at 3′ ends during the PCR process.

RNase protection assay

The segment RS4 (as noted above) was amplified from RNA isolated from 03RAT cells by reverse transcription and PCR, yielding two distinct bands. Each band was isolated from the gel and cloned into pGEM3zf(+) at the EcoRI/BamHI sites. Double-stranded plasmids, pK6.3 and pK6.4 containing the minor and major RS4 PCR fragments respectively, were linearized with EcoR1 for *in vitro* transcription (12) with 65 μCi of [α-^{32}P]C-TP (800 Ci/mmol, NEN). ^{32}P-labeled RNA probes 6.3 (642 nt), transcribed from pK6.3, were hybridized with 10 or 20 μg of total RNA in 80% formamide/1,4-piperazinediethanesulfonic acid buffer (pH 6.4) at 48° for 16 h and single-stranded RNA was digested with RNase A (Boehringer Mannheim, Indianapolis, IN) and RNase T1 (BRL, Gaithersburg, MD), then precipitated with ethanol (12). Protected RNA was redissolved and resolved by electrophoresis on 4% acrylamide/7M urea gels. Gels were dried and exposed to Kodak XAR film. Quantitation of relative amounts of protected RNA was done by densitometry of exposed film using a Quick Scan (Helena Laboratory, Beaumont, TX) and Fastscan computing densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

When the 3′ end of p53 cDNA was amplified by PCR, 2 distinct bands were generated in cDNA samples from 291 normal or 05RAT and 03RAT carcinoma cells of epidermis (Fig. 1). Sequence analysis of both bands revealed the major band (517

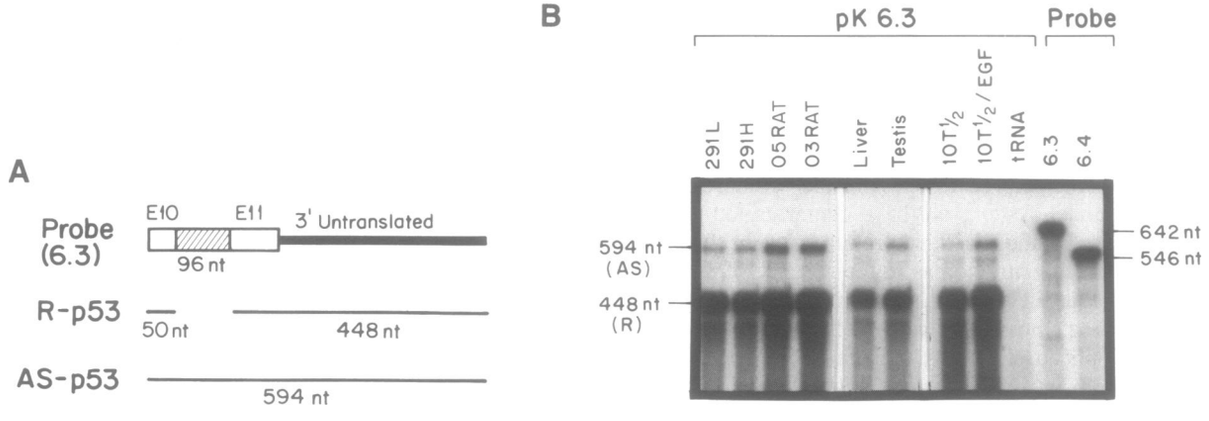

Fig.2. RNase protection analysis of AS-p53 and R-p53 transcripts. **(A)** Scheme of predicted fragments protected by AS-p53 and R-53 after RNase digestion. **(B)** RNase protection analysis: ^{32}P-labeled antisense RNA (6.3) from the plasmid containing the 3′ p53 cDNA segment (pK6.3) was hybridized at 48°C for 16 h with 10 μg of total RNA from normal 291L and 291H cells, 03RAT and 05RAT carcinoma cells, 15 μg from 10T1/2 fibroblast cells and 10T1/2 cells treated with EGF, 20 μg from mouse liver and testis, and 20 μg of yeast tRNA as a control in 80% formamide/1,4-PIPES buffer (pH 6.4) (12). Protected RNA after RNases A and T1 digestion was resolved on a 4% acrylamide/7M urea gel. Untreated riboprobes (6.3 and 6.4) served as molecular weight markers. The sizes of protected RNA are indicated along with the type of splicing (AS or R).

bp) representing the regularly spliced wild-type p53 (10) and the minor higher molecular weight band (613 bp) resulting from alternative splicing at the 3′ end of intron 10, identical to that described previously (3) (data not shown).

The general relevance of this finding and the relative amount of regularly spliced (R) and alternatively spliced (AS) p53 were examined by an RNase protection assay. The minor band (6.3) cloned in a pGEM3zf(+) vector (pK6.3) was used to make an antisense ^{32}P-labeled RNA probe. A scheme showing the predicted fragments protected from RNase digestion by R- and AS-p53 hybridization is shown in Fig. 2A. The proliferating (291L) and differentiating (291H) normal epidermal and carcinoma (05RAT, 03RAT) cells contained both R- and AS-p53 RNA in approximately a 3 to 1 ratio (Fig. 2B). AS-p53 was also present in mouse liver and testis at a similar ratio (Fig. 2B), confirming that AS-p53 is neither a culture artifact nor transformation-specific. It has been reported that the level of p53 RNA is elevated in mouse fibroblast cells in response to mitogenic stimulus such as TPA or serum (13). To investigate whether AS-p53 also responds similarly, quiescent murine 10T1/2 fibroblast cells were stimulated with EGF (20 ng/ml) as described (7) and examined by RNase protection. The levels of R-p53 and AS-p53 were each approximately 2 fold higher in EGF-treated cells compared to untreated 10T1/2 cells (Fig. 2B).

The AS-p53 RNA species are predicted to result in premature termination of p53 protein, making it 9 amino acids shorter and differing in 25 amino acids at the C-terminus. Thus the protein translated from AS-p53 RNA would not be recognized by monoclonal antibody PAb421 which recognizes C-terminal amino acid residues of p53. While immunoprecipition analysis is not strictly quantitative due to multiple factors which can affect the association of p53 with antibody, there appeared to be less p53 protein in complex with PAb421 than with PAb246 antibody, which recognize distinct epitopes (2), consistent with the observed ratio of R-p53 to AS-p53 RNA. Future studies of a distinct AS-p53 protein and its potential biological significance will be facilitated by specific antibody to AS-p53 protein.

DISCUSSION

The results presented in this report indicate that alternative splicing is a universal feature of p53 gene expression in normal cells of different tissue types and not a transformation-specific event. It is likely that the R-p53 and AS-p53 are differentially spliced from common precursors since 03RAT carcinoma cells are monosomic at chromosome 11 where the mouse p53 gene is found. This possibility is supported by the coordinated increase of the R-p53 and AS-p53 levels upon EGF treatment in 10T1/2 cells. This implies that the observed ratio of R-p53 to AS-p53 RNA is maintained post-transcriptionally.

The carboxyl terminus of the protein translated from AS-p53 is predicted to be quite distinct from that from R-p53 by having reduced basic charges, which could influence the secondary structure of p53. Furthermore, differences in the carboxyl terminus between AS-p53 and R-p53 proteins could lead to significant biochemical and biological changes. AS-p53 protein is expected to no longer have the site for RNA linkage (14) or phosphorylation by casein kinase II which may be important for growth stimulation by mitogens (15). It has been demonstrated that the carboxyl terminus of p53 is required for stable oligomer formation *in vitro* (16) between normal and mutant p53, thereby driving wild-type conformation into mutant conformation when the two forms are co-translated (17). Regarding these observations and recent studies demonstrating that wild-type p53 has a dual function both as a positive and negative growth regulator (18–20), more precise biochemical and biological characterizations of AS-p53 protein along with R-p53 protein appear to be critical in future studies of p53 function in normal cells and in oncogenesis.

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