MOLECULAR AND CELLULAR BIOLOGY, July 1984, p. 1402–1410 Vol. 4, No. 7
0270-7306/84/071402-09$02.00/0
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Inactivation of p53 Gene Expression by an Insertion of Moloney Murine Leukemia Virus-Like DNA Sequences

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Received 7 February 1984/Accepted 6 March 1984

Analysis of Abelson murine leukemia virus-transformed L12 cells which lack the p53 cellular encoded tumor antigen revealed alterations in the p53-specific genomic DNA sequences. The active p53 gene, usually contained in a 16-kilobase *Eco*RI DNA fragment of p53 producer cells, went through major alterations leading to the appearance of a substantially larger 28.0-kilobase p53-specific *Eco*RI fragment. Detailed restriction enzyme analysis, with genomic probes spanning throughout the whole active p53 gene, indicated that the L12 p53 altered gene contains all the exons and principal introns of the normal p53 16.0-kilobase gene. However, its structure was interrupted by the integration of a novel DNA segment into the noncoding intervening sequences of the first p53 intron. Analysis of the inserted sequences revealed close homology to Moloney murine leukemia virus. This Moloney leukemia murine virus-like particle resides in a 5′ to 3′ transcriptional orientation, similar to the p53 gene, permitting the transcription of aberrant fused mRNA molecules detected in these cells.

p53 is a cellular encoded protein that is overproduced in cancer cells. Accentuated concentrations of this protein were detected in a variety of cell lines and primary tumors of several species (6, 7, 9, 16, 17, 30, 33, 39), suggesting that p53 may play a role in neoplastic transformation. In tumor cells this protein was found in its phosphorylated form (13, 20, 30, 32), and in several instances it was found in a stable complex with viral tumor antigens (16, 17, 19, 37). A limited occurrence of p53 was also observed in nontransformed cells, such as normal thymocytes (34), primary embryonic fibroblasts (24), and NIH-3T3 fibroblasts (27). It was suggested that p53 may display a function in the normal cell cycle (21, 23, 24).

The function that p53 fulfills in transformed and nontransformed cells can be understood by comparing cells that express the protein and variant cells that do not express it. We have at our disposal a unique Abelson murine leukemia virus (Ab-MuLV)-transformed cell line, L12, which lacks detectable amounts of the p53 protein. Injection of these cells into syngeneic mice induced the development of tumors which were subsequently rejected, whereas other Ab-MuLV-transformed cells that were overproducing p53 developed into lethal tumors (31, 45). This observation suggests a correlation between expression of p53 in tumor cells and their capacity to exhibit a fully transformed phenotype, evaluated as development of lethal tumors in syngeneic mice.

In our previous studies employing specific cDNA probes (25, 26), we found that the inability of L12 cells to produce p53 is due to the absence of detectable mature p53-specific mRNA (46). Instead, these cells contain two major p53-specific mRNA species of a substantially larger size than the p53-specific mRNA in the p53 producing cells (46). The experiments that are presented here were aimed at characterizing the p53 gene of the L12 cells, in an endeavor to elucidate the complexed molecular mechanism responsible for the altered p53 mRNA pattern observed in L12 cells. We found that p53 expression in L12 cells was modified by an insertion of Moloney MuLV (Mo-MuLV)-like DNA sequences into the first intron of the principal active p53 gene in these cells.

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MATERIALS AND METHODS

Cell lines. The Ab-MuLV-transformed lymphoid cell lines used were 2M3/M, also containing the Moloney helper virus, 2M3, a Moloney nonproducer cell line derived from 2M3/M (both of BALB/c origin), and L12 and 230-23-8 (a gift from N. Rosenberg, Tufts University) of C_{57}L/J origin. Lymphoid cells were grown in RPMI 1640 medium enriched with 10% heat-inactivated fetal calf serum (Biolab, Israel) and 2×10^{-5} M beta-mercaptoethanol. Meth A chemically transformed cells of BALB/c origin were grown in RPMI 1640 medium supplemented with 10% heat-inactivated calf serum. Hybridoma cell lines RA3-2C2 (5, 34), PAb122 (11), and PAb421 (12) 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.

RNA and DNA blot analysis. RNA was prepared according to the method of Auffray and Rougeon (1) and selected for polyadenylated molecules by oligodeoxythymidylate cellular chromatography (2). Samples of 5 μg of polyadenylated RNA prepared from various cell lines were heated for 10 min at 60°C in 50% formamide–6% formaldehyde–running buffer (20 mM MOPS [morpholinepropanesulfonic acid] [pH 7.0], 5 mM NaAc, 1 mM EDTA). The samples were electrophoresed through a 1% agarose gel containing 6% formaldehyde. The RNA was transferred onto a nitrocellulose sheet (41) and hybridized to nick-translated (28) whole plasmid pp53-271A p53-specific cDNA, obtained from M. Oren, The Weizmann Institute (25, 26). Hybridization was for 16 h at 43°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution (8)–20 mM sodium phosphate (pH 7.0)–100 μg of salmon sperm DNA per ml–10% dextran sulfate. Hybridized filters were washed extensively at 50°C with 0.1× SSC–1% sodium dodecyl sulfate and autoradiographed. Samples of restriction enzyme-digested high-molecular-weight DNA (5 μg) were electrophoresed on 0.8% agarose gels, blotted onto nitrocellulose filters (40), and hybridized to nick-translated pp53-271A DNA (28). Autoradiography was performed at −70°C with an intensifier screen.

Preparation of high-molecular-weight genomic DNA. Cells were washed twice with phosphate-buffered saline, resuspended in lysis buffer (0.5% sodium dodecyl sulfate, 0.1 M

NaCl, 50 mM Tris-hydrochloride, 25 mM EDTA [pH 7.5], 0.2 mg of proteinase K per ml) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and incubated for 14 h at 37°C. The solution was extracted twice with an equal volume of redistilled phenol, followed by two extractions with an equal volume of ether. The deproteinated solution was incubated with 10 μg of heat-treated RNase (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C, followed by one extraction with an equal volume of redistilled phenol and two extractions with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA solution was adjusted to a final concentration of 0.3 M NaCl, and two volumes of cold ethanol were added. The DNA precipitate that formed was isolated by a sealed Pasteur pipette and resuspended in 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.5) at a final concentration of 1 mg/ml.

Isolation of the cell line 230-23-8 p53 16.0-kb *Eco*RI genomic DNA fragment. 230-23-8 *Eco*RI-digested genomic DNA (200 μg), prepared to 20 mM with respect to EDTA (pH 7.5), was heated to 65°C for 15 min, cooled to 4°C, and applied to an 11.5-ml 15 to 40% linear sucrose gradient (1 M NaCl, 10 mM Tris-hydrochloride, 5 mM EDTA [pH 7.5]) in a Beckman SW41 centrifuge tube. The gradient was centrifuged at 30,000 rpm for 20 h at 20°C. Fractions of 0.5 ml were collected, and samples were assayed by the Southern blot procedure (40) for the two *Eco*RI-derived p53 genomic fragments utilizing the cDNA clone pp53-271A (25, 26). Peak fractions containing the 16.0-kilobase (kb) *Eco*RI genomic fragment were pooled, concentrated by ethanol precipitation, and resuspended in 10 mM Tris-hydrochloride–0.1 mM EDTA (pH 7.5) at a final concentration of 1 mg/ml.

Isolation of the cell line L12 p53 5.5-kb *Hin*dIII and 9.0-kb *Hin*dIII genomic DNA fragments. L12 *Hin*dIII-digested genomic DNA (200 μg) was heated to 65°C for 15 min, cooled to 4°C, and fractionated on an 0.8% agarose (Seakem) gel in TAE (40 mM Tris-acetate, 2 mM EDTA [pH 8.3]). The bands containing the 5.5- and 9.0-kb *Hin*dIII fragments were cut out, electroeluted, and concentrated by an Elutip (Schleicher & Schull, Inc., Keene, N.H.) The Elutip was regenerated between usages by a 2 M sodium acetate (pH 5.0) wash. The DNA was concentrated by ethanol precipitation and resuspended in 10 mM Tris-hydrochloride–0.1 mM EDTA (pH 7.5) at a final concentration of 1 mg/ml.

Cloning and isolation of p53 genomic DNA fragments from a bacteriophage library. The 16.0-kb *Eco*RI fragment was ligated to the genomic arms of the charon 4A vector at a 2/1 molecular ratio of arms to insert (18). The 5.5-kb and 9.0-kb *Hin*dIII fragments were ligated to the *Hin*dIII-digested charon 21 vector in two independent reactions at a 2/1 molecular ratio of arms to insert. The ligated vectors were packaged according to the method of Maniatis et al. (18) by utilizing the Amersham lambda-in vitro packaging kit. Approximately 10^6 PFU from each library were plated and screened according to the procedure of Benton and Davis (3). The charon 4A *Eco*RI library probed with a 350-base pair *Pst* insert of clone pp53-271A. The charon 21 libraries were probed with a 2.0-kb *Hind-Xho* fragment of clone p8R2.H. Positive hybridizing plaques were plaque purified, and small-scale preparations were analyzed by restriction enzyme digest on 0.8% agarose gels (18).

Subcloning into plasmid pBR322. Positive phages containing the 230-23-8 16.0-kb *Eco*RI fragment were double digested with *Hin*dIII and *Eco*RI and ligated to either *Hin*dIII- or *Hin*dIII- and *Eco*RI-digested, calf intestine phosphatase-treated pBR322. Positive phages containing the L12 5.0-kb *Hin*dIII or 9.5-kb *Hin*dIII fragment were digested with *Hin*dIII and ligated to *Hin*dIII-digested, calf intestine phosphatase-treated pBR322. Ligated DNA was used to transform *Escherichia coli* HB101 according to the procedure of Cohen et al. (4). Subclone identification was determined by restriction enzyme analysis on small-scale overnight cultures (18). Column chromatograph (Bio-Rad A-50)-purified plasmids were used directly for nick translation (28). Selected internal restriction enzyme fragments, as specified below, were isolated by agarose gel electrophoresis, electroelution, and Elutip (Schleider & Schull) concentration.

Nucleic acid sequencing. The 5' *Hin*dIII-*Pvu*II fragments of plasmids p8R2.H and pL2.H were subcloned into the *Sma*I-*Hin*dIII site of M13-Mp8,Mp9 (22) and sequenced by the method of dideoxy chain termination (36).

RESULTS

Isolation and characterization of the p53-specific 230-23-8 16.0-kb *Eco*RI genomic fragment. The properties of L12, an Ab-MuLV-transformed variant cell line studied here, are summarized in Table 1. These cells expressed the "abl"-specific p120 product, as well as Mo-MuLV-specific structural proteins. However, immunoprecipitation of biosynthetically labeled L12 cell lysates has clearly indicated the absence of detectable p53 molecules that are recognized by several types of anti-p53 monoclonal antibodies or specific polyclonal serum (31, 32). In addition, these cells lacked the expression of a mature 2.0-kb p53 mRNA molecule and instead expressed two new species of 3.5- and 6.5-kb polyadenylated mRNA hybridizing p53 cDNA probes (46). Analysis of genomic DNA by using p53 cDNA probes showed the existence of two noncontiguous p53 genes, one which is probably a pseudogene contained within a 3.3-kb *Eco*RI fragment and a second within a 16.0-kb *Eco*RI fragment (25, 46). We found that L12 cells contained the common 3.3-kb *Eco*RI fragment but lacked the 16.0-kb *Eco*RI fragment. Instead these cells exhibited a 28.0-kb *Eco*RI fragment hybridizing to p53 cDNA probes, which implies the absence of the normal 16.0-kb *Eco*RI p53 homology. The difference in size of the *Eco*RI fragments was not due to mouse strain-specific restriction site polymorphism since 230-23-8 Ab-MuLV-transformed cells of the same C_{57}L/J genetic origin displayed the usual 16.0-kb band (46).

Detailed genomic analysis with p53 cDNA probes indicated identical restriction enzyme patterns derived from the 230-23-8 16-kb *Eco*RI p53 producer and the L12 28-kb *Eco*RI nonproducer p53 gene upstream and downstream from an estimated 10.0-kb area of rearrangement located at the 5' proximal region of the L12 p53 gene. However, the spacious placement of the exons, as reflected by the cDNA probes throughout the 16.0-kb *Eco*RI fragment, complicated the pinpointing of the exact region and structural identity of the rearrangement. The ultimate localization and characterization of the rearranged region necessitated, therefore, the use of DNA fragments that probed in more detail the 5' proximal region (including exons and introns) of the L12 p53 gene. Therefore, we isolated the 230-23-8 16.0-kb *Eco*RI p53 genomic fragment from a lambda bacteriophage charon 4A library of the producer cell line, 230-23-8, employing specific p53 cDNA probes (obtained from M. Oren) (25, 26). The genomic p53 clone that was obtained was further digested with *Hin*dIII and subcloned in pBR322.

The cloned 230-23-8 16.0-kb *Eco*RI fragment designated λ8R and its subcloned fragment in pBR322, as well as the flanking genomic regions, as deduced by Southern blot analysis, are shown schematically in Fig. 1. The principal exon clusters are denoted (as asterisks) (47), and the sub-

TABLE 1. Properties of L12 Ab-MuLV-transformed cells

Biological property	Cell line				
	L12	230-23-8	2M3	2M3/M	Meth A
Transforming agent	Ab-MuLV	Ab-MuLV	Ab-MuLV	Ab-MuLV	methylcholanthrene
Genetic origin	$C_{57}L/J$	$C_{57}L/J$	BALB/c	BALB/c	BALB/c
Develops lethal tumors in syngeneic mice[a]	–[a]	+	+	+	+
Expresses the abl oncogene product (p120)[b]	+	+	+	+	–
Expresses Moloney-related viral proteins[c]	+	+	–	+	–
Contains the p53 protein[d]	–	+	+	+	+
Size(s) of p53 mRNA species[e]	3.5 kb, 6.5 kb	2.0 kb	2.0 kb	2.0 kb	2.0 kb
Sizes of *Eco*RI DNA fragments hybridizing p53 cDNA probes[f]	28.0 kb, 3.3 kb	16.0 kb, 3.3 kb	16.0 kb, 3.3 kb	16.0 kb, 3.3 kb	16.0 kb, 3.3 kb

[a] L12 cells develop local tumors in $C_{57}L/J$ syngeneic mice that are rejected by the host (31, 45).
[b] p120 protein was detected by immunoprecipitation of radioactive cell lysates with either goat anti-Moloney serum (45) or AbT serum (44).
[c] Moloney-related viral proteins were detected by immunoprecipitation of radioactive cell lysates with goat anti-Moloney serum.
[d] p53 protein was detected by immunoprecipitation of radioactive cell lysates with anti-p53 monoclonal antibodies (5, 11, 12, 34).
[e] Polyadenylated mRNA was hybridized with pp53-271A cDNA probe as described previously (46).
[f] *Eco*RI-digested DNA fragments were hybridized with the whole plasmid containing pp53-271A cDNA. The *Eco*RI fragments of L12 cells hybridizing with p53-specific DNA were originally estimated to be 23.0 and 3.3 kb in size (46). Our present measurements indicate that the sizes of p53-specific *Eco*RI fragments in L12 cells are 28.0 and 3.3 kb.

cloned genomic fragments are numbered accordingly (p8R1.H through p8R5.H). The p53 gene spans 14.0 kb of the 16.0-kb *Eco*RI fragment and contains all relevant exons, as well as a potential promoter-like region, as determined by cDNA clone analysis (26, 46). The first exon, found at the extreme 5′ proximal end, was most noticeably separated from the second exon and the major exon clusters by an intron of 6 kb (47). This was the only exon which corresponded to the aberrant polyadenylated p53 L12 mRNA species and related to the extreme 5′ region of the mRNA (46).

Southern blot analysis of the rearrangement in the p53-specific L12 28.0-kb *Eco*RI genomic fragment. The characterization of the rearrangement in the p53 gene in L12 cells was mainly based on Southern blot analysis that was performed by employing three probes appropriately spanning the entire p53 gene. The probes that were used included pp53-271A cDNA corresponding to the first, second, and third exon regions (25), subclone p8R2.H corresponding to the initial 2.5-kb 5′ proximal part of the first intron, and subclone p8R4.H probing the 3′ proximal part of the p53 gene (see Fig. 1). The intron-selective genomic subclone p8R2.H was reduced 500 base pairs at its 3′ end due to the presence of a repetitive sequence, retaining a *Hind-Xho* fragment. This probe specifically hybridized to the functional gene contained in the 16.0-kb *Eco*RI fragment but failed to hybridize to the 3.3-kb *Eco*RI fragment which was deprived of intron sequences (see Fig. 3; compare lanes a). Figure 2 clearly shows that the rearrangement lay at the 5′ proximal region of the L12 28-kb *Eco*RI fragment and was potentially contained within the first intron designated by subclone p8R2.H. Further analysis of DNA fragments generated by *Hin*dIII, *Bam*HI, and *Bgl*II restriction enzymes, which did not cut within the first 2.0 kb of the p53 intron, revealed the nature of the rearranged sequences. Results shown in Fig. 3 demonstrate that the rearrangement was contained solely within the first 2.0 kb of the 6.0-kb intron, as represented by the two *Hin*dIII novel fragments (5.5 and 9.0 kb), which indicated the presence of a 12.0-kb novel DNA fragment within the 5′ proximal region of the first intron in the L12 28.0-kb *Eco*RI fragment.

The deduced structural organizations of the normal 230-23-8 and the abnormal L12 p53 genes derived by Southern blot analysis with various subcloned p53 genomic fragments corresponding to exons and introns and p53 cDNA as hybridization probes are illustrated in Fig. 4. The p53 gene residing in the 3.3-kb *Eco*RI fragment, which was considered to be a nonfunctional pseudogene due to its paucity of introns, close resemblance in the restriction map to the cDNA, and lack of part of the first exon (25, 46), was not altered in any manner in the L12 p53 nonproducer cells. On the other hand, the p53 gene residing in the 16.0-kb *Eco*RI fragment, which was considered as the active gene, was altered in the L12 p53 nonproducer cells by integration of a

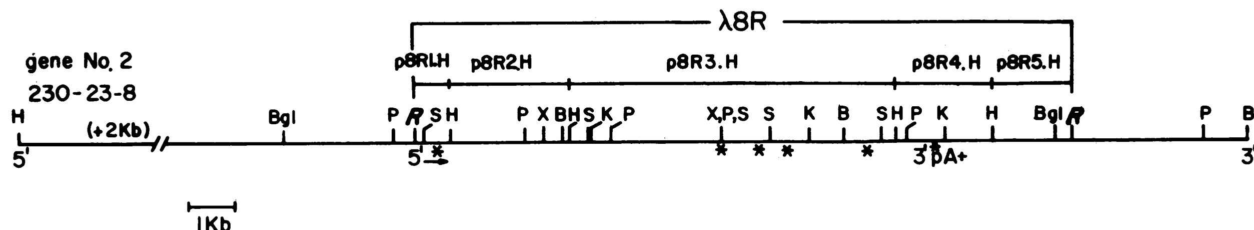

FIG. 1. Structure of the functional active p53 gene. The restriction enzyme map of p53 gene contained in a 16.0-kb *Eco*RI fragment and the cellular flanking sequences was derived by analysis of genomic 230-23-8 DNA and the cloned λ8R fragment. The *Eco*RI 16.0-kb fragment was initially cloned in a lambda charon 4A vector with the pp53-271A cDNA as a specific screening probe. The cloned λ8R was digested with *Hin*dIII and *Eco*RI restriction enzymes, and the generated fragments (noted on the figure as p8R1.H through p8R5.H) were further subcloned into pBR322. The principal exons, denoted as asterisks, were derived based on p53-specific cDNA probes (25, 47). Abbreviations for the restriction enzyme used are: B, *Bam*HI; Bgl, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; R, *Eco*RI and X, *Xho*I.

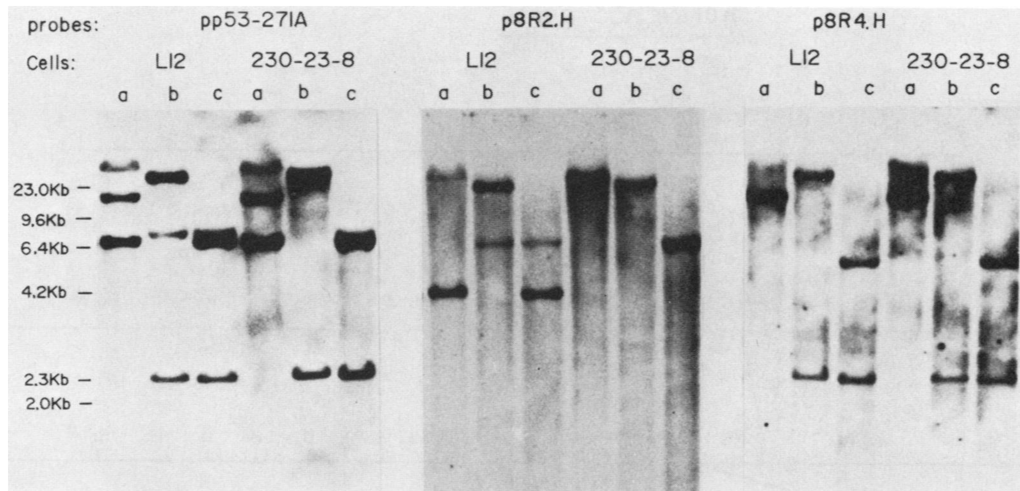

FIG. 2. Analysis of p53 genomic DNA fragments detailing the exon and intron regions. DNA samples of L12 p53 nonproducer and 230-23-8 p53 producer Ab-MuLV-transformed cell lines were digested and double digested with the following enzymes: *Bam*HI (lanes a), *Bgl*II (lanes b), and *Bam*HI plus *Bgl*II (lanes c). The DNA blots that were obtained were initially hybridized with pp53-271A cDNA probes. The same filters were then extensively washed with 0.5 N NaOH for 30 min at room temperature to remove all radioactive signals and were subsequently hybridized with the *Hind-Xho* fragment of the p8RH.2 genomic clone (p8RH.2) and with the p8RH.2 p53 genomic fragment which does not contain any repetitive sequences. The markers used were *Hin*dIII-digested fragments of lambda DNA (Bio-Lab).

12-kb DNA insert into the first intron. The comparable normal 2.0-kb intron region containing the 12.0-kb rearrangement did not reflect marked alteration. Indeed, the additional 12-kb DNA fragment appeared to reside neatly at the extreme 5′ end of the normal 2.5-kb *Hin*dIII intron region (see Fig. 1, clone p8R2.H).

Isolation and characterization of the foreign DNA element in the 28.0-kb *Eco*RI genomic fragment. Prompted by the above results, a literature survey of potential homology to retroviral-like particle insertion elements was undertaken. A Moloney-like particle insertion residing in a 5′ to 3′ transcription reading frame similar to the p53 gene was tentatively suggested. Characterization of the inserted DNA fragment that was detected in the first intron of p53 gene in L12 cells necessitated, however, its molecular cloning. The rearranged region was contained entirely within two *Hin*dIII genomic fragments, 5.5 and 9.0 kb (see Fig. 4). These were independently cloned from lambda bacteriophage Charon 21 libraries, with the *Hind-Xho* fragment of p8R2.H as the intron-specific probe. In agreement with our assumption, the subcloned genomic fragments demonstrated specific hybridization homology to a *Kpn-Kpn* fragment of the Moloney-specific long terminal repeat (LTR) (10) and a circular Mo-MuLV provirus cloned into the *Hin*dIII site of lambda phage vector DNA (E. Cannani, The Weizmann Institute). The two subcloned genomic p53 fragments that were obtained (pL1.H and pL2.H) were digested with several restriction enzymes, and the map that was derived (Fig. 5b′) indicated close homology to a Moloney-like viral particle residing in a 5′ to 3′ transcriptional orientation similar to the p53 gene. The Moloney-like insert shares homology with the Mo-MuLV upstream and downstream from a major area of polymorphism (3.3 kb) found between the *Hin*dIII and *Bgl*II sites of the Moloney virus map units 5.4 to 5.6 (10). In all, ca. 95% of the Moloney-like viral particle was conserved. Further evidence that the inserted sequence represented a Mo-MuLV-like particle was derived from analysis of the nucleic acid sequence at the 5′ junction of the p53 L12 intron and the predicted Moloney-like insert. For that purpose we sequenced the 5′ proximal *Hind-Pvu* fragments (see Fig. 4) of the 230-23-8 p53 intron from p8RH.2 and the L12 p53 intron from pL2.H which contained the intron-LTR junction region. The sequence that was obtained (Fig. 6) clearly indicated homology to the 5′ LTR of Mo-MuLV, retaining the typical 11-base-pair inverted repeat. A comparison between the p53 L12 intron and the 230-23-8 sequence at the junction indicated that Mo-MuLV did not cause any rearrangement in the p53 gene upon integration.

Northern blot analysis of the p53 and Moloney-specific polyadenylated mRNA in L12 and 230-23-8 cells. We observed previously that the aberrant L12 polyadenylated p53-specific mRNA was substantially larger (3.5 and 6.5 kb) than

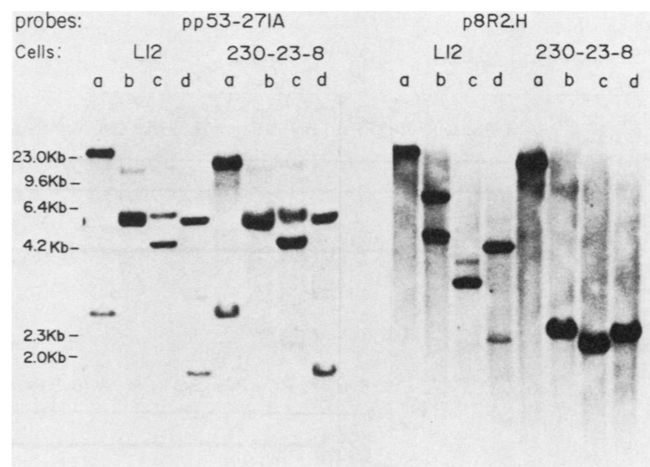

FIG. 3. Analysis of p53 genomic DNA fragments detailing the first intron. DNA samples of L12 p53 nonproducer and 230-23-8 p53 producer Ab-MuLV-transformed cells were digested and double digested with the following enzymes: *Eco*RI (lanes a), *Hin*dIII (lanes b), *Hin*dIII plus *Bam*HI (lanes c), and *Hin*dIII plus *Bgl*II (lanes d). The DNA blots that were obtained were initially hybridized with the pp53-271A cDNA probe and subsequently rehybridized with the *Hind-Xho* fragment of the p8R2.H genomic first intron (p8R2.H). (For more details see the legend to Fig. 2.)

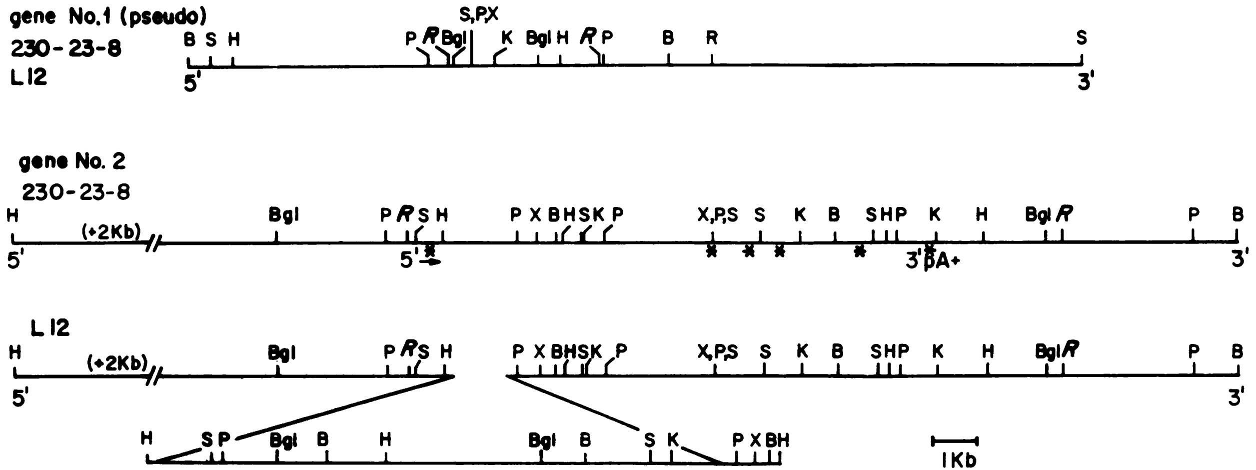

FIG. 4. Structural comparison of the p53 genes contained in L12 nonproducer and 230-23-8 producer Ab-MuLV-transformed cell lines. The structure of p53 genes was deduced from genomic blot restriction enzyme analysis by using p53-specific cDNA and genomic probes.

the mature p53 mRNA (2.0 kb). This aberrant mRNA species contained homology only with the extreme 5′ proximal end of the p53 mRNA, which was found to reside in the first exon of the normal 230-23-8 16.0-kb *Eco*RI p53 gene (46). The orientation and the position of the Moloney-like insert within the p53 intron of L12 cells supported a transcriptional mechanism that generated fused p53-Moloney mRNA. Therefore, to resolve the derivation of the unusual L12 polyadenylated p53 mRNAs, Northern blot analysis, with the p8RH.1 through p8RH.4 and pL2.H subcloned genomic probes, as well as the Moloney-specific sequences, was performed on total cellular polyadenylated mRNA isolated from L12 and 230-23-8 cells. Figure 7 shows that the aberrant L12 p53 polyadenylated mRNA species contained homologous sequences derived from the initial 3.0 kb of the p53 gene indicated by clones p8RH.1 and p8RH.2 (Fig. 7). The 6.5-kb p53 mRNA of L12 cells specifically hybridized with both probes, whereas the 3.5-kb L12 p53 mRNA

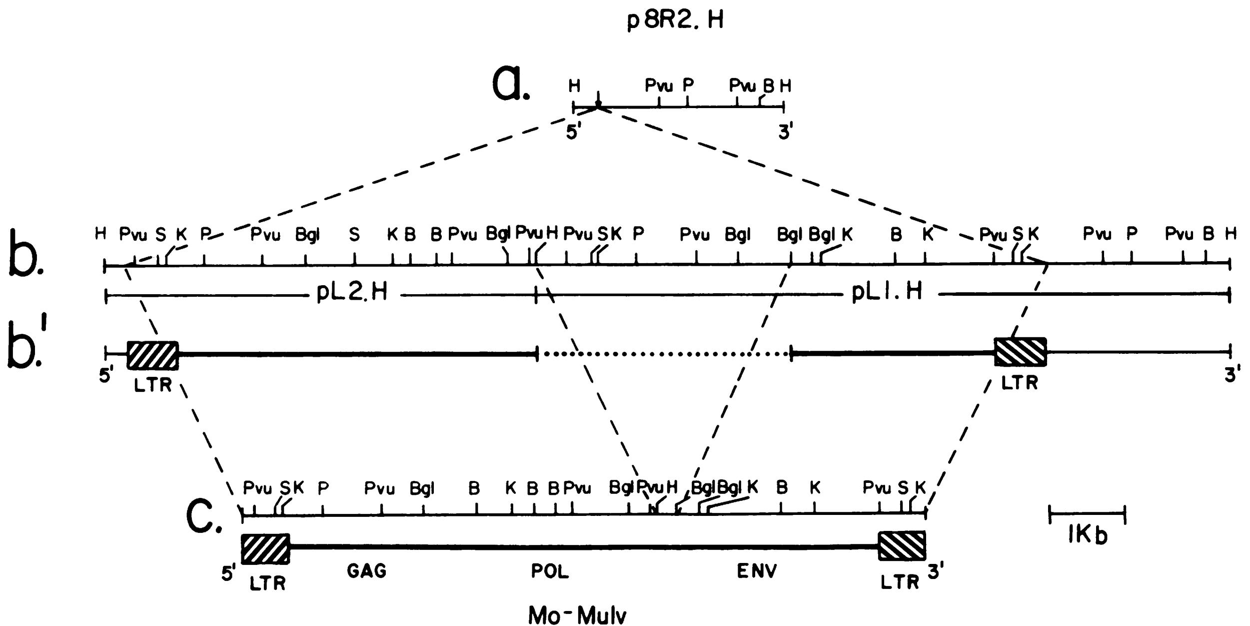

FIG. 5. Novel DNA sequences inserted into the first intron of the L12 rearranged p53 gene share homology with the Mo-MuLV. The rearranged intron of L12, represented by 5.5- and 9.0-kb *Hin*dIII genomic fragments, was initially cloned in Charon 21 and subsequently subcloned in pBR322, pL2.H (5.5 kb), and pL1.H (9.0 kb). The hybridization probe used was the *Hind-Xho* fragment of p8RH.2. (a) Restriction map of the *Hind-Hind* fragment containing the 5′ part of the first p53 intron of 230-23-8 cells (2.5 kb). (b) Restriction map of the *Hind-Hind* intron fragment of L12 cells containing the novel inserted sequences. (b′) Regions of homology to the intron p8R2.H (a) and to Mo-MuLV (solid line). The region of polymorphism is indicated by a dotted line. (c) Restriction map of Mo-MuLV (10, 42). The striated rectangles represent the LTRs of Mo-MuLV.

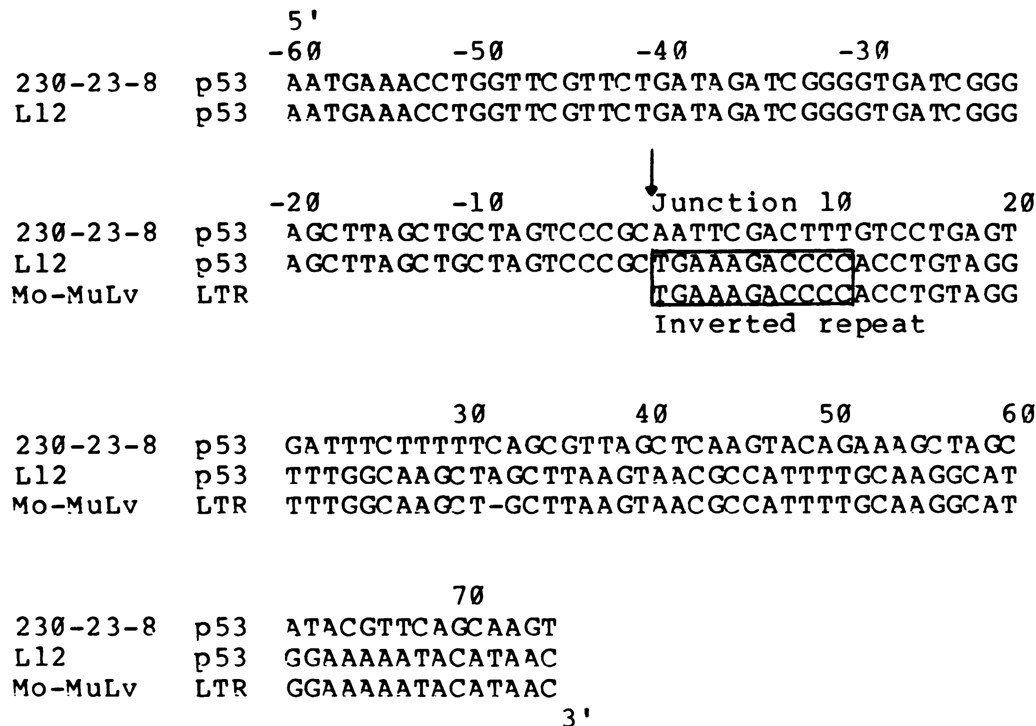

FIG. 6. Nucleic acid sequence of the L12 p53 intron and the Mo-MuLV-like particle junction. The 5′ *Hind-Pvu* fragments of the 230-23-8 and L12 p53 introns (see Fig. 4) were subcloned into M13-Mp8,Mp9 and sequenced (22, 36). The Mo-MuLV sequences were according to previously published data (43).

species hybridized to the p8RH.1 subclone only, which is in agreement with our previous observation (46). Larger polyadenylated mRNA terminal processed precursors common to the L12 and 230-23-8 cells hybridized the major internal intron and exon clusters (Fig. 7, probe p8RH.3). Subclone p8RH.4, corresponding to the last exon and the 3′ noncoding region (47), only hybridized to the mature p53 2.0-kb species expressed in the 230-23-8 p53 producer cells. Hybridization with the *Kpn-Kpn* Moloney LTR-specific probe or fragments of the pL2.H L12 p53 gene (*Hind-Pst* representing the LTR; *Pst-Hind* representing the *gag-pol* sequences) (see Fig. 6) reflected identical patterns (Fig. 7, probes pL2.H and Mo-MuLV). The Moloney LTR probe hybridized to the characteristic Moloney and Abelson transcripts which are common to the L12 and 230-23-8 cells. Furthermore, this probe revealed additional mRNA species unique to the L12 cells. Three of these novel Moloney-specific mRNA molecules of L12 cells comigrated with the aberrant 3.5-kb and the doublet 6.5- to 7.5-kb p53 mRNA species whose derivation with regard to the p53 gene has been demonstrated. The fourth prominent band that was detected by the *Kpn-Kpn* LTR Moloney probe is unique to the L12 cells and most likely was derived from the Moloney-like virus integrated into the p53 gene (denoted as an arrow in Fig. 7).

We concluded that the aberrant L12 polyadenylated p53-specific RNA was probably derived by a splicing event between the p53 first exon-intron and the inserted Moloney-like particle by using a polyadenylation site that was most likely donated by the 3′ LTR.

DISCUSSION

Analysis of genomic DNA by specific p53 cDNA probes (26) has indicated the presence of two noncontiguous p53 genes (25, 45, 46). In the variant L12 Ab-MuLV-transformed cells, we observed that the principal active p53 gene contained in a 16.0-kb *Eco*RI fragment went through major alterations, leading to the appearance of a substantially larger 28.0-kb p53-specific *Eco*RI fragment. However, the pseudogene of these cells, contained in a 3.3-kb *Eco*RI fragment, was intact. Preliminary karyotype analysis indicated that L12 cells apparently have the normal diploid number of chromosomes. The fact that L12 cells lack the 16.0-kb *Eco*RI fragment implies that these cells are devoid of the normal p53 homolog chromosome. This suggests that both homologs of chromosome 11, which contain the p53 gene (35), are rearranged. Preliminary analysis of the altered p53 gene in L12 cells with p53 cDNA probes has mapped the rearrangement to the 5′-proximal part of this gene (46). Definition and characterization of the rearrangement region was presently achieved by using genomic p53 DNA probes. Hybridization of DNA fragments with clone p8R2.H, which corresponded to the 5′ proximal part of the first intron of the active p53 gene, indicated the presence of a 12.0-kb DNA insert. On the basis of detailed analysis with probes spanning throughout the whole p53 gene, we concluded that the L12 p53 gene contained all exons and the principal introns of the normal p53 16.0-kb gene. However, its structure was interrupted by the integration of a novel DNA insert into the noncoding sequences. Subcloning of the inserted DNA fragment and its analysis revealed close homology to Mo-MuLV. Furthermore, analysis of nucleic acid sequence at the 5′ LTR junction region indicated that the Moloney particle integrated into the first intron of the L12 p53 gene, retaining the typical inverted repeat sequences of the Mo-MuLV LTR (see Fig. 6) without any appreciable rearrangement in the p53 intron flanking sequence. This Moloney-like viral particle residing in a 5′ to 3′ transcriptional orientation similar to the p53 gene contained a 3.3-kb region of polymor-

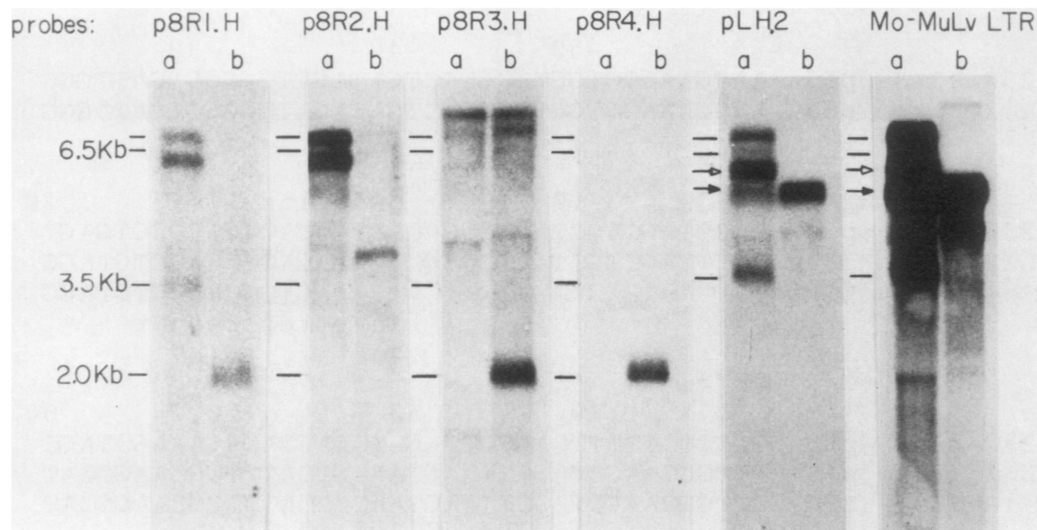

FIG. 7. Analysis of p53 mRNA species in L12 p53 nonproducer (a) and 230-23-8 p53 producer (b) cell lines. Samples of polyadenylated mRNA were fractionated by formaldehyde agarose gel electrophoresis and hybridized with the following genomic probes: p8R1.H, p8R2.H, p8R3.H, p8R4.H, and *Pst-Hind* fragments of clone pLH.2, as well as a Mo-MuLV LTR probe obtained from E. Cannani. The Mo-MuLV-specific bands that comigrated with the p53-specific mRNA are denoted as (—). The comigrating Moloney-MuLV and Abelson-MuLV mRNA expressed in L12 and 230-23-8 cells are denoted as (➔). The unique Mo-MuLV L12 mRNA is denoted as (–▹).

phism detected between map units 5.4 and 5.6. The modifications observed in the inserted Moloney-like particle compared with functional Mo-MuLV may be attributed to a possible rearrangement which occurred while the Moloney-like particle was integrating into the p53 sequences. However, it is yet to be determined whether the Moloney-like insert in the p53 intron is a functional virus.

The Ab-MuLV-transformed cell lines that were studied here were established by infecting bone marrow cells with a stock containing Ab-MuLV and Mo-MuLV, which usually yielded tumor cells containing both viruses (29). It is possible, therefore, that this initial step of viral infection, while establishing the L12 cells, included a rare event whereby Mo-MuLV was integrated into the first intron of the p53 gene. Results that were obtained with a Moloney-specific DNA probe (Ms-4, hybridizing specifically to Mo-MuLV at 4.95 to 5.4 map units; obtained from I. Verma, The Salk Institute) clearly indicated that L12 cells contain, in addition to the rearranged inserted Mo-MuLV, a normal-sized Moloney provirus present in most Ab-MuLV transformed cells (data not shown).

Inactivation of cellular genes by retrovirus insertion has been reported recently in several instances. Kuff et al. (15) have demonstrated the presence of A-type particles in the intervening sequences of mutated immunoglobulin genes. Jenkins et al. (14) have shown that insertion of retroviral genome into the germ line of mice was associated with spontaneous mutations. Varmus et al. (42) induced reversion in culture of transformed cells by integration of Mo-MuLV into the *gag* envelope region of the avian sarcoma virus. Furthermore, Schnieke et al. (38) have shown that integration of Moloney proviral genomes into the alpha (I) collagen gene leads to its complete block. These observations, in agreement with our own, support the notion that modifications in the coding or intervening sequences by insertion of various DNA elements may alter eucaryotic gene expression.

Analysis of polyadenylated mRNA species in L12 cells has shown that the aberrant p53 mRNA species share homology with Mo-MuLV sequences. This may suggest that these unique mRNA transcripts are a fusion product that initiates in the first exon of the p53 gene and terminates at a polyadenylation site contained within the Moloney-MuLV-like insert. The existence of a fusion p53-Moloney protein is presently being explored.

In summary, we described here an Ab-MuLV-transformed variant cell line which expresses a functional oncogene product, p120 (see Table 1), but lacks detectable concentrations of p53 cellular encoded protein. It is conceivable that the apparent absence of detectable amounts of this protein in L12 cells is caused by the insertion of a Moloney-MuLV-like particle into the first intron noncoding sequences of the principal active p53 gene. However, because the entire p53 gene remained basically intact, it is still conceivable that L12 cells may produce minute amounts of p53 which are not detected by in vivo labeled immunoprecipitating protein or by specific p53 mRNA hybridization, thus enabling these cells to enter into the cell cycle and replicate, as suggested by others (21, 23, 24). Alternatively, it is possible that L12 cells utilize a metabolic pathway which overcomes the requirement of p53 expression for cell division.

Our goal is to introduce a functional p53 gene into L12 cells and test whether expression of a functional p53 gene will change the phenotype of these cells, from cells which develop rejectable tumors in syngeneic mice into cells which are lethal for a syngeneic host. This approach may give a clue to the role p53 fulfills in cancer cells.

ACKNOWLEDGMENTS

This work was supported by a grant from the Israel Cancer Research Foundation and a grant from Leo and Julia Forchheimer Center for Molecular Genetics, The Weizmann Institute of Science. V.R. is the incumbent of the Norman and Helen Asher Career Development Chair.

We thank M. Oren of the Department of Chemical Immunology, The Weizmann Institute of Science, for fruitful collaboration and discussion during the performance of these experiments and S. Admon for excellent technical assistance.

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