DFMO and interferon and, ultimately, whether these observations can be extended to human neoplasias. In preliminary studies we have obtained similar results with metastic Lewis lung tumor (3LL) in mice. However, in L1210 leukemia (a condition in which the tumor cells are rapidly dividing), the two agents did not show any striking antitumor activity. Hence, the effectiveness of DFMO and interferon may vary with tumor growth rate and type.

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15. We thank S. Baron for the generous gift of mouse interferon, I. Fidler for supplying the B16 melanoma cell line, C. C. Chang, A. Hagan, and A. L. Rosenberger for technical assistance, and L. Parrott and B. J. Taylor for secretarial assistance.
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11 August 1982; revised 22 October 1982

The Human c-*ras*$_1^H$ Oncogene: A Mutation in Normal and Neoplastic Tissue from the Same Patient

Abstract. *The c-ras$_1^H$ oncogene can be distinguished from its normal cellular counterpart by the loss of a restriction endonuclease site. This sequence alteration is the basis of a rapid screening method for the presence of this oncogene. DNA's from 34 individuals were screened by this method, and all were homozygous for the normal allele. In contrast, DNA from a patient's bladder tumor, as well as DNA from his normal bladder and leukocytes, were heterozygous at that restriction endonuclease site. Further restriction enzyme mapping pinpointed the change in the mutant allele as being one of two nucleotides, either of which would change the 12th amino acid (glycine) in the normal c-ras$_1^H$ gene product. Point mutations in the codon for this amino acid have previously been described in a bladder tumor cell line and in the viral oncogene v-ras^H. These results indicate that the patient carried a c-ras$_1^H$ oncogene in his germ line, raising the possibility that the c-ras$_1^H$ oncogene confers a predisposition to neoplasia.*

DNA-mediated gene transfer (transfection) studies have shown that DNA from many human tumors—or from cell lines derived from tumors—transform NIH 3T3 mouse fibroblasts, whereas normal human DNA generally does not transform these cells (*1–3*). The DNA from transformed NIH 3T3 cells can be used for serial passage of the phenotype of transformation. This result led to the concept that a discrete genetic element, an oncogene, is responsible for initiation or maintenance (or both) of the transformed state.

One of these oncogenes, which was cloned from the related human bladder tumor cell lines EJ and T24 (*3, 4*), is closely related to the oncogene of the Harvey sarcoma virus (v-*ras*^{H}), as well as to a homologous genetic element present in normal human DNA (c-*ras*$_1^H$) (*5*). The biological differences between the bladder oncogene and its normal cellular counterpart were investigated by forming recombinants between segments of the viral and cellular genes and assaying these for the ability to transform cells (*6*). The segment harboring the transforming phenotype was thus mapped to a 351–base pair fragment extending from an Xma I site to a Kpn I site (Fig. 1). This fragment contains 26 nucleotides of a 5′ untranslated sequence, the first exon encoding 37 NH_2-terminal amino acids,

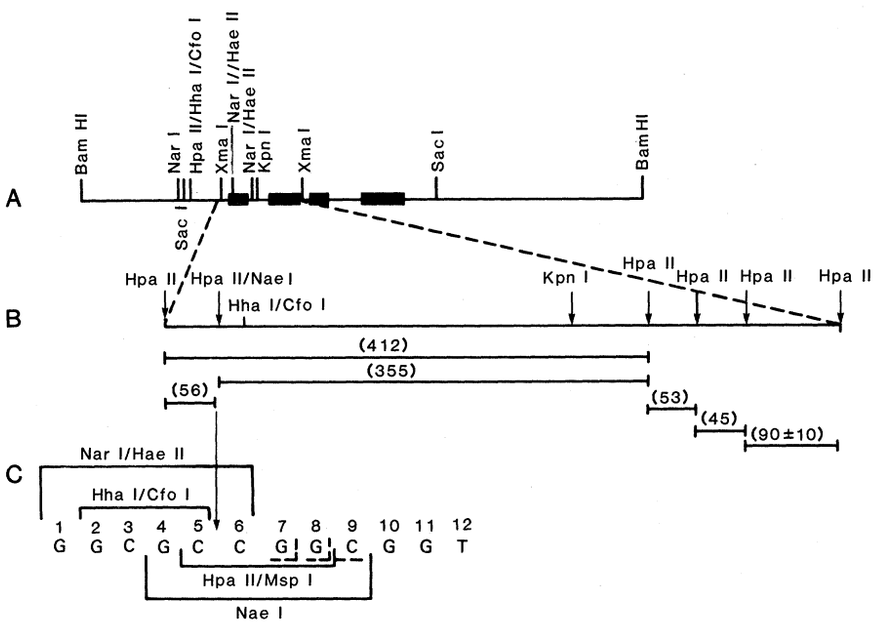

Fig. 1. Restriction map of the c-*ras*$_1^H$ gene. (A) The restriction map of the 6.6-kilobase Bam HI fragment of the c-*ras*$_1^H$ gene. The black boxes depict the coding exons. (B) The restriction enzyme map of the 600–base pair Xma I fragment. The lengths in base pairs of the various restriction fragments are shown in parentheses. The arrow from (B) to (C) extends through the Hpa II/Nae I site. (C) The nucleotide sequence surrounding that site. The numbers above the sequence have been arbitrarily assigned for reference in the text. The dotted line underscores GGC, the codon for glycine at amino acid 12. Brackets enclose the recognition sites for the indicated restriction enzymes. This figure was assembled from data in (*6*).

and 200 nucleotides from the first intron. Nucleotide sequence analysis revealed a single base change, from T to G (T, thymine; G, guanine), as the sole difference between the normal gene and the oncogene in this segment (*6*). This transversion would lead to the substitution of valine for glycine in the oncogene, a change that results in a detectable difference in the gene product, a 21,000-dalton protein (p21) (*7*), which then migrates more slowly in sodium dodecyl sulfate–polyacrylamide gels (*6*). Thus, this oncogene differs from its normal allele by a point mutation.

The alteration in nucleotide sequence in the human ras_1^H bladder oncogene fortuitously resulted in the obliteration of a restriction enzyme cleavage site [both Msp I (or Hpa II) and Nae I cleave only the normal oncogene] (*6*). This single nucleotide substitution provides the basis for a simple screening procedure that can distinguish the normal gene from the oncogene. Restriction enzyme cleavage of DNA, followed by Southern transfer (*8*) and annealing with a c-ras_1^H–specific probe should distinguish the normal gene, which is sensitive to restriction enzyme cleavage, from the mutated oncogene, which is resistant.

Since it is no longer possible to acquire normal tissue from the patient from whom the T24 cell line was established, we obtained both normal and abnormal tissue from another patient with a bladder tumor. This patient (EK) was a 59-year-old white male who underwent cystectomy for a papillary transitional cell epithelioma of the bladder. Both normal and abnormal tissue were obtained from the patient at the time of surgery for experiments described below. The analysis of such tissue relies on the assumption that this patient will have a defect in the c-ras_1^H gene at the Msp I restriction site. The subsequent data document that this assumption was reasonable.

The protocol for these studies involves complete digestion of cellular DNA samples with Hpa II or its isoschizomer Msp I (Fig. 1). If the normal allele is present, a DNA fragment of 355 nucleotides will be revealed by hybridization with a probe spanning the region of interest. If the sequence is mutated at the Hpa II/Msp I site, as it is in the c-ras_1^H oncogene, the loss of the central Msp I site will result in a fragment of 412 nucleotides. Because resistance to some endonucleases can result from the presence of methylated bases, we adopted the following strategy to assay for the presence of methylation: Msp I can cleave ^{m}CCGG or CCGG (C, cytosine; ^{m}C, methylated cytosine), while Hpa II can cleave C^{m}CGG or CCGG (*9, 10*). Identical results with both enzymes would exclude the possibility that resistance to cleavage is due to either of the singly methylated sequences indicated above, but the doubly methylated sequence ^{m}C^{m}CGG could account for resistance to both nucleases. The presence of such a double methylation at these positions (bases 5 and 6 on Fig. 1), however, would interfere with digestion by Hha I at its recognition site GCGC (bases 2 to 5 on Fig. 1), which overlaps the Msp I/Hpa II site. Hha I is unable to cleave if either or both C's are methylated (*9, 11*), but Cfo I cuts GCGC regardless of methylation (*12*). Identical cleavage patterns with both of these enzymes would indicate that methylation is not present at bases 3, 5, or 6.

A comparison of cleavage patterns can also pinpoint the site of the mutation to within two nucleotides that comprise the first two bases coding for amino acid 12 (glycine, whose codon is GGN, where N is any nucleotide). Absence of the Msp I/Hpa II site could be due to a mutation at any base within the recognition sequence CCGG, at positions 5 to 8 on Fig. 1. If Hha I and Cfo I both cleave the DNA, the alteration must then be in CGG at positions 6 to 8. This set of nucleotides overlaps the recognition sequence for the restriction endonuclease Nar I (*13*) or Hae II (GGCGCC, at positions 1 to 6 on Fig. 1). Thus, if Nar I cuts the mutant allele, its recognition sequence must be intact, and position 6 must also be a C. Hence, resistance to Msp I/Hpa II digestion could only result from a change in G at position 7 or 8. These are the crucial nucleotides in the glycine codon GGN, and any alteration at either site would encode a different amino acid.

In control experiments, exhaustive digestion with Hpa II or Msp I of DNA from the bladder tumor lines T24 or EJ, as well as the NIH 3T3 line transformed by this oncogene, generated a 410-nucleotide band (Fig. 2). In contrast, DNA from two normal kidneys, a Burkitt's lymphoma, two spleens, and the peripheral leukocytes of nine normal individuals were cleaved to a fragment consisting of 355 nucleotides (Figs. 2 and 3; other data not shown). These results demonstrate the validity of the experimental approach and suggest that both copies of c-ras_1^H alleles in many normal persons are sensitive to cleavage at the Hpa II/Msp I site.

Extensive digestion of the DNA from the bladder carcinoma of EK with either

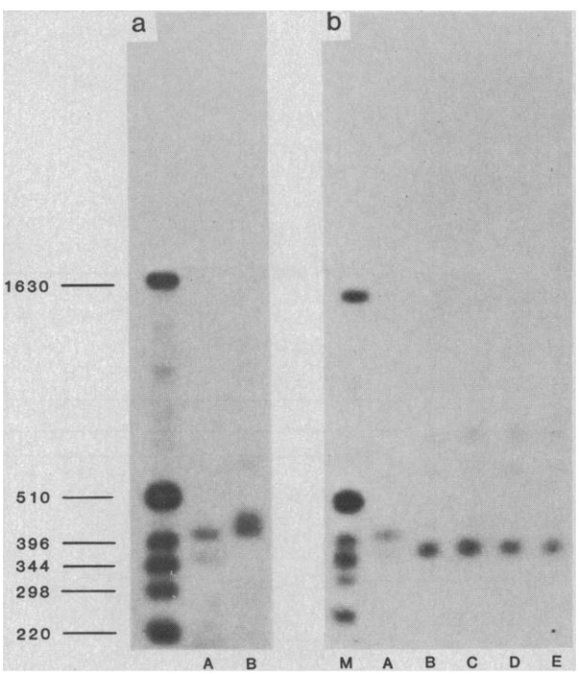

Fig. 2. Restriction enzyme analysis of tumor and normal DNA's. High molecular weight DNA was prepared from various sources (*18*) and 5 to 20 μg was digested to completion with Msp I and Hpa II (New England Biolabs). Samples were subjected to electrophoresis on 1.4 percent agarose gel at 100 mA, transferred to 0.1-μm nitrocellulose paper by the method of Southern (*8*), and hybridized [25 ml of triple-strength standard saline citrate; fivefold-strength Denhardt's solution; 0.1 percent sodium dodecyl sulfate; sheared calf thymus DNA (100 μg/ml); and 10 percent (weight to volume) dextran sulfate] for 3 to 4 hours at 68°C. The Xma I fragment of c-ras_1^H (see Fig. 1) was purified from an agarose gel (*19*) and labeled by nick translation (*20*) using α-^{32}P-labeled deoxycytidine triphosphate. The probe was added to the hybridization buffer (5×10^5 cpm/ml) and annealed at 68°C for approximately 16 hours. The filter was washed, dried, and placed against Kodak XAR-5 film for 16 hours to 1 week. The DNA sources were as follows. (a) (Lane M) ^{32}P-labeled marker obtained by Hinf I digestion of pBR322, with sizes as indicated. (Lane A) Xma I digest of two plasmids, one containing the Bam HI fragment of the c-ras_1^H oncogene from cell line EJ in pBR322, and a second with the normal c-ras_1^H gene (EC) in pBR322. This produces a 412-nucleotide band from the resistant line (EJ) and a 355-nucleotide fragment from the sensitive line (EC). (Lane B) T24 human bladder carcinoma cell line (*1*). (b) (Lanes M and A) Same as above. (Lanes B to E) Leukocytes from normal individuals.

Hpa II or Msp I alone (data not presented), or both together, (Figs. 2 and 3) gives rise to two major fragments that hybridize to the ^{32}P-labeled c-ras_1^H probe. These fragments of 410 and 355 nucleotides are of approximately equal intensity. Digestion with Kpn I, Msp I, and Hpa II results in reduction of both bands by 85 nucleotides, as predicted by the sequence (Fig. 3; data not shown). Digestion of this DNA with either Cfo I or Hha I yielded the identical fragment, 340 nucleotides long, as expected from the sequence (Fig. 4). If the site was absent, a band of 360 nucleotides was generated. This result taken together with the results of the Msp I and Hpa II cleavage indicate that methylation is not responsible for the inability to cleave the resistant allele. Cleavage by Hha I and Cfo l confirms that nucleotide 5 (Fig. 1) is a C.

Cleavage with Nar I alone did not result in complete digestion, even after extensive incubation. However, with approximately 75 percent digestion, only bands 400 nucleotides long were seen. If the site had been altered, a band at 492 would have been seen (data not shown). Analogously the cleavage pattern generated by Hae II, which recognizes PuGCGCPy (Pu, a purine; Py, a pyrimidine), results in a band at 340 instead of 432, indicating that the nucleotide at position 6 must be either a C or a T (data not shown). These results taken together strongly suggest that the mutation in EK's DNA must be at either position 7 or 8 and would therefore result in a change in the codon for amino acid 12.

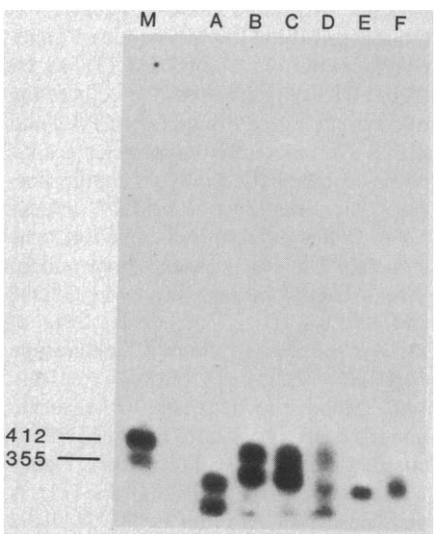

Fig. 3. Cleavage of EK DNA from bladder carcinoma, normal bladder, and leukocytes. DNA was prepared, digested, and blotted as described in Fig. 2. The lanes contain DNA from the indicated sources, digested with the stated enzymes: (lane M) the marker, as in Fig. 2; (lane A) EK carcinoma, Kpn I, Hpa II, and Msp I; (lane B) EK carcinoma, Hpa II and Msp I; (lane C) EK normal bladder wall, Hpa II and Msp I; (lane D) EK leukocytes, Hpa II and Msp I; (lane E) leukocytes from normal control, Hpa II and Msp I; and (lane F) Burkitt's lymphoma cell line, Hpa II and Msp I.

A similar analysis performed on the DNA taken from either the patient's normal bladder adjacent to the tumor, or from peripheral blood leukocytes, showed the same two bands at 410 and 355 nucleotides, indicating the presence of the same two alleles as were present in the patient's carcinoma (Fig. 3; other data not shown). Thus, the alteration identified in this gene at the Nae I or Msp I site by restriction enzyme cleavage appears to be in the germ line and must have existed before development of the bladder carcinoma.

Investigations from a number of laboratories (*6*) demonstrated that a point mutation in the cloned T24 bladder oncogene (in the codon for amino acid 12) determines its ability to transform NIH 3T3 cells. However, since this oncogene was cloned from transformed cell lines, the point mutation could have been selected for growth in tissue culture and might, therefore, be an artifact of cell culture systems. We have now identified a mutation in the same codon (amino acid 12 in c-ras_1^H) in the DNA from the EK bladder tumor. Thus, it is tempting to speculate that there is an association between this point mutation in the c-ras_1^H gene and the bladder carcinoma. Although we have no information at present regarding the frequency of the mutant c-ras_1^H gene in bladder tumors, we do know that this change is infrequent in the general population since analysis of DNA from 34 individuals revealed the presence of the Msp I/Hpa II site. More extensive screening should provide an estimate of the frequency of polymorphism at this site.

Of particular importance in this study is the finding that DNA from normal EK tissues (bladder wall and leukocytes), like the DNA from the bladder carcinoma, contained a copy of both the mutant and the normal c-ras_1^H alleles. This result suggests that the mutant allele is present in the germ line of EK; analyses of DNA from family members will clarify this point. The mutant gene, while present in most of EK's tissues from birth, was insufficient to induce neoplasia early in his life. Perhaps the mutant allele was not expressed but was later activated in the bladder tumor tissue. Alternatively, a separate event involving another gene or set of genes may have been required for carcinogenesis in vivo. There is some evidence to support the notion that a series of events may be required for malignant transformation (*2*, *14–16*) and transformation of NIH 3T3 fibroblasts may represent only a subset of those events.

These results underscore some of the limitations of the NIH 3T3 assay system. While certain alterations that represent significant elements in oncogenesis (such as mutations in the c-ras_1^H gene) can be recognized by transformation of NIH 3T3 cells, other mutations (perhaps already present in the NIH 3T3 cell line) may be undetected. Thus, the presence of a mutant allele such as the altered c-ras_1^H gene may prove to be a necessary but by itself an insufficient factor for oncogenesis. If this mutation is genetically acquired, it might render that individual more susceptible to developing a malignancy. The active oncogene may not be tissue-specific but may predispose to many types of tumors, depending on the site. Such a model could explain the high frequency of multiple primary neo-

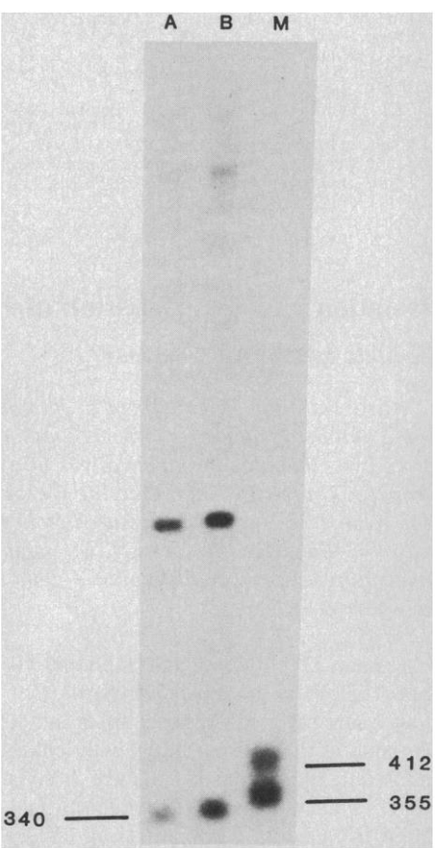

Fig. 4. Cleavage of DNA from the EK carcinoma with Cfo I or Hha I. The experiments were performed as described in Fig. 2. Lanes contain EK carcinoma DNA digested to completion with the indicated restriction enzymes: (lane A) Hha I; (lane B) Cfo I; and (lane M) Msp I and Hpa II.

plasms observed in certain persons (*17*). As more oncogenes are isolated and characterized, it may be possible to establish screening procedures to assess individuals at risk for various neoplastic diseases.

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15 November 1982; revised 27 December 1982

Isolation and Transmission of Human Retrovirus (Human T-Cell Leukemia Virus)

Abstract. *Nine new isolates of human T-cell leukemia-lymphoma virus (HTLV) were obtained from cells of seven patients with malignancies of mature T cells and from two clinically normal relatives of a T-cell leukemia patient. These people were from the United States, Israel, the West Indies, and Japan. The virus was detected in the fresh T cells and was isolated from the established T-cell lines. Each isolate is closely related to the first HTLV isolate, and all the new HTLV isolates were transmitted into normal human T cells obtained from the umbilical cord blood of newborns.*

Human T-cell leukemia-lymphoma virus (HTLV) is a type-C retrovirus that has been isolated from cells of adult patients in the United States with leukemias and lymphomas of mature T cells (*1–4*). This virus was subsequently isolated from adult T-cell leukemia (ATL) patients in Japan (*5, 6*). In this report we describe the isolation of nine new HTLV isolates from people in different parts of the world, show transmission of each of the isolates into human cord blood, and show that HTLV can be isolated not only from patients with aggressive adult T-cell leukemia but also from cases of less aggressive peripheral lymphomas and even from clinically "normal" individuals with abnormal T cells.

The new HTLV-positive T-cell lines were established from four patients in the United States, one in Israel, one in the West Indies, and three members of a family from northwest Japan (Table 1). The last three included a male patient (S.K.) with T-cell leukemia and his parents. His father (H.K.) and mother (T.K.) are clinically healthy, but the mother has abnormal lymphocytes with convoluted nuclei characteristic of leukemic T cells from patients with ATL (*7*). All the lines were derived from the peripheral blood or bone marrow of the patients by using T-cell growth factor (TCGF) as described (*8, 9*). The cells showed a decreased requirement for exogenous TCGF and potential for indefinite growth in vitro. Over 80 percent of the cells were positive for TCGF receptors (reactive with antibody prepared against the activated T cells, anti-TAC) (*10*). All T-cell lines formed rosettes with sheep erythrocytes (E-rosette positive); and they were negative for surface immunoglobulins (IgA, IgG, and IgM), Epstein Barr virus nuclear antigen (EBNA), and terminal deoxynucleotidyl transferase (TdT). In addition, the cell lines reacted with most pan-T monoclonal antibodies (up to 100 percent). No reactions were observed with monoclonal antibodies against B cells (BA-1) and thymus (OKT-6 and NAI/34) T cells. Thus, studies with both conventional and monoclonal antibody T-cell markers indicate that the HTLV-positive cell lines represent mature T cells.

The surface phenotype of these HTLV-producing cell lines was further characterized by using the monoclonal antibodies OKT-4, Leu-3A, OKT-8, and Leu-2A. Five of the seven lines from patients with leukemia consistently exhibited only the "helper-inducer" phenotype (OKT-4$^+$, Leu-3A$^+$) (Table 1). About 20 to 30 percent of the cells of the two remaining cell lines, from P.L. and S.K., also reacted with monoclonal antibodies supposedly typing a "suppressor-cytotoxic phenotype" (OKT-8$^+$, Leu-2A$^+$). Cell lines from H.K. and T.K., the clinically healthy parents of patient S.K., reacted either only with OKT-8 and Leu-2A (H.K.) or, in the case of T.K., exhibited a "double" phenotype (OKT-8$^+$, Leu-2A$^+$, OKT-4$^+$, Leu-3A$^+$). Thus, the data support observations that primary tumor cells of HTLV-positive malignancies are mature T cells with the OKT-4, Leu-3A subtype (*11*). A high percentage of the cells (69 to 92 percent) are TCGF receptor positive as estimated by the direct response of these cells to purified human TCGF (*12*) and by their reactivity with a monoclonal antibody to TCGF receptor (anti-TAC) (*13*). All cell lines are diploid, possess distinct histocompatibility antigen (HLA) profiles, and match the karyotype of the primary donor cells.

The cell lines were analyzed for the presence of HTLV by competition radioimmunoprecipitation assay (RIPA)