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Human-Tumor-Derived Cell Lines Contain Common and Different Transforming Genes

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Summary

We have screened different cultured cell lines established from human tumors for the ability of their DNAs to induce transformed foci in NIH/3T3 cells. Based on restriction endonuclease digestions and the presence of human sequences in mouse transformants, we conclude that five of these human tumor cell lines contain a gene or genes capable of transforming mouse cells and that at least three different transforming genes are present in these five lines. Three cell lines, two derived from lung carcinomas and one derived from a colon carcinoma, transfer the same or closely related human genes. If these transforming genes are mediating the tumorigenic state of the human cells, then our results indicate that overlapping pathways leading to tumorigenesis may arise independently.

Introduction

DNA-mediated gene transfer is a powerful tool for genetic analysis. Certain cultured cell lines stably incorporate and express foreign genes in DNA presented as a calcium phosphate coprecipitate (Wigler et al., 1977; Andersson et al., 1979), and even single-copy genes can be so transferred when total genomic DNA is used as donor (Wigler et al., 1978, 1979a; Peterson and McBride, 1980). Thus complementation of a biochemical defect or transfer of a growth-altered phenotype is possible when genomic DNA from an appropriate source is used. In this manner, Shih et al. (1979) demonstrated the morphological transformation of NIH/3T3 mouse cells using DNA from methylcholanthrene-transformed mouse fibroblasts, suggesting the existence of dominant-acting transforming genes in those cells. More recently, Shih et al. (1981) demonstrated that DNAs extracted from a variety of rodent tumor cell lines and from one human tumor cell line are capable of transforming NIH/3T3 cells. Similarly, Krontiris and Cooper (1981) have demonstrated transformation of NIH/3T3 cells with DNA from human tumor cell lines. Studies such as these may provide profound insights into the origins of human cancer. We report studies on DNA transfer from 21 different human tumor cell lines with NIH/3T3 as recipient. Based on the presence of human sequences in mouse transformants, we conclude that five of these 21 tumor cell lines contain a gene or genes capable of transforming mouse cells. At least three different transforming genes are present in these lines, and three cell lines, one derived from colon and two from lung carcinomas, transfer the same or a closely related human gene or genes.

Results

Screening Human-Tumor-Derived Cell Lines

For our transformation studies, we have used as donors DNAs from cell lines derived from many types of human tumors. Extensive studies, most recently an analysis of electrophoretic mobility of polymorphic enzymes (Wright et al., 1981), have shown that cellular contamination between these lines is not evident. The origins of these cell lines are listed in Table 1. As recipient, we have used the NIH/3T3 cell line (Jainchill et al., 1969). The transformation assay is based on the development of a dense focus of morphologically altered cells appearing against the background of a monolayer of semiconfluent cells several weeks after treatment with donor DNA. This assay, commonly used to demonstrate transformation by DNA of viral origin, was first used by Weinberg and colleagues to demonstrate the existence of transforming genes of cellular origin (Shih et al., 1979). High molecular weight DNAs (greater than 50 kb) were prepared from each line, and DNA transfer was performed by the calcium phosphate coprecipitation method as described in Experimental Procedures. Since we have observed variation in the ability of some DNA preparations to serve as gene donors, we monitored all DNAs for their ability to serve as donors of the thymidine kinase (*tk*) gene to LMtk$^-$ cells. Only experiments with good *tk* donors are reported (see Table 1).

Detection of Transformed Foci after Transfer with DNA from Human Tumor Cell Lines

Two predominant types of foci were observed in NIH/3T3 cells after treatment with DNA (see Figure 1). Type I foci consisted of cells that were spindle shaped and refractile by phase contrast microscopy. Although the cells grew on one another, large intercellular spaces were a hallmark of these foci in their early stages. These foci resembled those observed when NIH/3T3 cells were treated with DNA from cells transformed by either Harvey murine sarcoma virus or Rous avian sarcoma virus. Type II foci consisted of piled up cells that were sometimes spindle shaped and disoriented; but the cells were not refractile, and the foci never had large intercellular spaces. Other types of foci were seen, which lacked one or more of the characteristics of the type I focus. In our screening, we picked cells from foci of all types and grew them

Table 1. Transformation Efficiencies of DNA from Human Tumor Cell Lines

Cell Line[a]	Origin	References	Transfer Efficiency		Total Number of		Human Sequ.[f]	Retrans.[g]
			tk[b]	Foci[c]	Expts.[d]	Foci[e]		
T24	Bladder carcinoma	Bubenik et al., 1973	1.5 ± 0.5	2.0 ± 1.0	2	>300	7/7	3/3
Calu-1	Lung carcinoma	Fogh and Trempe, 1975	2.0 ± 1.0	0.15 ± 0.1	3	35	7/7	5/5
SK-LU-1	Lung carcinoma	Fogh et al., 1977a	2.2 ± 0.4	0.08 ± 0.02	3	15	11/12	3/3
SK-CO-1	Colon carcinoma	Fogh and Trempe, 1975	1.6 ± 0.6	0.05 ± 0.03	3	14	4/4	3/3
SK-N-SH	Neuroblastoma	Biedler et al., 1973	1.0 ± 0.3	0.05 ± 0.03	4	18	2/3	2/3
SK-HEP-1	Liver adenocarcinoma	Fogh and Trempe, 1975	1.75	~0.005	2	1	1/1	1/1
SW-1088	Astrocytoma	Fogh et al., 1977b	1.50	~0.005	2	1	1/1	1/1
J82	Bladder carcinoma	Fogh, 1978	0.42	<0.005[h]	2	2	0/2	0/2
RT4	Bladder carcinoma	Rigby and Franks, 1970	1.8	<0.005[h]	2	1	0/1	0/1
C-4-II	Cervix carcinoma	Auersperg, 1964	0.5	<0.005[h]	2	3	0/3	0/2
RMPI-2650	Nasal septum carcinoma	Moore and Sandberg, 1964	2.1	<0.005[h]	2	1	0/1	0/1
IMR-32	Neuroblastoma	Tumilowicz et al., 1970	2.5 ± 0.8	<0.005[h]	2	1	0/1	0/1
575A	Bladder carcinoma	Fogh, 1978	0.75 ± 0.05	<0.005	2	0		
SK-BR-3	Breast carcinoma	Fogh and Trempe, 1975	0.4 ± 0.2	<0.002	4	0		
734 B	Breast carcinoma	Soule et al., 1973	0.8 ± 0.2	<0.003	3	0		
Cama-1	Breast carcinoma	Fogh et al., 1977a	0.5	<0.003	3	0		
HT-29	Colon carcinoma	Fogh and Trempe, 1975	0.3	<0.005	2	0		
SW-594	Fibrosarcoma	Fogh et al., 1977a	0.75 ± 0.05	<0.005	2	0		
T-98	Glioblastoma	Fogh et al., 1977a	2.2 ± 0.6	<0.005	2	0		
SK-MES-1	Lung carcinoma	Fogh and Trempe, 1975	0.4 ± 0.1	<0.005	2	0		
Caki-2	Renal carcinoma	Fogh and Trempe, 1975	0.7	<0.005	2	0		
Controls	Human placenta DNA		0.5	<0.008[h]	2	2	0/2	0/2
	NIH/3T3 DNA		0.2 ± 0.1	<0.0005[h]	18	4		0/3

Transfection assays were performed by the addition of 30 μg DNA to each of three plates of NIH/3T3 cultures, and foci were scored after 2 to 3 weeks, as described in Experimental Procedures. In many experiments, more of the same DNA precipitate was added to LMtk$^-$ cells and tk$^+$ colonies selected in HAT medium.

[a] The names of the cell lines are listed, and their origin. The properties of these cell lines are summarized in Wright et al., 1981.

[b] HAT-resistant colonies per microgram DNA. One value is listed if *tk* activity was analyzed once or the average value if analyzed twice.

[c] The average values of foci per microgram from several independent experiments are listed. Foci containing no human sequences are not counted. See text for details and footnote h below.

[d] Each experiment tested 90 μg DNA as described above.

[e] Total number of type I foci obtained in the total number of experiments.

[f] Presence of human sequences in primary transformants as analyzed by blot hybridization with the blur 8 recombinant plasmid as probe. Number scored positive/number analyzed.

[g] Ability to retransform NIH/3T3 cells with DNA from focus-derived cells. Number of type I foci scored positive/number analyzed.

[h] These transformation efficiencies have been calculated discounting type I foci, since DNA from none of these contained human sequences or was able to retransform 3T3 cells.

into mass culture for further characterization. Cells derived from type I and type II foci bred true, retaining their original morphological features upon mass culture. However, only DNA derived from type I foci were capable of reproducibly inducing foci in NIH/3T3 in a second round of DNA transfer experiments. Moreover, there was a correlation between the incidence of type I focus induction and the choice of the human donor DNA, while no consistent correlation was observed for the other types of foci.

To clarify which foci types arose from stable DNA transfer, we examined DNA from cells derived from all foci types for the presence of human sequences. We know from our previous studies on DNA transfer that cells which take up a selectable biochemical marker also incorporate variable amounts of additional DNA (up to 10^4 kb) from the calcium phosphate coprecipitate (Perucho et al., 1980; Wigler et al., 1979b). It therefore seemed likely that NIH/3T3 cells transformed with human DNA would contain copious human sequences.

We examined DNA from cells derived from NIH/3T3 foci for the presence of human repetitive sequences by blot hybridization. As controls, we examined ran-

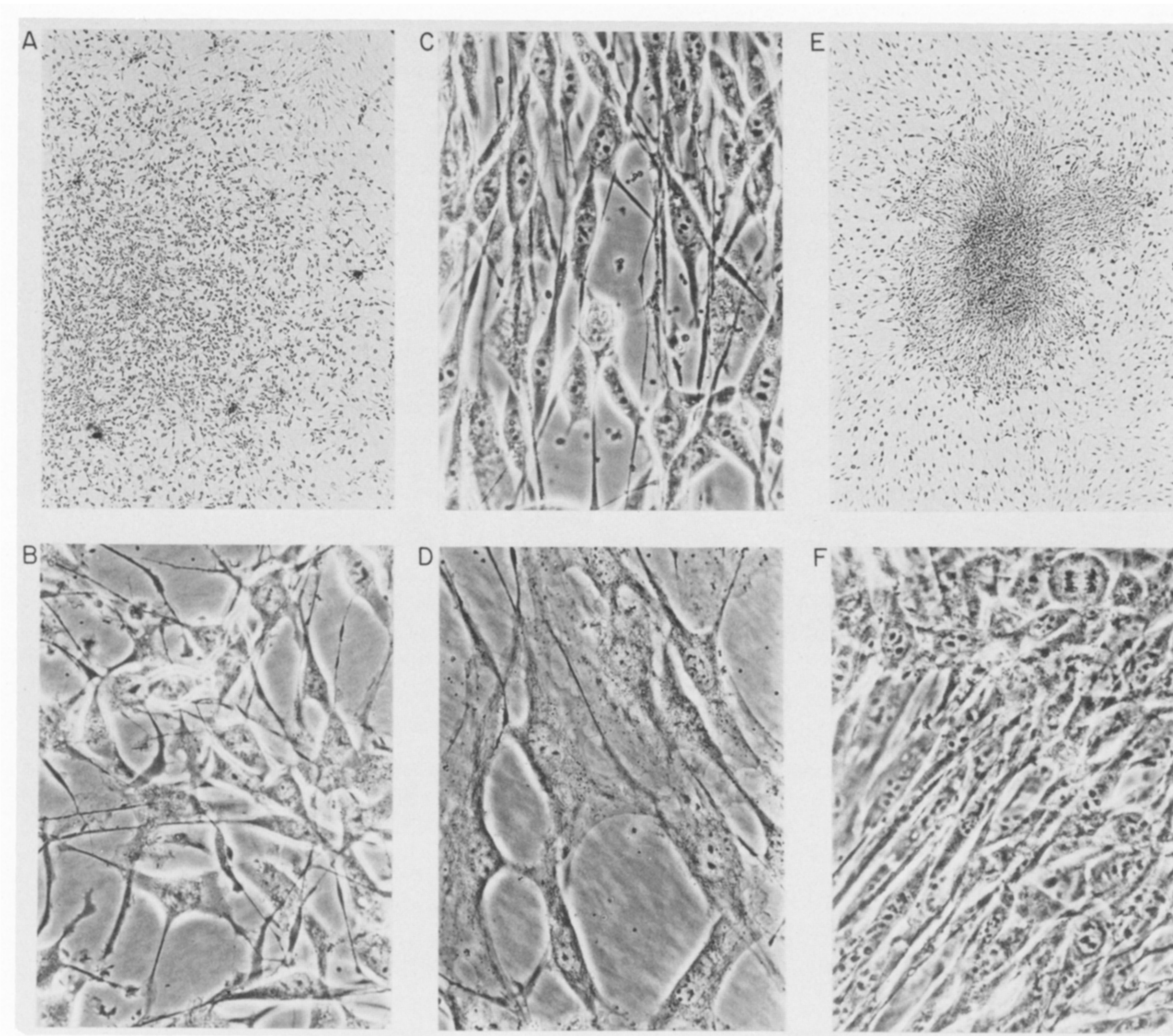

Figure 1. Morphology of NIH/3T3 Cells from Type I and II Foci Induced by DNA of Different Human Tumor Cell Lines

Culture dishes containing foci of transformed NIH/3T3 cells were washed in PBS, fixed with 2% glutaraldehyde in PBS for 30 min at room temperature, washed again in PBS and photographed with a Zeiss photomicroscope II. (A and E) Low magnification pictures (12.5×) of type I (A) and II (E) foci induced by DNA from the T24 bladder carcinoma cell line. (B and F) High magnification picture (200×) of cells from the same type I (B) and II (F) foci. (C) High magnification picture of cells from a type I focus induced by DNA from the Calu-1 lung carcinoma cell line. (D) High magnification picture of mouse background NIH/3T3 cells.

domly picked clones of NIH/3T3 cells from transfer experiments after exposure to human DNA. The probe in these hybridizations was ^{32}P-labeled blur 8 plasmid DNA. Blur 8 is a recombinant clone of pBR322 plasmid containing a human DNA sequence belonging to the interspersed, highly repeated Alu family (Jelinek et al., 1980). Figure 2 shows typical results of such studies. Blots of 3T3 DNA (see Figure 2, lane A, and Figure 4, lane 0) occasionally show hybridizing bands. These are not reproducible (see Figure 5, lane A, and Figure 6, lane 11) and presumably reflect minor variations in hybridization conditions between experiments. Most cells derived from type I foci contained copious Alu family sequences (see Table 1 and Figures 2, 4 and 6), while cells derived from other types of foci (0/10) and the randomly picked clones (0/18) did not. Type I foci (15/15) containing human sequences induced with DNA from different human tumor cell lines were highly tumorigenic in nude mice, while our NIH/3T3 cells were not (unpublished observations).

We conclude from these studies that most type I foci arise from true DNA transfer events, while foci of other morphological types do not. In these experiments in which human DNAs are used as donors, we define primary transformants as cells derived from type I foci that have acquired human sequences. In subsequent experiments (see below), DNA from primary transformants have been used to transform 3T3 cells in a second round of DNA transfer. We refer to

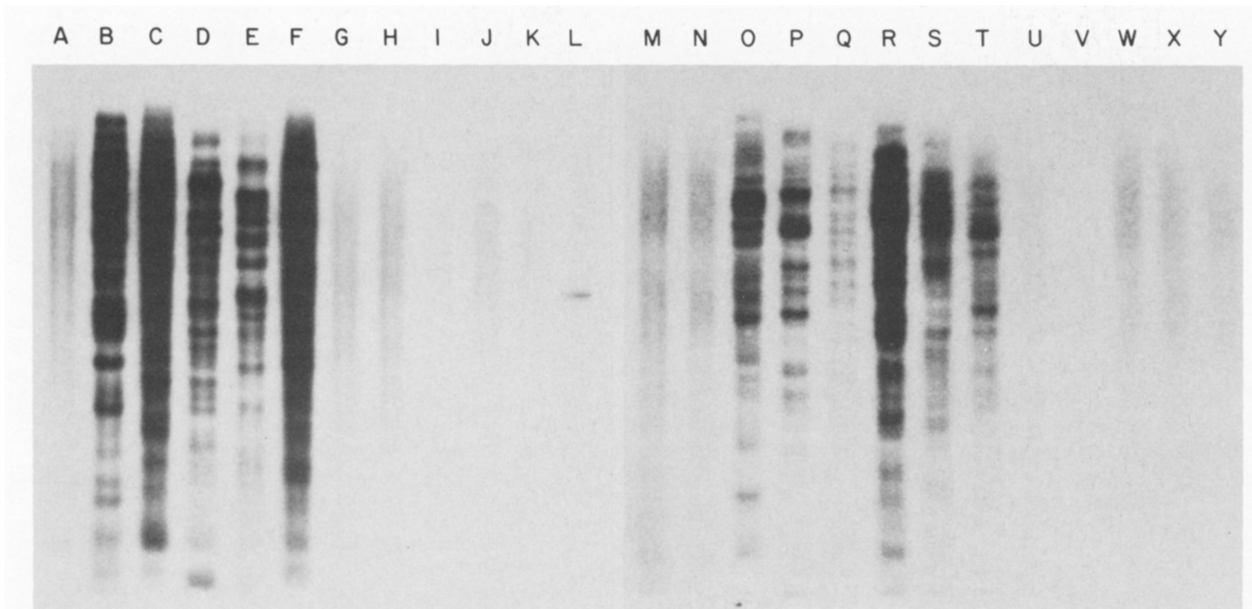

Figure 2. Presence of Human Sequences in NIH/3T3 Transformants and Random Subclones

High molecular weight DNA was prepared from individual type I foci (lanes B to F, O to Y), from type II foci (lanes M and N) and from random subclones (lanes G to K) of NIH/3T3 cells after transfection with DNA from different human tumor cell lines. Ten micrograms of each were digested with Eco RI, subjected to electrophoresis in 1% agarose gels and analyzed by blot hybridization with the blur 8 recombinant plasmid (3 $\times$ 10^8 dpm/μg) as probe. Lane A, NIH/3T3 parental line DNA; lanes B to F, five type I foci derived from the SK-LU-1 lung carcinoma cell line; lanes G to K, cells from the same culture dishes from which the previous type I foci were isolated were picked with cloning cylinders from different areas and replated, and individual colonies were picked after 10 days, grown into mass culture and analyzed; lane L, 50 pg Eco RI-cleaved blur 8 plasmid, with 10 μg NIH/3T3 DNA as carrier; lanes M and N, two type II foci that arose after transfection with DNA from the T24 bladder carcinoma cell line; lanes O, P and Q, three type I foci derived from the same line; lanes R, S and T, three type I foci derived from the Calu-1 lung carcinoma cell line; lanes U to Y, type I foci derived from lines J82 (lane U), RT4 (lanes V and W), RPMI 2650 (lane X) and IMR32 (lane Y), which were all scored as negative (see Table 1).

cells from the resulting type I foci as secondary transformants.

Efficiencies of Focus Induction with DNAs from Human Tumor Cell Lines

We have determined the efficiencies of focus induction on 3T3 cells after transfer of DNA from 21 different human-tumor-derived cell lines. These results are shown in Table 1. Only type I foci have been scored, and in many cases cells from these foci have been tested for the presence of human sequences.

DNA from a single cell line, T24, derived from a bladder carcinoma, induced foci at a frequency greater than one focus per microgram DNA. DNAs from primary transformants of T24 induce foci at the same frequency or at a frequency a few fold higher than T24 DNA itself. Several other bladder cell lines tested were negative for foci induction, including J82, which Krontiris and Cooper (1981) previously reported as positive. The reasons for this discrepancy are unclear.

DNA from four other cell lines, derived from two distinct lung carcinomas (Calu-1 and SK-LU-1), a colon carcinoma (SK-CO-1) and a neuroblastoma (SK-N-SH), gave rise to transformed foci at frequencies from 0.05 to 0.2 foci per microgram DNA. The frequency of transfer with these DNAs is thus lower by a factor of 10–50 than the frequency of transfer with T24 DNA. DNAs from primary transformants derived from these donors induce foci in NIH/3T3 at the same frequency or at a higher frequency than the original donor DNA.

DNAs from two cell lines, derived from a hepatoma (SK-HEP-1) and an astrocytoma (SW-1088), have given rise to only one focus each that contains human Alu sequences (a frequency less than one focus per 180 μg DNA cumulative). DNAs from the primary transformants induced by these donors were quite efficient as donors in second round transformations. The origin of these primary transformants is unclear. One possibility is that the primary transformants contain transforming genes which have arisen by rearrangement of normal genes during the process of DNA transfer and uptake (Cooper et al, 1980).

The rest of the cell lines analyzed have never been observed to give rise to foci containing human Alu sequences in several independent assays (over 180 μg DNA tested per cell line), despite high transfer frequencies of the *tk* gene. Included in this group are several donors that gave rise to type I foci in scattered experiments. In all cases, these foci did not contain detectable human Alu sequences, and DNA from them could not induce secondary transformants in NIH/3T3.

Restriction Endonuclease Sensitivity of DNA from T24 and One of Its Primary Transformants, and a Primary Transformant from SK-N-SH

One method for obtaining information about putative transforming genes is restriction endonuclease digestion of donor DNA followed by transformation assays. DNAs from T24 and one of its primary NIH/3T3 transformants, T24-a2, were cleaved with a variety of restriction endonucleases. These cleaved DNAs were assayed for their ability to induce foci in NIH/3T3, as described in Experimental Procedures. The results are presented in Table 2. Both donors have the same profile of restriction endonuclease sensitivity. This indicates that the same gene is present in the primary transformant as is present in the original donor. This gene has a spectrum of sensitivity different from that reported for the DNA from another bladder-carcinoma-derived cell line, EJ (Krontiris and Cooper, 1981). Transformation studies with sucrose gradient, size-fractionated Eco RI-digested T24-a2 DNA indicate that all the transforming activity of the total digest migrates as a single peak from 8 to 15 kb in size (Figure 3). These results indicate that the transforming principal is a discrete entity and not a multitude of potential transforming genes contained in the T24 donor.

A similar restriction analysis was performed on an NIH/3T3 transformant, SK-N-SH-a1, derived from the neuroblastoma cell line SK-N-SH (Table 2). The data clearly indicate that the gene contained in this line differs from that contained in T24.

Table 2. Effect of Restriction Endonuclease Digestion on Transforming Activities of T24 Bladder Carcinoma, T24-a2 and SK-N-SH-a1 Primary Transformant DNAs

Restriction Endonuclease	T24 DNA (foci/75 μg)	T24-a2 DNA (foci/20 μg)	SK-N-SH-a1 DNA[a] (foci/25 μg)
None	11	6	18
Bam HI	15	8	0
Bgl II	7	4	ND
Hind III	13	10	0
Eco RI	7	34[a]	0
Xho I	12	5	ND
Kpn I	0	0	ND
Xba I	0	0	ND
Sal I	ND	ND	35

[a] These DNAs were tested in a separate experiment.

DNA from T24 human bladder carcinoma cell line (75 μg), from T24-a2 primary transformant (20 μg) and from SK-N-SH-a1 primary transformant induced by the neuroblastoma cell line SK-N-SH (25 μg) was digested with one of the indicated restriction endonucleases. Cleaved DNA was purified and mixed with a twofold excess of NIH/3T3 carrier DNA. DNAs were applied to cultures of NIH/3T3 cells following calcium phosphate precipitation, and cultures were scored for transformed foci 17 days later.

ND: not done.

Presence of Human Alu Family Sequences in Secondary Transformants

As we have already indicated, primary transformants contain human Alu family sequences. Some or all of these sequences arise by cotransfer and are not necessarily linked to the transforming gene or genes in the original donor. If any of these sequences are closely linked to the transforming gene or genes, we would expect that they would be retained in secondary transformants derived from independent primary transformants. We would expect unlinked sequences to be lost upon secondary transformation. To examine this possibility, we analyzed DNAs from secondary transformants by Southern hybridization for the presence of human Alu family sequences. The results of these experiments are shown in Figures 4, 5 and 6.

From our two lung donors, Calu-1 and SK-LU-1, we derived a number of primary transformants. DNA from

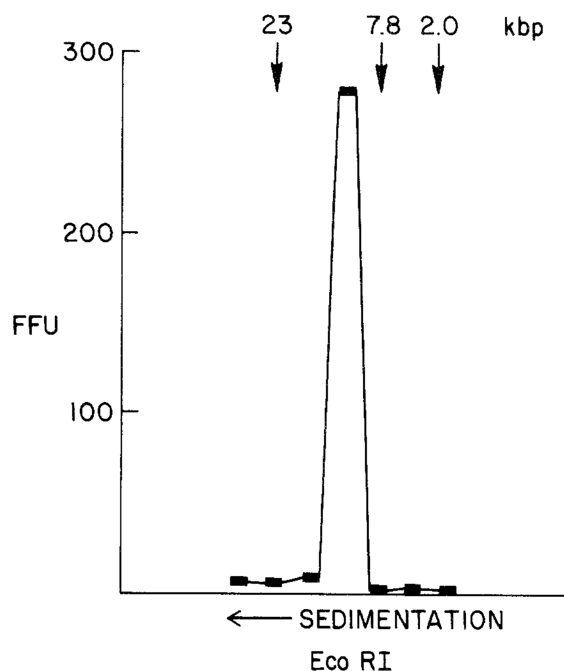

Figure 3. Transformation of 3T3 Cells with Sucrose-Gradient-Fractionated T24-a2 DNA

Eco RI-cleaved DNA (180 μg) from the 3T3 primary transformant cell line T24-a2 was sedimented through a 36 ml 15% to 40% w/v sucrose gradient (in 100 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA) at 23,000 rpm for 27 hr at 20°C in a Beckman SW27 rotor. We collected 1.5 ml fractions and determined the molecular weight range of DNA fractions by subjecting fraction aliquots to agarose gel electrophoresis, using Hind III-cleaved λ DNA as marker. Approximate molecular weights of DNA molecules through the gradient are shown at top of figure. The DNA fractions in the 1–30 kb size range were adjusted to 30 μg by the addition of NIH/3T3 carrier DNA, and pairs of adjacent fractions were pooled and assayed for their ability to transform NIH/3T3 cells. The figure shows the number of foci obtained (FFU) from each pair of fractions. Unfractionated Eco RI-cleaved T24-a2 DNA was also tested for transforming activity. The transforming efficiencies of fractionated versus unfractionated DNA indicated that there was no loss in transforming activity following gradient fractionation.

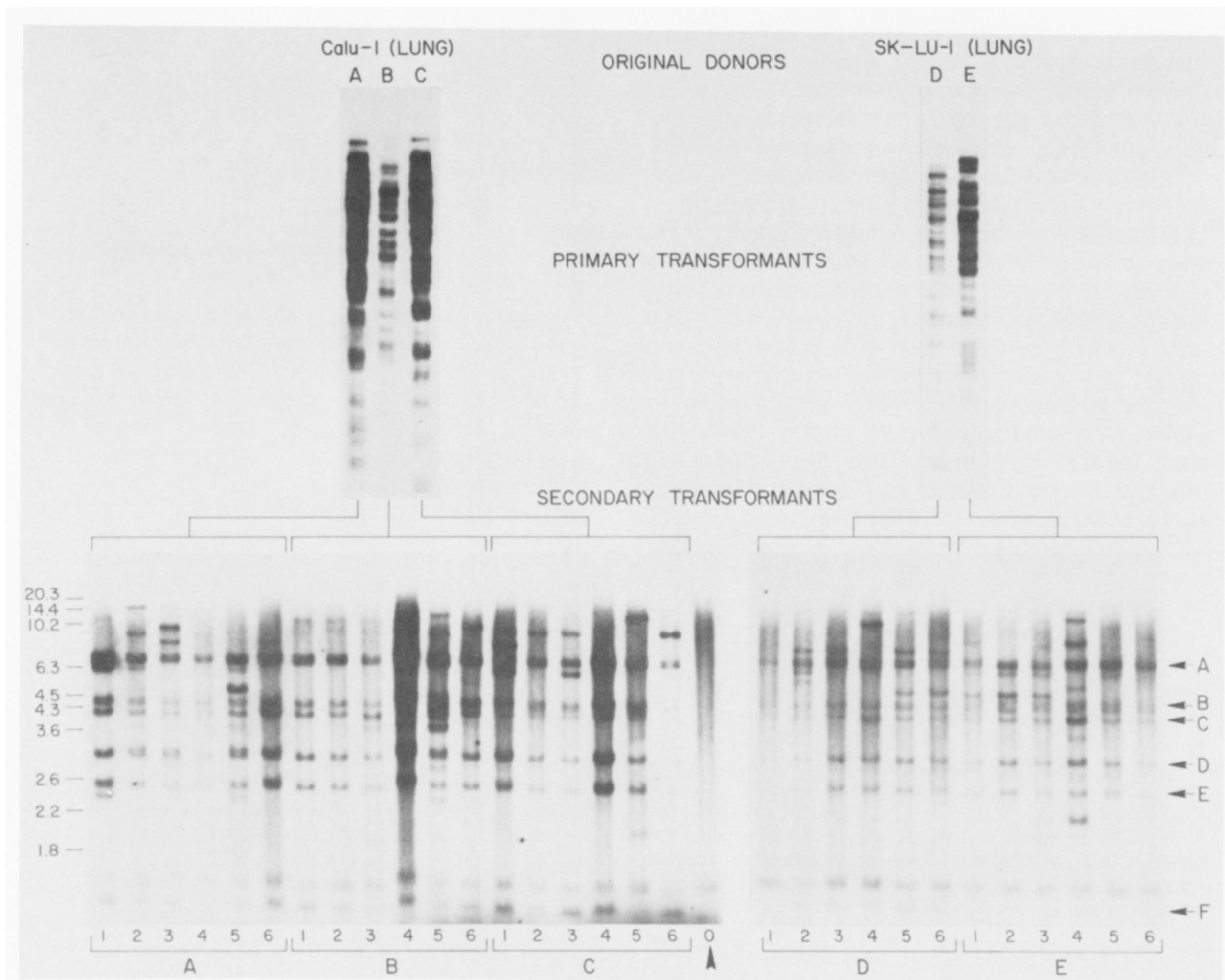

Figure 4. Presence of Human Sequences in NIH/3T3 Secondary Transformants Derived from Two Different Human Lung Carcinoma Cell Lines

Thirty different secondary transformants, six each induced by three different primary transformants derived from line Calu-1 (A, B and C) and two different primary transformants derived from line SK-LU-1 (D and E) were analyzed for the presence of human sequences as described in Figure 2. Lane 0: NIH/3T3 DNA. All lanes contain 10 μg DNA, cleaved with Eco RI. At left of the figure are indicated the position and molecular weights in kb of adenovirus 2 DNA Bam HI and Eco RI restriction fragments. For arrows on right, see text.

five of these, three derived from Calu-1 and two from SK-LU-1, were used in a second round of transfer experiments to derive a total of 30 secondary transformants, six from each primary transformant DNA. DNAs from all 30 secondary transformants and the five primary transformants were digested with Eco RI, blotted and probed for Alu family sequences with the blur 8 Alu family clone. The results are shown in Figure 4. DNA from the five primary transformants contain copious Alu family sequences. Each has a different blot pattern, indicating that they arose from independent transfer events. Blots of DNAs from secondary transformants, however, contain many fewer bands specific for the Alu family sequence, and their patterns are quite similar. Several conserved bands are present in all lanes and are labeled A through F on the extreme right in Figure 4. A band migrating slightly slower than band F is also present in all lanes, including the NIH/3T3 control lane. It is not reproducibly present in other blots of these DNAs. (See, for example, Figure 5, lanes B and C, and Figure 6, lanes 1–10.) The presence of this band is therefore not specific for transformation of NIH/3T3 cells with DNA from these cell lines. An additional band of approximately 1.8 kb between bands E and F cannot be clearly seen on this blot, but is visible in Figure 5, lanes B and C, and Figure 6, lanes 1–10. This band may be specific for transformation of NIH/3T3 with these donors. Band A appears as a doublet in shorter exposures of the blot shown in Figure 4. A few additional bands are seen in most lanes, but for these no consistent pattern of retention is discernible. In summary, there are seven to eight Eco RI restriction fragments, of 25–30 kb cumulative size, which appear to be specific for transformation of NIH/3T3 cells with DNA from the Calu-1 and SK-LU-1 cells.

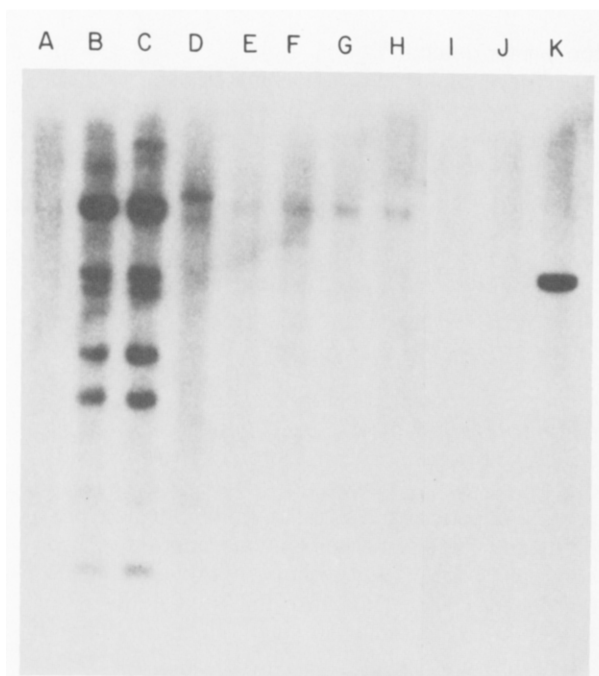

Figure 5. Presence of Human Sequences in NIH/3T3 Secondary Transformants Derived from Different Human Tumor Cell Lines

Different secondary transformants were analyzed for the presence of human sequences as described in Figure 2. All lanes contain 10 μg DNA, cleaved with Eco RI, as follows: lane A, NIH/3T3 DNA; lanes B and C, secondary transformants from the Calu-1 lung carcinoma cell line (corresponding to lanes A4 and B1 of Figure 4); lanes D to H, secondary transformants from the SK-N-SH neuroblastoma cell line (these derive from a single primary transformant); lanes I and J, secondary transformants from the T24 bladder carcinoma cell line (these secondaries derive from two different primary transformants); lane K, 50 pg of blur 8 plasmid cleaved with Eco RI, with 10 μg of NIH/3T3 DNA as carrier.

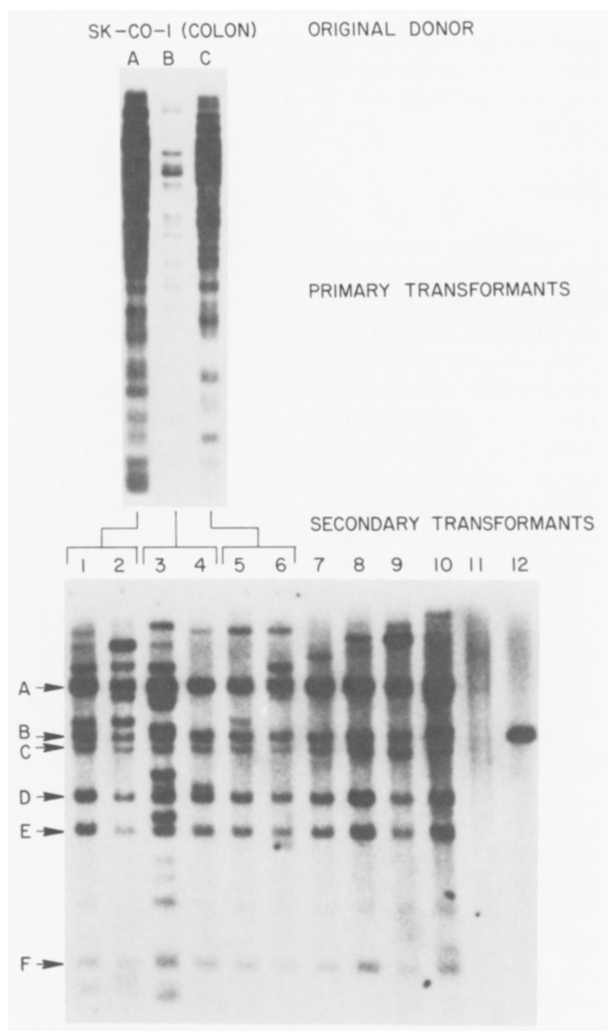

Figure 6. Presence of Human Sequences in NIH/3T3 Secondary Transformants Derived from a Human Colon and Two Lung Carcinoma Cell Lines

Six different secondary transformants (lanes 1 to 6), two each induced by DNA from three primary transformants derived from the line SK-CO-1 (lanes A, B and C), were analyzed for the presence of human sequences as described in Figure 2. For comparison, four different secondary transformants derived from the lines Calu-1 (lanes 7 and 8) and SK-LU-1 (lanes 9 and 10) are included. These secondary transformants correspond to lanes A4, B1, D4 and E5 from Figure 4. Lane 11, NIH/3T3 DNA; lane 12, 50 pg of the blur 8 plasmid cleaved with Eco RI and 10 μg of NIH/3T3 DNA as carrier. All lanes contain 10 μg DNA, cleaved with Eco RI.

Similar experiments were performed with secondary transformants derived from the SK-N-SH neuroblastoma and the T24 bladder carcinoma cell lines. The Alu family blot pattern of Eco RI–digested DNA of secondary transformants from the neuroblastoma cell line show a single band of about 7 kb conserved in four of five secondary transformants derived from a single primary transformant (Figure 5, lanes D–H). The intensity of these bands is consistently lower than that of the bands of DNA derived from secondary transformants of the lung carcinoma cell line donors (lanes B and C). This may reflect weaker homology between Alu sequences present in the neuroblastoma secondary transformants and our specific Alu probe. Alternatively, the transforming elements may be highly amplified in the secondary transformants derived from the lung carcinoma donors. DNA from two secondary transformants derived from the T24 bladder carcinoma cell line contains no bands hybridizing to the Alu family probe (lanes I and J). In another study (data not shown), no consistent pattern of retention of Alu sequences was seen in 13 secondary transformants derived from four independent primary transformants.

The blot pattern of Eco RI–digested DNA from secondary transformants of the SK-CO-1 colon carcinoma cell line, together with those of the lung carcinoma cell lines, is shown in Figure 6. These DNAs show the same conserved pattern of restriction fragments with homology to the Alu family as we observed in Figure 4. These conserved sequences probably are tightly linked to the transforming gene or genes in the original human cell donors. Such sequences, for example, might reside in the 5′ or 3′ regions flanking these genes, within introns or between a cluster of

essential genes. We therefore conclude that the three donor cell lines SK-LU-1, Calu-1 and SK-CO-1 contain and transfer the same or very similar transforming genes.

Discussion

Evidence has been presented that several cell lines derived from human tumors contain specific genes capable of transforming NIH/3T3 cells. First, some donor DNAs reproducibly induce transformed foci. Similar results have been reported previously by other investigators (Shih et al., 1981; Krontiris and Cooper, 1981). Second, transforming activity of DNA from one donor line, T24, displays a distinct profile of sensitivities to restriction endonuclease digestion. The DNA from a T24 primary transformant displays an identical profile. Moreover, all the transforming activity in an Eco RI digest of this primary DNA migrates as a single peak in sucrose gradients. These results suggest the existence of a single dominant-acting gene in the T24 donor. Third, primary transformants induced by human DNAs have acquired human sequences. Similar results have been obtained by others (Shih et al., 1981). Moreover, transformation induced by DNA from certain donors (SK-LU-1, SK-CO-1 and Calu-1) is always accompanied by the p1 transfer of a specific set of human sequences. We have no evidence that any donor contains more than one distinct transforming element.

We have observed different transformation efficiencies using DNAs from different human-tumor-derived cell lines (see Table 1). One explanation for these differences may be the size of the transforming genes involved. The T24 transforming gene is probably smaller than 15 kb (see Figure 3), whereas the transforming gene or genes of the lung and colon carcinoma cell lines may well exceed 30 kb, as described previously. Alternatively, the low efficiencies with some donors may reflect a need to transfer two unlinked genes in order to induce transformation. We have observed that transfer of two unlinked human genes, *tk* and *aprt*, occurs at a frequency that is lower than that of either gene alone by a factor of only 50–200 (our unpublished observations). DNAs from some cell lines were consistently negative for transformation in several independent assays, despite high transfer efficiencies for the human *tk* gene. The average size of the DNA molecules from most of these cell lines was large enough to have allowed our detection of transforming elements of sizes comparable to those of the lung and colon carcinoma cell lines. However, we cannot rule out the possibility that some of these negative cell lines contain even larger transforming elements. Alternatively, negative donors may lack genes capable of transforming NIH/3T3 cells, or contain a set of unlinked genes that together could transform 3T3 cells, but at an efficiency of focus induction too low for detection.

We have identified three distinct transforming principals among the human tumor cell lines we have studied. Since secondary transformants from the T24 bladder carcinoma cell line apparently lack conserved restriction fragments with homology to human Alu sequences, its transforming principal differs from that in the one colon and two lung carcinoma donors. By the same reasoning, the transforming principal of the SK-N-SH neuroblastoma cell line differs from that of the colon and lung carcinoma cell lines. Moreover, the DNA from a primary transformant of the neuroblastoma donor has a different profile of sensitivity to restriction endonuclease digestion than has T24 (Table 2), and these lines are therefore likely to contain distinct genes. By the criterion of sensitivity to restriction endonucleases, the transforming principal of the EJ bladder carcinoma cell line, as reported by Krontiris and Cooper (1981), differs from that of the T24 bladder cell line. Thus two cell lines derived from tumors of the same tissue may contain two different transforming genes.

Our data indicate, however, that the one colon and two lung carcinoma cell lines contain identical or very similar transforming genes. DNA from all secondary transformants of these donors have conserved Eco RI restriction fragments with homology to human Alu sequences. These secondary transformants contain at least seven such fragments with a cumulative size of 25–30 kb. We interpret this result to mean that the transforming gene or genes of these donors span at least this size. Our finding that different cell lines can contain similar or identical transforming genes is not without precedent. Shilo and Weinberg (1981) have presented evidence that independently derived methylcholanthrene-transformed mouse fibroblasts contain similar or identical transforming genes.

It may be coincidence that transforming genes are present in certain human tumor cell lines, and that these genes bear no special relationship to the generation of the tumors from which they ultimately derived. Alternatively, the expression of these genes within tumor cells may mediate the malignant phenotype. If so, our results suggest that overlapping or identical pathways leading to human cancer may arise independently.

Experimental Procedures

Culture and Identity of Cell Lines

NIH/3T3 (Jainchill et al., 1969) and LMtk$^-$ cells (Kit et al., 1963) were maintained as monlayer cultures in Dulbecco's medium supplemented with penicillin (100 μg/ml) and streptomycin (100 μ/ml) and 10% bovine calf serum. The NIH/3T3 stocks were kept frozen, and thawed cells were passaged when they reached a density of 10^5 cells per 10 cm plate. NIH/3T3 cells were not kept for longer than 2 months of continuous passage. Cells derived from human tumors were cultured in the medium-serum combination indicated to be optimal for growth (see Table 1). Cultured cells, transformed by DNA treatment, were obtained from foci by cloning with use of glass

cylinders. Such cultures were maintained in Dulbecco's medium supplemented with antibiotics and 5% bovine calf serum.

Preparation of Cellular DNAs

Confluent plates of cells were washed in isotonic phosphate-buffered saline (PBS), scraped into PBS and centrifuged at 2000 rpm for 5 min. After decanting, cell pellets were stored at $-20°C$. To prepare DNA, we rapidly thawed cell pellets and resuspended them in 10× volume of 150 mM NaCl, 10 mM EDTA, 10 mM Tris–HCl (pH 7.5). Proteinase K was added to a final concentration of 200 μg/ml, and cells were lysed by the addition of SDS to 0.4%. Lysed cells were heated to 65°C for 15 to 30 min, then incubated overnight at 37°C. Equal volumes of 650 mM NaCl, 10 mM EDTA and 10 mM Tris–HCl (pH 8.0) were added, and the lysates were gently extracted two to three times with an equal volume of buffer-equilibrated phenol. The aqueous phase was extracted once more with an equal volume of a mixture of phenol, chloroform and isoamyl alcohol (25:24:1) and once more with chloroform and isoamyl alcohol (24:1) before precipitation with two volumes of absolute ethanol. The DNA precipitates were removed with a glass rod and washed successively with two changes of 70% ethanol and two changes of 100% ethanol. The precipitates were then air-dried after which they were dissolved in 1 mM Tris–HCl (pH 7.5), 1 mM EDTA at a DNA concentration of 0.3–1.0 mg/ml.

All cellular DNAs were sized by gel electrophoresis in 0.5% agarose gels. Uncut and restriction-cleaved λ DNAs were used as size markers.

Transfer of Thymidine Kinase and Transforming Genes with Cellular DNAs

All DNA transfers used the calcium phosphate precipitation method (Graham and van der Eb, 1973) as modified by Wigler et al. (1979b).

Transfer of the thymidine kinase (*tk*) gene utilized LMtk$^-$ cells as recipients, and tk$^+$ colonies were selected in HAT medium (Szybalski et al., 1962).

Transfer of genes capable of inducing morphological transformation utilized NIH/3T3 cells as recipients. Precipitated cellular DNA (30 μg) in a volume of 1 ml was added to a 100 mm culture dish containing 5×10^5 to 10^6 cells in 10 ml of Dulbecco's medium and 10% calf serum. Cells were incubated at 37°C in presence of the precipitate, which was removed after 8 to 12 hr. After an additional 12 to 24 hr, each plate of treated cells was trypsinized and seeded into three to five 100 mm dishes in Dulbecco's medium and 5% calf serum. Cultures were refed with this medium at intervals of three to four days, until scoring for foci at days 14–21. 3T3 cultures fed with Dulbecco's medium and 5% calf serum do not maintain a confluent monolayer, so that the transformed foci eventually appear on a background of semiconfluent normal cells. Under these culture conditions, transformed foci are more clearly discernible, and the incidence of spontaneous focal overgrowths is lower than on cultures maintained in the same medium with 10% calf serum.

Detection of Human Repetitive DNA Sequences in 3T3 Cells Transformed with DNA from Human Tumor Cell Lines

Cellular DNAs were cleaved with restriction endonucleases (purchased from New England BioLabs and Bethesda Research Laboratories) according to suppliers' instructions. Cleaved DNA was subject to electrophoresis through neutral 1% agarose gels (McDonell et al., 1977), and DNA was transferred to nitrocellulose by the method first described by Southern (1975).

The recombinant DNA plasmid blur 8 was donated by W. Jelinek. Blur 8 is a pBR322 recombinant plasmid containing a human 300 bp DNA sequence that is a member of the highly repeated Alu sequence family (Jelinek et al., 1980). Blur 8 DNA was labeled with ^{32}P by nick translation (Maniatis et al., 1975), with all four α-^{32}P-dNTPs, to a specific activity $> 2 \times 10^8$ dpm/μg. Hybridization was performed at a probe concentration of 5 ng/ml at 67°C as previously described (Wigler et al., 1979b). Filters were washed four times for 20 min each at 67°C with 2× SSC (300 mM sodium chloride, 30 mM sodium citrate), 20 mM sodium phosphate, 0.06% sodium pyrophosphate and 0.05% SDS (pH 7.0), and one time in the same buffer, except with 1× SSC. After being blot-dried, filters were exposed at $-70°C$ for 16 to 48 hr with Kodak XR-5 film with intensifying screens.

Sensitivity of T24 DNA Transforming Activity to Restriction Endonuclease Digestion

DNAs from the human tumor cell line T24 and from a NIH/3T3 primary transformant (T24-a2), derived by transfer with T24 DNA, were cleaved with restriction endonucleases in the presence of 1 μg λ DNA per 100 μg cellular DNA. Completeness of digestion was monitored by gel electrophoresis and Southern filter hybridization of reaction aliquots, with ^{32}P-labeled λ DNA as probe. In all reactions, digestion was >95% complete.

Digested DNAs were extracted with a mixture of phenol, chloroform and isoamyl alcohol and were ethanol-precipitated. Dissolved DNAs were mixed with a twofold excess of NIH/3T3 carrier DNA, then assayed for their transforming activity upon calcium-phosphate-mediated transfer to NIH/3T3 cells.

Sucrose Gradient Fractionation of Eco RI-Cleaved T24-a2 DNA

Eco RI-cleaved T24-a2 DNA (600 μg) was added to 2.25 ml 10% w/v sucrose, 70 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA. We layered 0.75 ml of the DNA (200 μg) atop each of three 36 ml 15% to 40% w/v sucrose gradients in 100 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA. Gradients were ultracentrifuged in a Beckman SW27 rotor at 23,000 rpm for 27 hr at 20°C. Aliquots of gradient fractions were subjected to agarose gel electrophoresis along with Hind III-cleaved λ DNA to determine the molecular weight range of DNA in each gradient fraction.

After fractionated DNAs were concentrated by ethanol precipitation, 30% of each fraction was mixed with at least a twofold mass excess NIH/3T3 carrier DNA and was monitored for transforming activity upon transfer to NIH/3T3 recipient cells. cells.

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Note Added in Proof

A similar study of transforming genes in human tumor cell lines has been reported by Murray et al. (Cell *25,* 355–361, 1981). A pattern of conserved Eco RI fragments containing Alu sequences was observed in secondary transformants derived following DNA transfer from the colon carcinoma cell line SW-480. This pattern closely resembles what we have observed with one colon and two lung carcinoma donors. We obtained secondary transformants from R. Weinberg's laboratory and verified that these four human cell lines transfer the same or closely related genes.