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Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene

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A cDNA clone for human p53 cellular tumor antigen has been isolated and characterized. This clone contains the complete 3'-untranslated region and most of the open reading frame for the protein. Nucleotide sequence analysis revealed that p53 mRNA contains an Alu repeat in the 3'-untranslated region. Hybridization selection experiments showed this clone was capable of selectively binding p53 mRNA. *In vitro* translation of SV80 mRNA resulted in the synthesis of two immunoreactive p53 polypeptide species. Northern blot analysis showed that human p53 mRNA was 2.8 kb in length and was present in cell lines containing high and low levels of p53 protein. There appears to be only a single p53 gene in human cells and Southern blot analysis demonstrated no major genomic rearrangements or amplification of the p53 gene in the transformed cell lines examined.

Key words: Alu repeats/cDNA clone/human/mRNA/p53

Introduction

A variety of transformed cell lines and tumours of primate and murine origin have elevated levels of the cellular phosphoprotein p53 (for review, see Crawford, 1983). This protein is also found in normal primate and murine fibroblasts (Benchimol *et al.*, 1982; Chen *et al.*, 1983; Thomas *et al.*, 1983). However, the levels of p53 protein in these non-transformed cells are ~100-fold less than in their corresponding transformed cells (Benchimol *et al.*, 1982; Thomas *et al.*, 1983). Several human transformed cell lines such as HeLa and EJ contain little or no detectable p53 (Benchimol *et al.*, 1982; Thomas *et al.*, 1983). Thus, although p53 levels are generally elevated in transformed cells, this alone cannot be considered as a marker for transformation.

In cells transformed by SV40 or adenovirus, p53 is specifically associated with the viral tumour antigens, SV40 large T and adenovirus Elb 58k protein (Lane and Crawford, 1979; Linzer and Levine, 1979; McCormick and Harlow, 1980; Sarnow *et al.*, 1982). These physical associations increase the half life of p53 from ~15 min to >20 h, thus elevating the cellular concentration of p53 (Oren *et al.*, 1981). The association of p53 with viral proteins necessary for transformation suggests a role for p53 in the transformation process. The precise function of p53 in either normal or transformed cells is not known, but the evidence suggests that p53 plays a role in regulating cell division, probably acting during the entry into the cell cycle (G_1) from the resting state (G_0) (Milner and Milner, 1981; Mercer *et al.*, 1982, 1984; Milner, 1984; Reich and Levine, 1984). Therefore, altering the expression of such a protein may disrupt the normal regulation of cell division.

Although a considerable amount is known about the expression of p53 mRNA and the genomic organization of p53 genes in mouse cells (Oren and Levine, 1983; Oren *et al.*, 1983; Zakut-Houri *et al.*, 1983; Pennica *et al.*, 1984; Benchimol *et al.*, 1984a), much less is known about human p53. This is primarily because mouse p53 cDNA cross-reacts poorly with human p53 nucleotide sequences. This paper reports the isolation and characterization of a cDNA clone specific for human p53. This cDNA clone contains the entire 3'-untranslated region and most of the open reading frame coding for the p53 protein. Using the cDNA clone as a probe, we have inititated a study of the expression of the human p53 gene. In addition, evidence obtained by *in vitro* translation and Northern blot analysis is presented, indicating that p53 mRNA in human SV80 cells exists as two distinct species. The human p53 cDNA clone has also been used to compare the genomic organization of the p53 gene in transformed and normal cells.

Results

Approximately 10^6 colonies of a human cDNA library (Okayama and Berg, 1983) were grown on nitrocellulose and prepared for hybridization to nick-translated insert from plasmids HU7-1 and HU2-6. Plasmids HU7-1 and HU2-6 contain portions of the human p53 genomic coding region free of repetitive sequences (P. Lamb, in preparation). Eight reproducibly hybridizing colonies were picked, colony purified and grown to prepare large amounts of plasmid DNA.

The plasmid vector used in the construction of the library is such that the cDNA insert can be cut out with *Xho*I. In all cases, digestion of the purified plasmid DNAs with *Xho*I yielded two fragments indicative of an internal *Xho*I site in the cDNA. The plasmid with the longest insert (designated p102) was chosen for further characterization by nucleotide sequencing. The large *Xho*I fragment from plasmid 102 is 1.9 kb long and contains ~200 bp of vector DNA at the 5' end. The smaller *Xho*I fragment is 0.6 kb in length and contains ~100 bp of vector DNA at the 3' end.

A restriction map and sequencing strategy of the cDNA insert of plasmid 102 is shown in Figure 1 together with the location of the p53-specific genomic DNA fragments used as hybridization probes to screen the library. As expected, complete nucleotide sequence homology was found between the indicated genomic fragments and the corresponding cDNA fragments (data not shown). Most important, extensive sequence homology with a mouse p53 cDNA clone (Benchimol *et al.*, 1984a) was observed, confirming the identity and p53 specificity of plasmid 102 (Figure 2).

A single open reading frame was located which extended for 881 nucleotides from the 5' end, sufficient to code for 293 amino acids (Figure 2). In comparison with mouse cDNA, which codes for 390 amino acids, we would calculate that

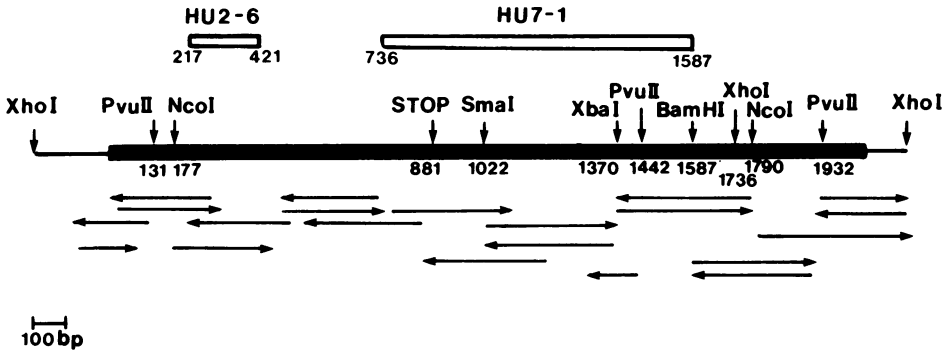

Fig. 1. Restriction enzyme map and sequencing strategy of human p53 cDNA 102. The solid thick line represents the cDNA and the solid thin line the pcD vector sequences. The upper open boxes show the position of the corresponding genomic fragments labelled HU2-6 and HU7-1 used to screen the library.

~300 bp at the 5′ end of the open reading frame are not present in plasmid 102. The nucleotide homology between the human and mouse open reading frame is 83% whereas the inferred amino acid sequence homology in this region is 84%. Many of the amino acid differences between mouse and human are conservative changes. Thus the overall conformation of most of the protein would be expected to be similar.

The 3′-untranslated region of human p53 mRNA is different from the mouse with regards to both nucleotide sequence and length. The length of the 3′-untranslated region of human p53 mRNA, as determined from the cDNA sequence, is ~1.2 kb. The corresponding region in mouse p53 mRNA is 400 bp in length (Zakut-Houri *et al.*, 1983; Pennica *et al.*, 1984). There are two small conserved regions within the 3′-untranslated region between nucleotides 1448 and 1570 (74% homology with mouse) and from nucleotide 1937 through to the polyadenylation signal at nucleotide 2056 (67% homology with mouse). An Alu repeat sequence was located in the 3′-untranslated region of human p53 mRNA between the two regions of sequence homology to the mouse noted above. The Alu repeat is in the opposite transcriptional orientation to the p53 mRNA. No Alu sequences are present in mouse p53 mRNA (Zakut-Houri *et al.*, 1983; Pennica *et al.*, 1984; Benchimol *et al.*, 1984a).

To determine the size of human p53 mRNA and to compare its size with mouse p53 mRNA, poly(A)$^+$ RNA was fractionated on a glyoxal agarose gel, transferred to Biodyne A nylon membrane and hybridized with the large *Xho*I fragment insert of plasmid 102 and a mouse p53 cDNA clone (Benchimol *et al.*, 1984a). The results shown in Figure 3, lane 1 indicate that human p53 mRNA is 2.8 kb in length. The mobility of mouse p53 mRNA, shown on the same gel (Figure 3, lane 2), corresponds to a length of 2.0 kb in agreement with previous reports (Oren *et al.*, 1983; Reich *et al.*, 1983; Benchimol *et al.*, 1984b). Thus, human p53 mRNA is ~800 bp longer than mouse p53 mRNA primarily as a result of a much larger 3′-untranslated region.

When the washing stringency of a similar Northern blot was reduced, a band of ~500 bases became apparent (Figure 3, lane 3). Since the 3′ end of the large *Xho*I fragment contains ~100 nucleotides of the Alu sequence (see Figure 1 and 2), this band probably represents Alu RNA polymerase III transcripts which are about the same size in human cells and may contain A-rich regions capable of binding to oligo(dT)-cellulose (Jelinek and Schmid, 1982).

We also find that p53 mRNA from human SV80 cells consists of two species that can be resolved by agarose gel electrophoresis (Figure 3, lanes 1 and 3). When the p53 protein from SV80 cells is fractionated on an SDS-polyacrylamide gel following metabolic labelling and immunoprecipitation, two polypeptides are also observed (Crawford *et al.*, 1981). The nature of the p53 protein doublet observed in many human cells is not understood. It is tempting to speculate that each polypeptide is encoded by one of the mRNA species we observe. To pursue this idea further, plasmid 102 was immobilized on a nitrocellulose filter and used to hybrid select p53 mRNA. Purified p53 plasmid 102 and a control plasmid containing no insert (pAT153) were bound to nitrocellulose filters, and used to select mRNA from total human SV80 poly(A)$^+$ mRNA. The filters were washed and the RNA eluted by boiling. Eluted RNA was translated *in vitro* in a rabbit reticulocyte lysate containing [^{35}S]methionine, followed by immunoprecipitation with anti-p53 serum. The results presented in Figure 4 (lane 3) indicate that plasmid 102 specifically selected mRNA, when translated *in vitro* followed by immunoprecipitation with immune serum, gave a doublet of ~53 000 daltons.

We conclude that plasmid 102 is capable of hybrid selecting p53 mRNA that encodes both polypeptide species of the p53 doublet. Because post-translational modifications are not known to occur normally in rabbit reticulocyte lysates, we favour the hypothesis that each polypeptide is encoded separately by one of the two mRNA species which are both capable of specifically hybridizing to plasmid 102. We have re-examined the seven other plasmid clones by restriction enzyme mapping and partial sequence analysis and find no gross differences that might indicate the presence of a second p53 mRNA species in our collection of cDNA clones. Clearly, the nature and significance of the two p53 polypeptides and two p53 RNA species remain to be resolved.

We have used insert derived from plasmid 102 as a nucleic acid hybridization probe to detect p53 mRNA in a variety of human transformed cells. Messenger RNA was isolated from SV80, C33I and Raji cells which all contain high levels of p53 protein and from HeLa cells which contain no detectable p53 (Benchimol *et al.*, 1982; Thomas *et al.*, 1983). Northern blot analysis (Figure 5, panel A) shows that p53 mRNA was present in all the cell lines. The SV80 p53 mRNA (lane 1) appears as a single band on this gel which was run for a shorter time than that shown in Figure 3. HeLa cells, which contain no detectable p53 protein, do contain p53 mRNA, (Figure 5, lane 4) although it may be present at a lower level than in the other cell lines. *In vitro* translation of HeLa and SV80 mRNA followed by immunoprecipitation with anti-p53 serum was performed to determine whether HeLa mRNA could be translated into p53 protein. Anti-p53 serum specifically

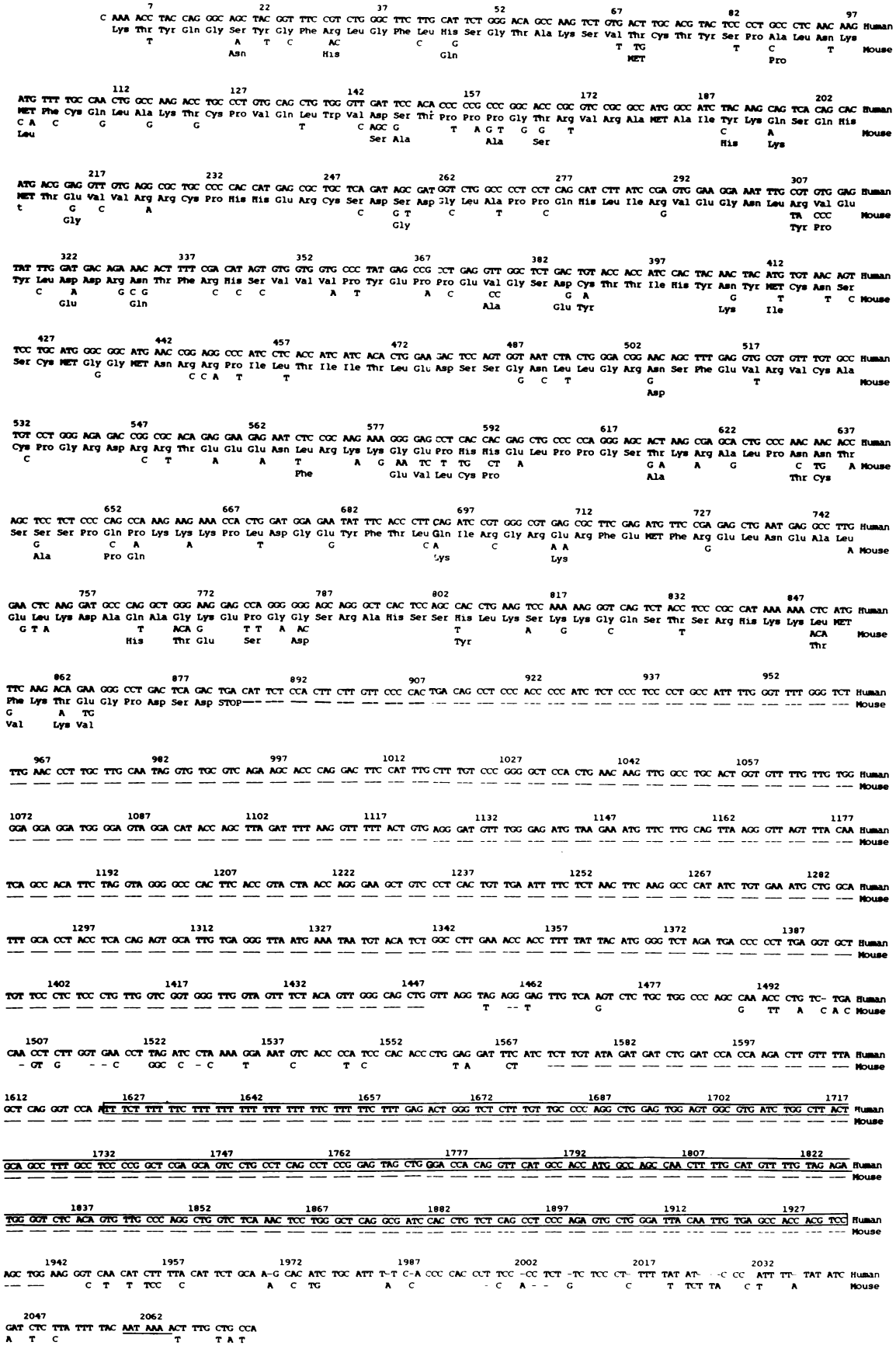

Fig. 2. Nucleotide sequence of human p53 cDNA. The nucleotide sequence of the p53 cDNA is given in parallel with the amino acid sequence predicted from the open reading frame. Nucleotide and amino acid differences between the human p53 cDNA and the comparable mouse p53 cDNA (shown below the human p53 cDNA sequence) are noted. The numbers used indicate the positions in the human p53 cDNA. Hyphens denote the sequences which are not in mouse or human p53 cDNA. The boxed region contains the Alu repeat sequences. The AATAAA polyadenylation signal sequence is underlined.

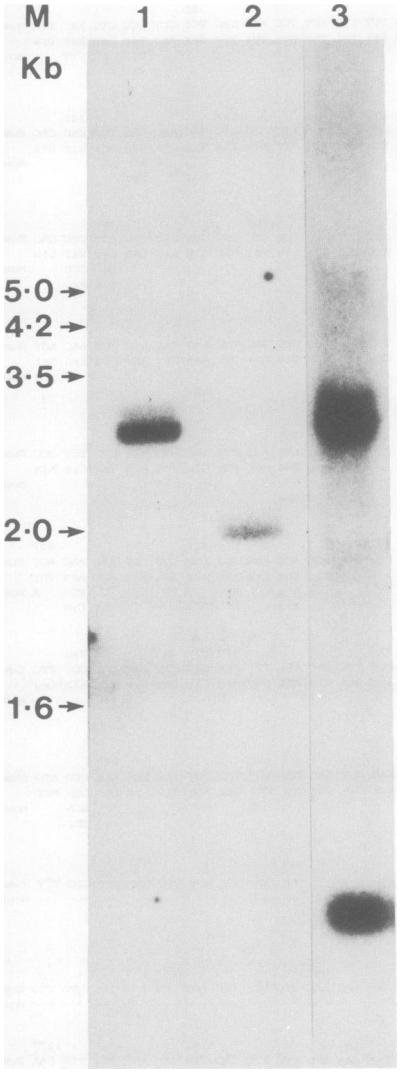

Fig. 3. Northern blot analysis of human and mouse p53-specific mRNAs. The filter was hybridized simultaneously with radiolabelled large *Xho*I-*Xho*I fragment (Figure 1) and a plasmid containing mouse-specific p53 cDNA (Benchimol *et al.*, 1984a). **Lane 1** and **2**, 10 μg of human SV80 and 10 μg of mouse SVA31E7 poly(A)$^+$ mRNA, respectively. **Lane 3**, 10 μg of SV80 poly(A)$^+$ mRNA hybridized with radiolabelled large *Xho*I-*Xho*I fragment and washed using reduced stringency conditions. **Lane M**, *Hind*III and *Eco*RI digested lambda DNA fragments were run on the same gel and stained with ethidium bromide to act as mol. wt. markers.

precipitated p53 from both SV80 and HeLa *in vitro* translation products (Figure 5, panel B, lanes 7 and 9) whereas the non-immune serum did not (lanes 6 and 8). As an internal control, monoclonal antibody PAb419 was used to immunoprecipitate SV40 large-T antigen from SV80 translation products. This confirms that HeLa p53 mRNA is translationally functional.

Southern blot analysis was performed on genomic DNA isolated from normal foetal liver tissue, SV80, Raji, C33I and HeLa cells to establish if p53 genes are rearranged or amplified in these human transformed cells. The DNA samples were cut with *Eco*RI or *Pvu*II, the fragments separated by electrophoresis and then blotted onto nitrocellulose filters. Blots were hybridized with the *Nco*I/*Xho*I fragment located between nucleotides 177 and 1736 of plasmid 102 (Figure 1). This fragment was chosen because it contained no GC polylinker or SV40 DNA sequences although it does contain some Alu sequence. Under high stringency

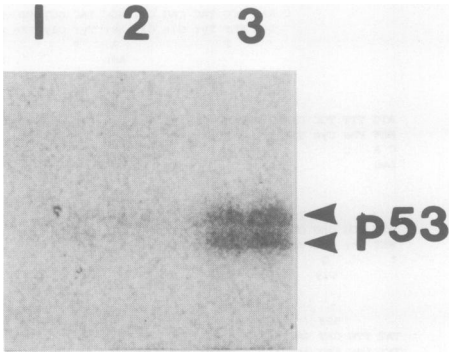

Fig. 4. Hybridization selection with human p53 cDNA 102. Plasmid DNA was bound to nitrocellulose filters and hybridized to 100 μg of poly(A)$^+$ mRNA from SV80 cells. Selected RNA was translated *in vitro* in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine followed by immunoprecipitation and analysis by SDS-polyacrylamide gel electrophoresis. **Lane 1**, control translation containing no mRNA and immunoprecipitated with anti-p53 serum. **Lanes 2** and **3**, polypeptides immunoprecipitated with anti-p53 serum from translation of mRNA selected by a control plasmid pAT153 without insert or p53 plasmid 102, respectively. The arrows denote the position of p53 polypeptides.

hybridization and washing conditions only specific bands are apparent (Figure 6, panel A). With the enzymes used, all the cell lines show similar restriction fragment patterns and band intensities. Strongly hybridizing and reproducible bands were obtained at 18 kb for the *Eco*RI-digested samples and 4.0 kb and 1.6 kb for the *Pvu*II-digested samples. Faintly hybridizing bands at 2.0 kb are not reproducibly observed and are most likely not p53 specific. Thus, there is no detectable gross alteration in the genomic organization of the p53 gene in these transformed cells. The simplicity of the Southern blot pattern indicates that the human p53 gene does not belong to a multigene family. In addition, the appearance of only one hybridizing 18-kb *Eco*RI fragment suggests that there is only a single human p53 locus. This is in contrast to the mouse where a processed pseudogene is contained within an additional 3.3-kb *Eco*RI fragment (Zakut-Houri *et al.*, 1983; Benchimol *et al.*, 1984a). The appearance of two *Pvu*II genomic fragments is consistent with this idea since the probe used in this experiment contains a *Pvu*II site.

The same blot was re-hybridized using the small *Xho*I fragment under the same conditions and this resulted in a smearing pattern with few discernable bands (Figure 6, panel B). Since the small *Xho*I fragment contains 200 bp of Alu sequence (Figure 2), this smear is presumably due to the Alu repetitive sequences within genomic DNA.

Discussion

The human p53 cDNA clone we have isolated covers most of the open reading frame for p53 and contains the complete 3′-untranslated region. The amino acid sequence of human p53 predicted from the cDNA sequence reveals strong homology (84%) with the mouse p53 primary amino acid sequence. The human p53 cDNA contains an Alu repeat sequence which is not present in mouse p53 cDNA (Zakut-Houri *et al.*, 1983; Pennica *et al.*, 1984; Benchimol *et al.*, 1984a). Alu sequences have previously been reported in human mRNA but the reason, if any, for their presence is not known (Jelinek and Schmid, 1982). The Alu sequence interrupts a region in human p53 mRNA which shows homology to mouse p53 mRNA and the structure of human p53 mRNA is considerably different from that of a mouse p53 mRNA,

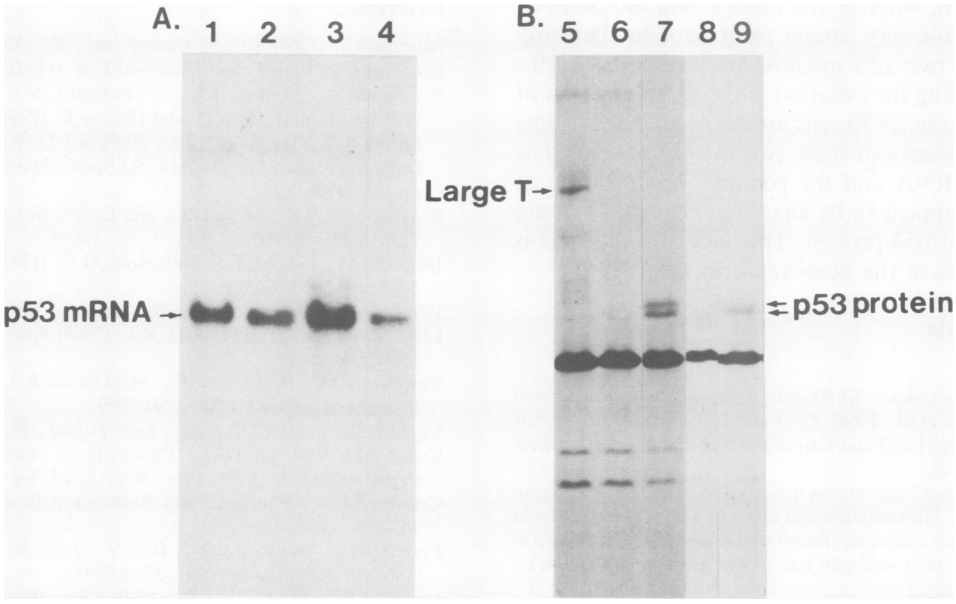

Fig. 5. Panel A. Northern blot analysis of p53 mRNA from transformed cell lines. The lanes contained 10 μg of poly(A)$^+$ mRNA from SV80 cells (**lane 1**), Raji cells (**lane 2**), C331 cells (**lane 3**) and HeLa cells (**lane 4**). **Panel B.** Detection of p53 protein synthesised *in vitro*. SV80 mRNA was translated as described for Figure 4 and immunoprecipitated with: anti-SV40 large-T monoclonal antibody PAb419 (**lane 5**), non-immune serum (**lane 6**), and anti-p53 serum (**lane 7**). HeLa mRNA was treated identically and immunoprecipitated with: non-immune serum (**lane 8**) and anti-p53 serum (**lane 9**).

whereas the actual coding sequence of the p53 protein is very similar.

In vivo labelling of p53 protein in SV80 cells with [^{35}S]methionine followed by immunoprecipitation reveals the presence of two species of p53 with different apparent mol. wts. (Crawford *et al.*, 1981; Thomas *et al.*, 1983). This may be due to post-translational modifications on the same primary amino acid sequence or to their having different but similar primary amino acid sequences. In this study, immunoprecipitates of p53 synthesised *in vitro* from total SV80 mRNA or hybrid-selected mRNA using plasmid 102 also show two mol. wt. species of p53. This, as well as the Northern blot data, presents evidence that p53 mRNA also consists of two species. This provides a possible explanation for the existence of two polypeptide species, each being derived from one of the mRNA species.

Northern blot analysis on mRNA isolated from the various transformed cell lines we used shows that they all contain p53 mRNA. The p53 protein levels in these cell lines vary by >100-fold (Benchimol *et al.*, 1982; Thomas *et al.*, 1983), yet this is not reflected at the mRNA level so the amount of p53 protein in these cells must be regulated post-transcriptionally. Similar observations have been made in murine cell lines (Oren *et al.*, 1981; Benchimol *et al.*, 1984b). It is of particular interest that, although HeLa cells contain no detectable p53, they do contain p53 mRNA which is translationally active.

Southern blot analysis of the structural organization of the human p53 gene in normal foetal liver DNA and DNA from various transformed cell lines revealed no rearrangements or amplifications. Previous Southern blot analysis of p53-specific sequences in mouse cellular DNA using *Eco*RI digestions showed two fragments, one of 18 kb and another of ~3.3 kb (Oren *et al.*, 1983; Benchimol *et al.*, 1984a). Only a single 18-kb *Eco*RI fragment is apparent on Southern blots of human genomic DNA, suggesting the presence of a single locus and our data with *Pvu*II is consistent with this idea.

In conclusion, the structure of human p53 mRNA is different from the structure of mouse p53 mRNA in the

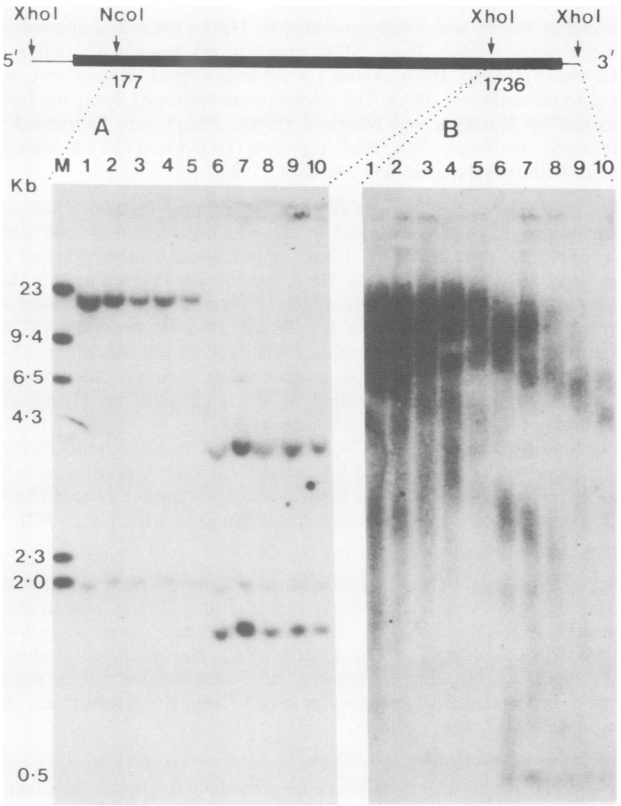

Fig. 6. Panel A. Southern blot analysis of genomic DNA from normal foetal liver and transformed human cell lines. DNA (10 μg) from normal foetal liver (**lanes 1** and **6**), SV80 cells (**lanes 2** and **7**), Raji cells (**lanes 3** and **8**), C331 cells (**lanes 4** and **9**) and HeLa cells (**lanes 5** and **10**) were digested with either *Eco*RI, **lanes 1–5**, or *Pvu*II, **lanes 6–10**. An *Nco*I-*Xho*I fragment located between nucleotides 177 and 1736 as shown in Figure 1 was nick translated (10^8 c.p.m./μg) and used as the hybridization probe. **Lane M**, *Hind*III-digested lambda DNA mol. wt. markers. **Panel B.** Same sample arrangement as in **Panel A** using as the hybridization probe (10^8 c.p.m./μg) an *Xho*I-*Xho*I fragment located from nucleotide 1736 through to the *Xho*I site within the vector as shown.

3'-untranslated region, whereas the human and mouse open reading frames code for very similar proteins. Our data suggest the existence of two p53 mRNA species in SV80 cells. Future studies, including the isolation and characterization of more cDNA clones from SV80 cells are necessary to elucidate the nature and significance of these two mRNA species. The expression of p53 mRNA and the genomic organization of the p53 gene do not appear to be altered in cells that contain high and low levels of p53 protein. This indicates that p53 is regulated in these cells at the post-transcriptional level.

Materials and methods

Cells and antibodies

The cell lines used were as follows: SV80 cells, a line of SV40 transformed human fibroblasts (Todaro *et al.*, 1966). C33I and HeLa, derived from cervical carcinomas (Auersperg, 1964) and Raji cells derived from a Burkitt lymphoma (Epstein *et al.*, 1966).

The pool of human anti-p53 serum was a mixture of sera, collected from five breast cancer patients. The detection and assay of anti-p53 antibodies in these sera have already been described (Benchimol *et al.*, 1982; Crawford *et al.*, 1982). PAb419 monoclonal antibody was L19 of Harlow *et al.* (1981).

Isolation of p53 gene fragment

Two genomic segments of human DNA containing sequences from the p53 gene were isolated by cross-hybridization to a mouse p53 cDNA (Benchimol *et al.*, 1984a) and cloned as plasmids pHU2-6 and pHU7-1 (P. Lamb, in preparation). Cloned genomic inserts were gel purified and labelled by nick translation (Rigby *et al.*, 1977).

Isolation of human p53 cDNA clone 102

The cDNA library was kindly provided by Hiroto Okayama and was constructed with mRNA from SV40 transformed human fibroblast cells (Okayama and Berg, 1983). About 1 x 10^6 transformed bacteria were plated out onto nitrocellulose filters. The colonies were replicated, lysed and fixed as described by Hanahan and Meselson (1980). Filters were hybridized with human p53-specific genomic DNA fragments (HU2-6 and HU7-1) using standard procedures (Hanahan and Meselson, 1980).

Analysis of nucleic acids by Northern and Southern blotting

Total cellular RNA was extracted in the presence of guanidinium isothiocyanate (Chirgwin *et al.*, 1979). RNA samples were chromatographed twice over oligo(dT)-cellulose. Genomic DNA was prepared according to standard procedures (Blin and Stafford, 1976). Hybridization probes were nick translated to a specific activity of 1 x 10^8 c.p.m./μg (Rigby *et al.*, 1977).

For Northern blots, 10 μg of polyadenylated RNA was denatured in 1.0 M glyoxal and fractionated on a 1% agarose gel in 10 mM sodium phosphate buffer pH 7.0 then blotted on to Biodyne A nylon membranes. Hybridization conditions were as described by Thomas (1980).

For Southern blots, genomic DNA was digested to completion with the appropriate restriction enzyme and fractionated on a 0.8% agarose gel in Tris-acetate-EDTA buffer. DNA was blotted onto nitrocellulose filters (Southern, 1975) and hybridized using standard conditions (Maniatis *et al.*, 1982).

Hybridization selection and in vitro translation

10 μg of plasmid DNA were linearized by digestion with *Sal*I, heat denatured and baked onto 1 cm squares of nitrocellulose filter. Filters were hybridized with 100 μg of total poly(A)$^+$ mRNA, washed and eluted as previously described (Ricciardi *et al.*, 1979). Eluted mRNA was translated *in vitro* in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine and the products immunoprecipitated with polyclonal anti-p53 serum (Crawford *et al.*, 1982), then subjected to electrophoresis.

DNA sequence analysis

Nucleotide sequence determinations were carried out by the dideoxy chain termination method (Sanger *et al.*, 1977) in the presence of dideoxynucleoside triphosphates and [^{35}S]thio dATP (Biggin *et al.*, 1983). The resulting DNA products were run on buffer gradient gels (Biggin *et al.*, 1983).

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