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Molecular Basis for Heterogeneity of the Human p53 Protein

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The human p53 tumor antigen comprises several physically distinct proteins. Two p53 proteins, separable by polyacrylamide gel electrophoresis, are expressed by the human transformed cell line SV-80. The individual cDNAs which code for these proteins were isolated and constructed into the SP6 transcription vector. The proteins encoded by these clones were identified by in vitro transcription with the SP6 vector and translation in a cell-free system. p53-H-1 and p53-H-19 cDNA clones code for the faster- and slower-migrating p53 protein species, respectively, of SV-80. The in vitro-expressed proteins of p53-H-1 and p53-H-19 had the same antigenic determinants and were structurally indistinguishable from their in vivo counterparts. By expressing defined restricted cDNA fragments in vitro, the region of heterogeneity between the respective cDNAs was located at the 5′ end of the cDNAs. Exchanging the 5′ fragments of interest and expressing the chimeric clones in vitro confirmed that the DNA heterogeneity was responsible for the difference in the electrophoretic mobility of these proteins. The sequences of the two cDNAs revealed a single base pair difference (G versus C) in the coding region of the clones. This sequence difference resulted in an arginine being coded for in clone p53-H-1 and a proline being coded for at the equivalent position in clone p53-H-19. This variation accounted for the change in the electrophoretic mobility of the individual p53 protein species.

The cellular protein p53 is expressed at low levels in nontransformed cells. When quiescent cultures of such cells are stimulated to proliferate, the amount of p53 is elevated transiently (19, 21–23, 28). In contrast, various types of transformed cells express the protein at elevated levels constitutively (3, 4, 9, 12, 16, 29, 30), which suggested that the protein is involved in transformation.

p53 was shown to be a transforming protein by using the L12 nonproducer murine cell line (32, 33, 36). These cells induce tumors in syngeneic hosts, which subsequently regress. When p53 expression was reconstituted in the cells by the transfection of a functional p53 gene, it induced lethal tumors in the hosts (32, 33). p53 was therefore necessary for expression of the fully transformed phenotype of the cell line. The protein was also shown to complement an activated Ha-*ras* gene in the tumorigenic conversion of rat embryo fibroblasts (5, 25) and rat adult chondrocytes (10), demonstrating another facet of the oncogenic character of p53.

p53 expression is also enhanced in a number of human transformed cell lines. A salient feature of these cell lines is that they express more than one discrete p53 protein (3). When human primary tumors were screened for p53 with anti-p53 monoclonal antibodies, they too were found to contain several p53 species (27). The study of human p53 has been facilitated by the cloning of human cDNAs (7, 17, 34, 36, 37). Analysis of human genomic DNA with these p53 cDNAs revealed a single p53 gene (7, 17, 36, 37). It was therefore of interest to study the molecular mechanism by which the single p53 gene codes for more than one p53 protein.

As the mature eucaryotic cell mRNA is invariably translated into a single polypeptide chain, it was expected that the p53 protein heterogeneity may be controlled by either transcriptional or posttranslational modifications.

A cDNA library derived from the SV-80 cell line, which expresses in vivo two discrete forms, was therefore screened to isolate the individual cDNAs, each of which encodes one of the two p53 proteins. Sequence analysis of cDNA clones coding for each of the corresponding two p53 proteins revealed a single base pair difference between the two cDNAs which accounted for the protein heterogeneity. The expression of multiple p53 species is probably a consequence of gene polymorphism.

MATERIALS AND METHODS

Cells. Human cell lines were grown in RPMI 1640 medium enriched with 10% heat-inactivated fetal calf serum (Biolab, Israel). Hybridoma cell lines producing anti-p53 antibodies were grown in RPMI 1640 medium enriched with 20% heat-inactivated fetal calf serum supplemented with 20 mM L-glutamine and 20 mM sodium pyruvate.

Antibodies. Monoclonal anti-p53 antibodies were obtained from the established hybridoma cell lines PAb122 (6) and PAb421 (8). Monoclonal antibodies were obtained from supernatants of the hybridoma cell lines or from ascitic fluid of syngeneic mice injected intraperitoneally with the hybridoma cell lines. Antibodies were purified and concentrated by binding to Sepharose-protein A columns (Sigma Chemical Co., St. Louis, Mo.).

In vitro transcription-translation assay. The pSP65 plasmids containing the p53 inserts were linearized by *Hin*dIII or restricted at unique sites within the cDNA and extracted with phenol prior to precipitation. Approximately 1 μg of the linearized plasmid was transcribed by SP6 RNA polymerase (Anglian, Biotech) (34). A fraction of the reaction mixture was used directly for translation in a rabbit reticulocyte lysate system (Anglian, Biotech) (26).

Immunoprecipitation of p53 protein. A monolayer of SV-80 cells at the logarithmic stage of growth was washed several times in phosphate-buffered saline and 1.5 ml of Dulbecco modified Eagle medium without methionine, enriched with 10% dialyzed heat-inactivated fetal calf serum, and 250 μCi

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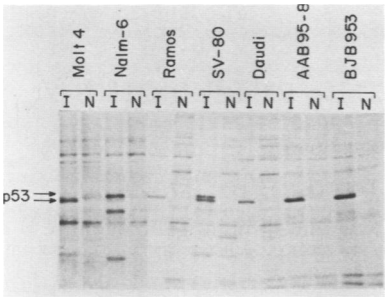

FIG. 1. In vivo expression of two p53 protein species. Equal amounts of TCA-insoluble protein, obtained from various human cell lines, metabolically labeled with [^{35}S]methionine, were immunoprecipitated with anti-p53 monoclonal antibody PAb421 (lanes I) or with normal serum (lanes N). The arrows point to the faster- and slower-migrating p53 proteins.

of [^{35}S]methionine (Amersham) was added. Cells were incubated for 1 h at 37°C, washed in phosphate-buffered saline, and extracted into 2 ml of lysis buffer (10 mM Na_2HPO_4-NaH_2PO_4 [pH 7.5], 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) at 4°C. Labeled cell lysates were precleared by repeated absorption on *Staphylococcus aureus* and nonimmune serum. Equal amounts of trichloracetic acid (TCA)-insoluble radiolabeled protein were immunoprecipitated with anti-p53-specific antibodies. Antigen-antibody complexes were collected on *S. aureus* cells (11). SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (14).

Two-dimensional PAGE. The protein sample was denatured with nonequilibrium pH gradient electrophoresis (NEPHGE) sample buffer (8 M urea, 2% Nonidet P-40, 0.4% Ampholine [LKB], pH 3.5 to 10, and 5% β-mercaptoethanol). The sample was then loaded on a NEPHGE tube gel as described by O'Farrell et al. (24). NEPHGE was performed for 6 h at 500 V. The gel was extruded from the tube, equilibrated for 2 h in SDS sample buffer, and layered on an SDS-polyacrylamide slab gel.

DNA sequencing. DNA sequences of the human cDNAs were determined by the dideoxy sequencing methods of Sanger et al. (31). Synthetic 17-mer oligonucleotide primers were prepared with a synthesizer and used to sequence the cDNAs cloned in the M13 vectors mp8 and mp9. The 17-mer M13 universal primer was used to sequence cDNA fragments (7) subcloned in the M13 vectors. [^{32}P]dATP (Amersham) was used in the sequencing reactions, and the products were analyzed on 6 and 8% polycrylamide sequencing gels.

RESULTS

In vivo expression of two p53 protein species. Human transformed cell lines express p53 species which are distinguished by their rates of migration in polyacrylamide gels. Two p53 species were immunoprecipitated from the SV-80 cell lysate with anti-p53 monoclonal antibodies (Fig. 1). The signal intensities of the respective radiolabeled proteins indicated that they were expressed to the same extent. Nalm-6 cells, by comparison, expressed the slower-migrating species more than the faster-migrating species (Fig. 1). Other cell lines expressed only the slower-(BJB953) or the faster (Molt4)-migrating p53 species. Given that the human cells contained a single p53 gene, the expression of multiple p53 species may be due to transcriptional or post-translational control.

Isolation of p53 cDNA clones encoding two p53 species. If the differential expression of the single p53 gene in SV-80 cells is a result of transcriptional control, it is expected that these cells will express more than one p53 mRNA species, and thus, a cDNA library should contain all of the representative cDNA clones. A λgt10 SV-80 cDNA library (34, 36) was therefore screened for p53 cDNAs which code for each of the two p53 products with a full-length p53 cDNA (p53-H-7) isolated previously (36). The various p53 cDNA clones were subcloned in the transcription vector pSP65 and characterized by in vitro transcription and translation in a cell-free reticulocyte cell lysate (26). Clones p53-H-1, p53-H-7, and p53-H-8, although each of different overall size, directed the synthesis of the faster-migrating p53 protein found in SV-80 cells. Clone p53-H-19 coded for the slower-migrating p53 species (Fig. 2). By comparing the migration of the in vitro-synthesized products with the in vivo products, it appeared that each of the cDNAs contained a complete coding region. The disparity in migration rates of the in vivo proteins was maintained in the in vitro system.

Structural comparison of the SV-80 p53 proteins. The cDNA-encoded products were the major protein species expressed in the in vitro transcription-translation system. Both protein species were immunoprecipitated with the anti-p53 monoclonal antibodies PAb421 (Fig. 3A, lane I), as were the in vivo-labeled controls (Fig. 3A, lane I SV-80). Smaller proteins were also found to be immunoprecipitated with the anti-p53 monoclonal antibodies. These proteins, which constituted a minor fraction of the translation products, are thought to be encoded by shorter mRNA produced by inefficient in vitro transcription or by premature termination occurring in vitro (Fig. 3A).

Two-dimensional PAGE analysis of the in vitro-synthesized proteins showed that each of the in vitro-synthesized p53 proteins was composed of an identical complement of subspecies with the same relative isoelectric points (Fig. 3B). The results of the two-dimensional PAGE analysis were identical to those obtained with the in vivo-synthesized proteins (data not shown).

A comparison of the partial proteolytic peptide maps of the SV-80 and cDNA-encoded proteins showed how the in vivo and in vitro translation products were related (Fig. 4). The partial peptide maps were obtained by digesting the SV-80 p53 doublet and the individual cDNA products with increasing concentrations of *S. aureus* V-8 protease (Fig. 4, lanes b to d). The in vitro-synthesized proteins gave different partial peptide maps which, when combined, constituted the partial peptide map of the native p53 protein pair of transformed SV-80 cells. p53 derived from transformed human Daudi cells migrated with the p53-H-1 cDNA-encoded species and gave a partial peptide map identical to that of the in vitro-synthesized product. On the basis of these structural analyses, therefore, the individual cDNA protein products were indistinguishable from their in vivo-synthesized counterparts.

The difference in migration of these p53 species was thought to reflect a structural difference in the coding regions of their respective cDNAs. To locate the region of heterogeneity between the cDNA clones, the in vitro transcription-translation assay was used to generate progressively smaller translation products. Figure 5 shows the truncated protein products resulting from restriction of the cDNA clones p53-H-1 and p53-H-19 within the coding region. The endonuclease *Hin*dIII cut only within the SP65 vector sequence,

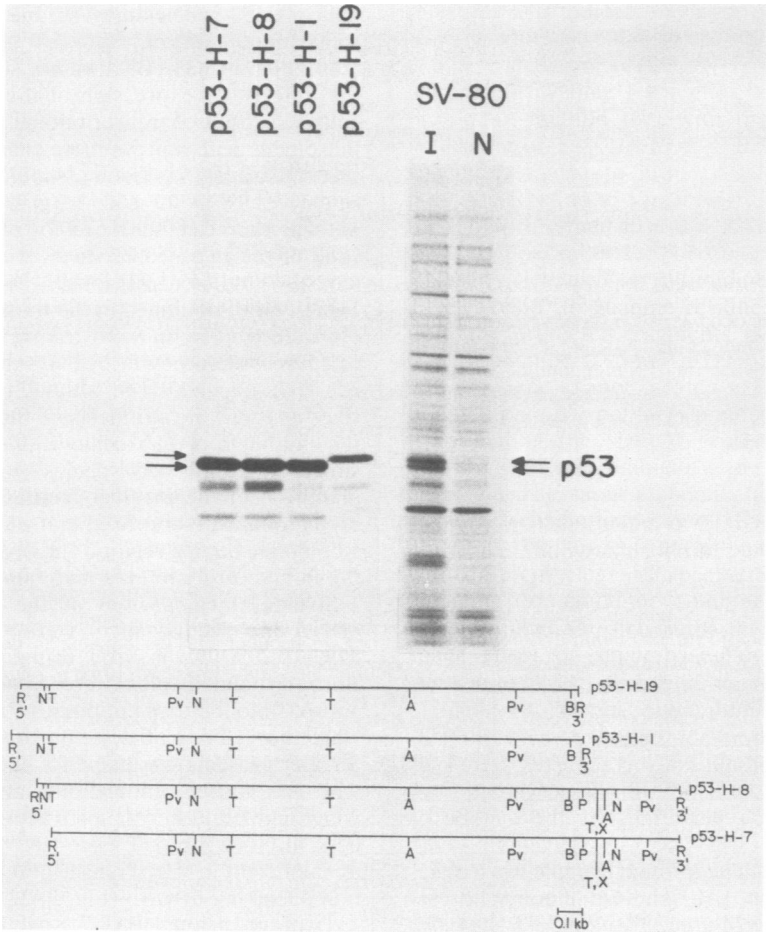

FIG. 2. Isolation of p53 cDNA clones expressing two p53 species. Restriction maps of four individual p53 cDNA clones are illustrated. Abbreviations: R, *Eco*RI; N, *Nco*I; T, *Taq*I; Pv, *Pvu*II; A, *Ava*II; B, *Bam*HI; P, *Pst*I; X, *Xho*I. The four cDNA clones containing *Eco*RI polylinkers were subcloned into the *Eco*RI site of the pSP65 transcription vector. The plasmids were linearized by *Hin*dIII digestion, transcribed in vitro, and then translated in the reticulocyte cell-free lysate in the presence of [^{35}S]methionine. Portions of radioactively labeled proteins of each group were separated on an SDS-polyacrylamide gel. In vivo-labeled transformed SV-80 fibroblasts were immunoprecipitated with specific anti-p53 monoclonal antibody PAb421 (lane I) or normal serum (lane N).

leaving intact the p53 coding sequences. *Stu*I cut at the 3′ end, and *Pvu*II cut at the 5′ end of the cDNA insert (Fig. 2). The size of the protein product was progressively reduced as restriction proceeded from the 3′ to the 5′ end of the cDNA insert, but the relative difference in migration rates was maintained (Fig. 5). The heterogeneity between the cDNAs was therefore understood to lie upstream from the *Pvu*II restriction site. To confirm this, the 5′ *Nco*I-*Nco*I fragments (400 base pairs [bp]) of the respective cDNAs were exchanged (Fig. 6). The result was that the p53-H-19 hybrid, containing the *Nco*I-*Nco*I fragment of clone p53-H-1, expressed the faster-migrating p53 species encoded by the parental p53-H-1 cDNA clone. The heterogeneity between the cDNAs was located, therefore, in the the 5′ coding region.

DNA sequence of two cDNAs expressing the two p53 protein species. The sequences of cDNAs p53-H-1 and p53-H-19, which had the same size and restriction map but coded for different p53 species, were determined to elucidate the heterogeneity between their translation products. The restriction map of both cDNAs and the sequencing strategy are shown in Fig. 7. The entire cDNA, contained in a 2.3-kilobase (kb) *Eco*RI fragment, and the 600- and 300-bp *Taq*I fragments were subcloned separately in the M13 vectors mp8 and mp9 (20). The 17-mer M13 universal primer and 17-mer synthesized oligonucleotides homologous to internal stretches of the cloned fragments were used to determine the cDNA sequences by the dideoxy method of Sanger et al. (31). The coding and 5′ noncoding sequences of each cDNA are shown in Fig. 8. In the 5′ noncoding region, p53-H-19 was shorter than p53-H-1 by 3 bases. A continuous reading frame extended for 393 codons in both cDNAs. Translation was presumed to begin at base 1 (the first ATG after base −125). A proposed consensus sequence for eucaryotic initiation sites, CTGCCATGG (13), suggested that translation was initiated from the same proximal ATG in each clone. The reading frame of each cDNA was terminated by a stop codon (TGA) at base 1180. It was found that the DNA sequences of the respective reading frames were identical but for a single change at base 215. At this point p53-H-1 had a guanine, whereas p53-H-19 had a cytidine. This guanine in clone p53-H-1 generated an *Fnu*DII restriction site (recognition sequence, CGCG) which was absent from clone p53-H-19. This difference was exploited to corroborate the sequence data by restriction analysis (data not shown). The predicted amino acid sequences of the open reading frames

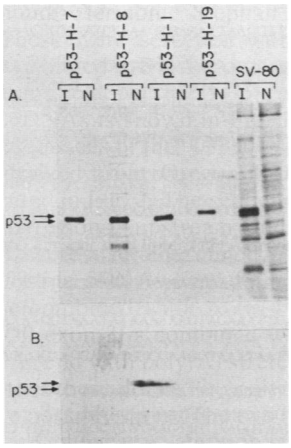

FIG. 3. Immunoprecipitation of the in vitro-transcribed and -translated p53 proteins by specific monoclonal antibodies. (A) [^{35}S]methionine-labeled proteins obtained from the various p53 cDNA clones (p53-H-7, p53-H-8, p53-H-1, and p53-H-19) generated by the in vitro transcription-translation assay were immunoprecipitated with specific anti-p53 monoclonal antibody PAb421 (lanes I) or normal serum (lanes N). (B) Immunoprecipitated proteins obtained from clones p53-H-19 and p53-H-1, representing the slower- and faster-migrating proteins, respectively, were subjected to two-dimensional PAGE analysis. The arrows point to the p53 proteins.

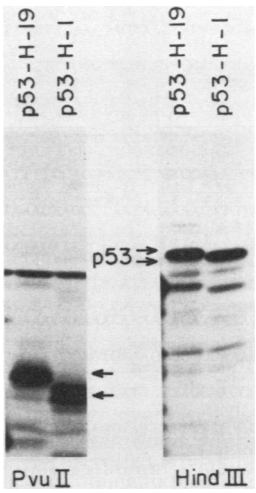

FIG. 5. Mapping the region of heterogeneity between the slower- and faster-migrating p53 species. The p53 proteins generated by p53-H-19 and p53-H-1 represent the slower- and faster-migrating proteins, respectively. The full-length proteins were generated when the p53-SP65 plasmids were linearized with *Hin*dIII. *Pvu*II yielded, as expected (see Fig. 2), smaller-sized proteins. The arrows point to the full-length and truncated p53 proteins obtained.

are also shown in Fig. 8. This change resulted in an arginine being expressed by clone p53-H-1 and a proline by clone p53-H-19. The calculated molecular weights were 43,739, and 43,680, respectively. This difference could not account for the variation in electrophoretic mobility of these proteins.

Whereas a single base pair difference distinguished p53-H-1 from p53-H-19, two apparently nonsignificant base differences in the reading frames of both cDNAs compared with the published sequences were found. In p53-H-1 bases 234 and 235 were guanine and adenine, respectively. The equivalent bases in the published sequences (7, 37) were adenine and guanine, respectively. The consequence of these base differences is that p53-H-1 and p53-H-19 expressed an alanine followed by a threonine, whereas the published sequences coded for two consecutive alanines. In p53-H-1, base 818 was a guanine and the equivalent base in the published sequence was an adenine. p53-H-1 and p53-H-19 therefore expressed an arginine, whereas the published sequences coded for a histidine. The differences between the sequences presented here and the published sequences resulted in conservative changes in the respective p53 translation products and therefore were thought not to be significant with respect to the physical behavior or function of the proteins.

By comparing the sequences presented in this report with

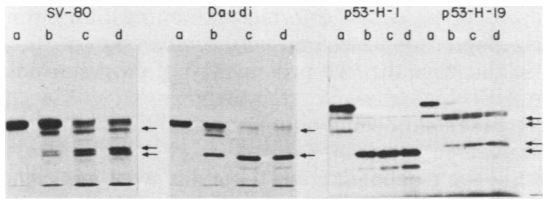

FIG. 4. Comparison of the partial proteolytic peptide maps of the in vivo- and in vitro-synthesized protein species. The partial peptide maps of p53 proteins from the following sources were compared: SV-80 transformed fibroblasts, which express the two p53 species in vivo; clones p53-H-19 and p53-H-1, which express in vitro the slower- and faster-migrating species, respectively; and the human transformed lymphoid cell line Daudi, which expresses the faster-migrating species in vivo. Each of the p53 species was radiolabeled with [^{35}S]methionine and immunoprecipated with the human-specific anti-p53 monoclonal antibody PAb421. The proteins were digested with increasing amounts of *S. aureus* V-8 protease: lanes a, no enzyme; lanes b, 1 μg; lanes c, 5 μg; lanes d, 10 μg. The arrows point to comigrating partially digested fragments generated from the various p53 proteins.

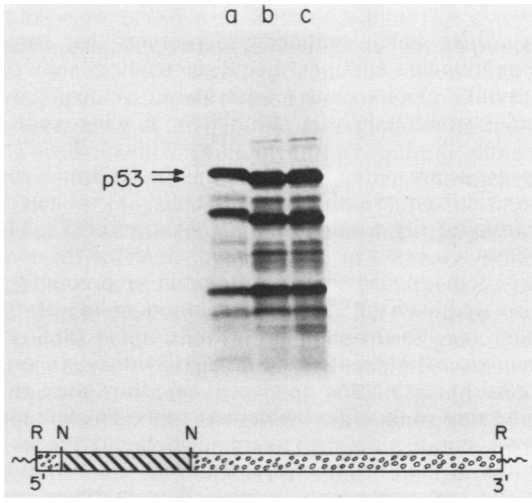

FIG. 6. Fragment exchange between p53-H-1 and p53-H-19 cDNA clones. The 5′ *Nco*I-*Nco*I 400-bp fragment (striped box) of the respective cDNA p53 clones was exchanged. The stippled region represents the remainder of the coding sequences. Abbreviations: R, *Eco*RI; N, *Nco*I. Lanes: Protein encoded by the intact p53-H-19 parental plasmid (lane a), protein encoded by the intact p53-H-1 parental plasmid (lane b), and protein encoded by a chimeric hybrid containing the *Nco*I-*Nco*I fragment of p53-H-1 and the remainder of the coding sequences from p53-H-19 (lane c).

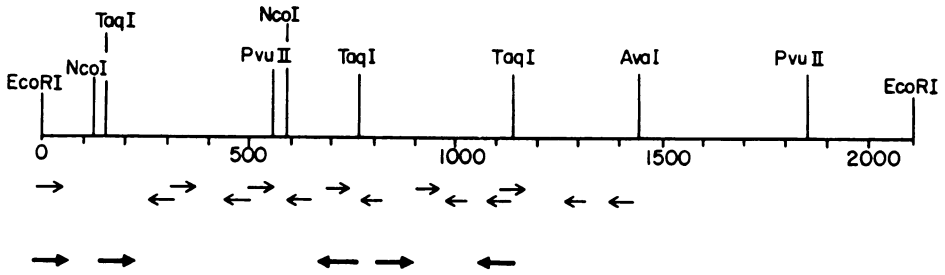

FIG. 7. Restriction enzyme map and sequencing strategy of human cDNAs p53-H-1 and p53-H-19. The entire cDNA, contained in the *Eco*RI fragment, was cloned in the M13 vector mp8. The two *Taq*I fragments were subcloned separately in the M13 vectors mp8 and mp9. The small arrows represent 17-mer synthetic primers, homologous to short regions of the cDNA. The large arrows represent the M13 universal primer, which is complementary to M13 vector DNA flanking the insertion site. The scale is shown in bases.

the published sequences (7, 37), it was evident that the translation products of the latter were exclusively the larger p53 species, equivalent to the protein encoded by p53-H-19. At the equivalent position in p53-H-19, the published sequences also coded for a proline.

DISCUSSION

The p53 gene product was shown to be associated with several biological functions. It was clearly demonstrated that p53 is an oncogene product which is directly involved in the process of malignant transformation (5, 10, 25, 32, 33). In addition, p53 was proven to constitute an essential cell cycle-dependent protein (19, 21, 22, 28). Both in the mouse and in the human genome, p53 was found to be encoded by a single p53 gene (7, 17, 34, 36, 38).

Our present studies on expression of the human p53 gene as well as our recent observations on expression of the p53 gene in mouse cells (1) strongly suggest that heterogeneity in the p53 protein population is mediated by the existence of several p53 transcripts. Recently, we observed that the mouse p53 is composed of more than one p53 protein species. By screening a mouse cDNA library we isolated p53 cDNA clones which encoded p53 proteins that contained different antigenic epitopes. Sequence analysis showed that these mouse cDNA clones represented authentic mRNA molecules generated by an alternative splicing mechanism (1).

The results presented here show that in the human genome there are at least two physically discrete p53 species which are translated from individual mRNA transcripts. Human p53 cDNA clones which expressed either of the two p53 proteins, indistinguishable from their in vivo counterparts, were isolated from an SV-80 library. Immunological studies, one- and two-dimensional SDS-PAGE, and partial proteolytic analyses showed that the in vitro-synthesized proteins were closely related and confirmed that they were genuine reproductions of their in vivo counterparts. The fact that the in vitro-generated proteins were identical to the in vivo-expressed protein strongly suggests that the variation between the two proteins is controlled by a transcriptional mechanism without further posttranslational modification.

Nucleic acid analysis indicated that these two p53 species varied in a single nucleic acid (G versus C), coding for a proline in one and arginine in the other. This may be a result of gene polymorphism, by which one p53 allele codes for one p53 species and the other, which contains another nucleic acid, codes for the second form. Arginine, which was expressed in the faster-migrating species (clone p53-H-1 base 215), has a large side chain and is positively charged in physiological conditions. In contrast, proline, the equivalent residue expressed by the slower-migrating protein (clone p53-H-19), has a small, nonpolar side chain. It is therefore possible that the tertiary structures of the respective proteins are altered, with attendant changes in detergent binding and charge-to-mass ratios. Another explanation, which is consistent with the discrepancy between the predicted size of p53 and its apparent size as determined from its electrophoretic mobility, was based on the relatively high proline content of the protein (2, 37). The distinguishing proline residue of clone p53-H-19 has a secondary rather than primary amino group. This almost rigid side chain of proline may be a factor in the slower migration of p53-H-19 than of p53-H-1.

This observation of a single nucleotide change was given further support by results from other laboratories. Lamb and Crawford (15) recently characterized a genomic p53 clone isolated from human liver DNA that has a G at the position equivalent to nucleotide 215 in p53-H-1, which encoded an arginine. In contrast, p53 cDNA clones isolated from an A431 cDNA library by Harlow et al. (7) and p53 cDNA isolated by Zakut-Houri et al. (37) contained at the same position a C and thus, like p53-H-19, code for a proline. In view of these findings, we infer that this heterogeneity in the various p53 clones represents an authentic variation in the p53 mRNA population.

A single amino acid change between protein species accounting for variations in their apparent molecular weights was also shown in other systems. Two classes of the dihydrofolate reductase enzymes exhibiting different molecular sizes were found to represent a conversion of a Leu into Phe. In this case the two proteins are of different biological nature (18).

Very little is known about the biochemical activity of p53 in transformed and nontransformed cells. If a critical determinant of the biochemical function of a protein is its conformation, the protein products of p53-H-1 and p53-H-19 could arguably represent different functional forms of the protein. The arginine residue which distinguishes p53-H-1 has a large, charged side chain, which is frequently found in the catalytic sites of enzymes or is itself the target for enzyme modification and cleavage. The side chain of proline, which occupied the equivalent position in p53-H-19, forms a relatively inert, closed circular structure. Consistent with this line of thought, the heterogeneity between the p53 species was found in a region previously credited with functional importance, albeit on a theoretical basis (2, 37).

Further studies are under way to establish the biological significance of the two p53 species.

FIG. 8. Nucleotide sequences of the upstream noncoding and coding regions of human p53 cDNA clones p53-H-1 and p53-H-19. The sequences shown were determined by the dideoxy method of Sanger et al. (31). The DNA sequence and corresponding amino acid sequence of p53-H-1 are shown in full. The DNA and amino acid sequences of p53-H-19 which are identical to those of p53-H-1 are represented as dots. The base pairs and the single amino acid which differ in p53-H-19 are indicated (boxed). The nucleotide sequence is numbered so that the first base of the ATG start codon is number 1.

ACKNOWLEDGMENTS

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