MAMMALIAN CELL GENETICS

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The use of DNA-mediated gene transfer led to the discovery of dominant-acting transforming genes in nonvirally transformed cells (Shih et al., *Proc. Natl. Acad. Sci. 76:* 5714 [1979]). For the past year, our major effort has been the isolation and characterization of transforming genes we previously identified in human neuroblastoma and bladder, lung, and colon carcinoma cell lines (Perucho et al., *Cell 27:* 467 [1981]). Considerable progress has been achieved in isolating the transforming gene common to lung and colon carcinomas. The transforming gene from a neuroblastoma cell line and its normal counterpart from placental DNA have both been fully cloned. Finally, the transforming gene of the bladder carcinoma cell line T24 and its normal counterpart have been cloned, sequenced, and compared, and we understand at the nucleotide level the difference between them. The transforming gene encodes a protein that differs at one amino acid from that encoded by the normal gene (Taparowsky et al., *Nature 300:* 762 [1982]). This difference accounts for its biological activity in the NIH-3T3 focus assay system. How this change in protein structure leads to cellular transformation is now our major concern.

Isolation and Characterization of the Transforming Gene of T24 Bladder Carcinoma Cells

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Last year, we reported isolating the transforming gene of T24, a human cell line derived from a bladder carcinoma (Goldfarb et al., *Nature 296:* 404 [1982]). The method of isolation was the tRNA suppressor method developed here by Kenji Shimizu. Hybridization studies of this gene indicated that it was human in origin and that its chromosomal locus displayed a high frequency of restriction fragment length polymorphisms (RFLPs) (Goldfarb et al., *Nature 296:* 404 [1982]). From this observation and blotting to human DNAs, we have been able to infer that this gene is present just once per haploid chromosome complement. This gene has been used as a probe by Ray White and colleagues (Howard Hughes Medical Institute in Salt Lake City, Utah) to demonstrate that hereditary predisposition to colon cancer is *not* linked to this gene (Barker et al. [1983, in prep.]).

Workers from other labs demonstrated that the T24 gene was the human homolog to the viral oncogene v-Ha-*ras*, encoding a 21,000-dalton protein (p21) (Der et al., *Proc. Natl. Acad. Sci. 79:* 3637 [1982]); Parada et al., *Nature 297:* 474 [1982]; Santos et al., *Nature 298:* 343 [1982]). This result has been confirmed by us (Shimizu et al., *Proc. Natl. Acad. Sci.* [1983, in press]) and provided the first instance of a gene, previously identified by its presence in oncogenic retroviruses, implicated in human cancer.

Our initial work on this gene has been to determine why it has transforming activity. The suppressor-rescued gene was used as probe to isolate the intact gene from both T24 DNA and placental DNA λ L47.1 libraries. No isolates from the placental DNA were capable of inducing foci on NIH-3T3 cells, whereas all isolates from the T24 library were efficient at focus induction in NIH-3T3. This indicated that the difference between the normal and transforming genes was indeed at the DNA level. By constructing chimeric genes—recombinants between the normal and tranforming genes—we were able to determine that a small 224-bp *Mst*II/*Xba*I fragment of the transforming gene contained the functionally altered sequence. Sequencing this region for the tranforming gene, the normal gene, and a cDNA copy to the transforming gene transcript indicated only one significant base change occurring in the coding portion of the first coding exon whereby the 12th N-terminal amino acid was changed from glycine in the normal to valine in the transforming *ras* p21 (Taparowsky et al., *Nature 300:* 762 [1982]). These sequence changes were also reported by others (Reddy et al., *Nature 300:* 149 [1982]; Tabin et al. *Nature 300:* 143 [1982]). Cotransformation into NIH-3T3 cells of either the normal or transforming gene indicated that indeed the *ras* p21 of T24 was 100–1000 times more efficient than the normal *ras* p21 at inducing the transformed phenotype in NIH-3T3 cells (Taparowsky et al., *Nature 300:* 762 [1982]). Taken together, at the DNA level, the molecular changes resulting in a cancer gene have been defined. Most importantly, these results indicate that, by a very minor alteration, a normal cellular protein can be converted to a transforming protein. Curiously, alterations in the 12th amino acid are also found in the viral *ras* oncogenes, suggesting this amino acid residue plays a critical role in the normal function of this protein. (Similar lesions were found in this gene isolated from the human bladder carcinoma cell line EJ. However, in collaboration with Jorgen Fogh and colleagues at the Sloan Kettering Cancer Research Institute in Rye, New York, we demonstrated that the cell line called EJ probably arose as a subline of T24.)

A complete cDNA to the T24 Ha-*ras* transcript was cloned and sequenced and compared with the genomic Ha-*ras* gene. The human Ha-*ras* gene has at least five exons, and the complete coding sequence is contained in four of these. We have not yet identified the promoter of the Ha-*ras* gene nor

have we located the 5′ untranslated sequences of the mature message. By making genomic/cDNA chimeras, we have established that introns are required for the efficient expression of this gene.

We made comparisons of the complete nucleotide sequence of the human Ha-*ras* gene with the viral Ha-*ras* gene, previously published by others (Dahr et al., *Science 217:* 934 [1982]). This resulted in discovering a remarkable conservation in amino acid sequence (Fasano et al., *J. Mol. Appl. Genet.* [1982, in press]). Although 30% divergence in neutral base positions was observed, only 3 amino acids out of 189 were different. In particular, there was complete conservation at the carboxyl end of the proteins, the regions where the viral Ki-*ras*, another gene in the *ras* family, and the viral Ha-*ras* diverge markedly (Dahr et al., *Science 217:* 934 [1982]; Tsuchida et al., *Science 217:* 937 [1982]). We conclude that this region may distinguish the physiologic functions of the *ras* genes.

Isolation and Characterization of the Lung and Colon Carcinoma Transforming Gene

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Previous work identified a large gene of human origin with transforming activity present in several lung and colon carcinoma cell lines (Perucho et al., *Cell 27:* 467 [1981]). Identification was made based on the presence of conserved *Eco*RI digest fragments containing human repeat sequences in NIH-3T3 cells transformed with DNA from these donors. The first fragment of this gene was cloned by screening λ Charon 4A libraries of NIH-3T3 secondary transformants derived from Calu-1, a human lung carcinoma cell line, with blur 8, a cloned representative of the *Alu* family of human repeat sequences (Shimizu et al., *Proc. Natl. Acad. Sci.* [1983, in press]). Subsequent fragments of this gene were obtained by chromosomal walking (Shimizu et al., and work in progress). The resulting isolation and mapping of over 20 overlapping phage inserts have led to the construction of a composite restriction map. Der et al. (*Proc. Natl. Acad. Sci. 79:* 3637 [1982]) demonstrated that another lung carcinoma cell line, LX-1, carried a transforming gene that was closely homologous to the viral Kirsten *ras* (Ki-*ras*) gene. The Ki-*ras* and Ha-*ras* genes are related members of a diverged family. They encode immunologically cross-reactive 21,000-dalton proteins and share considerable nucleotide homology. Hybridization studies on our cloned gene also indicated its close relation to Ki-*ras*, and its identity to the transforming gene of LX-1. Southern blotting with cloned viral Kirsten sequences as probe has identified the potential coding blocks of this gene. Although the gene product is the same size as the Ha-*ras* gene product, the human Ki-*ras* is 20 times the size (40 kbp) of the human Ha-*ras* gene and contains one more coding block. Preliminary sequence data indicates considerable homology between the second and third coding blocks of human Ki-*ras* and Ha-*ras*. Indeed, they appear to utilize the same splice site. We predict, therefore, that the human Ki-*ras* gene contains either a pseudo-exon or that exon choice exists, possibly in a developmentally regulated fashion, with an alternate third or fourth coding exon. As indicated from our previous studies, we believe these last two exons contain the protein determinants of physiologic specificity.

We have also begun to isolate the human Ki-*ras* gene from a placental DNA library but have not yet done sequence comparison of the normal and transforming genes to determine the potential candidate sites of functionally significant biological alteration.

Work from other labs has implicated this gene in pancreatic carcinoma and osteosarcomas (Pulciani et al., *Nature 300:* 539 [1982]).

Isolation and Characterization of the Transforming Gene of a Human Neuroblastoma Cell Line

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Transforming activity was found in the DNA derived from SK-N-SH, a human neuroblastoma cell line (Perucho et al., *Cell 27:* 467 [1981]). This past year, we succeeded in cloning the gene responsible for this activity (Shimizu et al., *Proc. Natl. Acad. Sci. 80:* 383 [1983]) using the methodology of tRNA suppressor rescue that had been developed here by Kenji Shimizu (see last year's report). Hybridization studies indicated that this gene was of human origin, since it had a normal counterpart in the DNA of all human material we examined. Further hybridization studies showed that the transforming gene of SK-N-SH was related to both the Ha-*ras* and Ki-*ras* genes. It encodes an immunologically cross-reactive 19.5-kD protein. Indeed, it constitutes a third member of the *ras* gene family, and all three *ras* genes are about equidistant in nucleotide divergence from each other. We have termed this gene N-*ras*. It is intermediate in size between Ha-*ras* and Ki-*ras*, a biologically active fragment being less than 12 kb and probably less than 9 kb. Its intron/exon structure has not yet been determined nor has sequence comparison to the other *ras* genes begun.

In contrast to T24, SK-N-SH contains both normal and transforming copies of this gene. The homolog cloned from placental DNA is also not active in the NIH-3T3 focus assay. Therefore, the difference between the transforming and normal genes lies at the nucleotide level. We are beginning to perform systematic recombination experiments between the normal and transforming versions of this gene to determine the nucleotide alterations that result in functionally altered biological activity. To facilitate this, we are in the process of isolating cDNA clones of the N-*ras* transcript. Preliminary results from

other labs have begun to indicate that this gene is implicated in a very wide range of tumors.

Other Assays for Transforming Genes

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It is a significant and curious result that all the transforming genes detectable by us with the NIH-3T3 focus assay are members of the *ras* gene family. On the one hand, this implies that the alteration by mutation of *ras* genes may be an important step in the development of many kinds of human cancer. On the other hand, it may point to a bias in the assay system that readily scores altered *ras* genes but is insensitive to many other tumor genes. For this reason, we have begun to develop another assay system, still utilizing NIH-3T3 cells as recipients for DNA-mediated transfer, but scoring for the transformation event by injecting cells exposed to DNA directly into nude mice. These experiments are being performed in collaboration with Jorgen Fogh of Sloan Kettering Cancer Research Institute, Rye, New York. Preliminary results indicate that this method will be sensitive to genes previously undetectable by the focus assay system.

Publications

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