Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3997–4001, June 1986
Medical Sciences

Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa

(DNA transfection/human solid tumor/oncogene)

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Contributed by Takashi Sugimura, December 31, 1985

ABSTRACT **DNAs from 21 human stomach cancers, 16 metastatic stomach cancers to lymph nodes, and 21 apparently noncancerous specimens of stomach mucosae from a total of 26 patients with stomach cancer were tested for their ability to induce neoplastic transformation of NIH 3T3 cells on transfection by the calcium phosphate precipitation technique. Three samples of DNA were shown to have transforming activity; one was from a primary stomach cancer of one patient, the second was from a noncancerous portion of stomach mucosa of the same patient, and the third was from a lymph node metastasis of stomach cancer from another patient. These transformants were tumorigenic in nude mice, and DNAs from the cells could induce secondary transformants. A portion of the transforming gene from the stomach cancer of one patient, which contained the sequences expressed in the NIH 3T3 transformants, was cloned. The transforming gene did not have any homology with the transforming sequences reported previously. We have applied the term *hst* to this novel human transforming gene. The transforming gene, *hst*, was found to be present in all the primary and secondary transformants induced by the other two samples of DNA.**

Development of the calcium phosphate precipitation technique for DNA-mediated gene transfer to NIH 3T3 cells has enabled us to detect cellular transforming DNAs in diverse human tumors, including carcinomas, sarcomas, and hematopoietic malignancies (for review, see refs. 1–3). However, in most of these studies, tissue culture cell lines have been used as sources of donor DNAs, and there have been few studies on the transforming activities of DNAs from fresh solid human tumors (4–10). Furthermore, except for oncogenes that have been designated B-*lym* (*BLYM*)‡ (11), *met* (*MET*)‡ (12), *mcf*-2, *mcf*-3 (13), c-*raf* (*RAF*)‡ (14, 15), *neu* (16), *dbl* (8), *ret* (10), and a transforming gene from a melanoma (17), all of the cellular transforming genes identified so far belong to the *ras* (*RAS*)‡ family of oncogenes, c-Ha-*ras*, c-Ki-*ras*, or N-*ras* (*HRAS*, *KRAS*, or *NRAS*),‡ all of which code for the guanine nucleotide binding protein, p21 (1–3). The transforming activities of c-Ha-*ras*, c-Ki-*ras*, and N-*ras* were found to be acquired by point mutations at codons coding for amino acid 12, 13, or 61 of p21 (1–3, 18). We have studied the transforming activities of DNAs from fresh samples of stomach cancers, which have the highest incidence of all cancers not only in Japan, but also in the world (19). We previously identified a transforming gene isolated from one of the stomach cancers as an altered form of c-*raf* (14). In the present study, we examined the transforming activities of 58 samples of DNAs obtained from 21 primary stomach cancers, 16 metastases to lymph nodes; and 21 noncancerous portions of stomach mucosae, from a total of 26 patients with stomach cancers. We found that three samples of DNA had transforming activity; one was obtained from a primary stomach cancer diagnosed as a moderately differentiated adenocarcinoma, the second from a noncancerous portion of stomach mucosa of the same patient, and the third from a lymph node metastasis from a mucinous adenocarcinoma of the stomach of a different patient. It was also shown that all the transformants induced by three different samples of DNAs contained the same DNA sequence. The transforming gene did not have any homology with the previously reported transforming genes, and we have applied the term *hst* to this novel human transforming gene from a human stomach cancer.

MATERIALS AND METHODS

Cells. NIH 3T3 and al-1 cells were kindly provided by M. Wigler. al-1 cells are secondary transformants of NIH 3T3 cells induced by transfection with DNA from a T24 bladder carcinoma (13). NTF-7 cells are NIH 3T3 cells transformed by transfection with an active N-*ras* clone.

Preparation of DNA. For preparation of high molecular weight DNAs from stomach cancer tissues, lymph node metastases from stomach cancers, noncancerous stomach mucosae, and tumors in nude mice, frozen tissues were ground in a steel Waring Blendor in liquid nitrogen. The tissue powder thus obtained or cultured cells were lysed with lysis buffer containing 1% $NaDodSO_4$, 0.1 M NaCl, and 50 mM EDTA (pH 7.8) and were digested with proteinase K at 200 μg/ml at 37°C for 16 hr. DNA was then purified by extraction with phenol/chloroform, treated with RNase, and extracted with phenol/chloroform as described (20).

Transfection Assays and Isolation of Transformants. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum under a humidified atmosphere of 5% CO_2 in air at 37°C. Medium supplemented with 5% calf serum was used for transformed NIH 3T3 cells. Transfection of DNA into NIH 3T3 cells was performed as described (21). NIH 3T3 cells, seeded at 2.5×10^5 cells per 10-cm Petri dish 48 hr before transfection, were transfected with 30 μg of DNA. In each experiment, 60 μg of DNA was applied to two recipient cultures. Transformed foci were scored 14–21 days after transfection. In each experiment, DNA from salmon testes or NIH 3T3 cells was used as a negative control, and DNA from al-1 cells was used as a positive control. Only results of experiments in which al-1 DNA gave more than 0.5 foci per μg of DNA were evaluated. Clones of transformed cells were obtained from foci by cloning with the use of cylinders, and all of the primary

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Abbreviations: kbp, kilobase pair(s); EBV, Epstein–Barr virus.
‡Locus designations in human gene nomenclature assigned to date are shown in parentheses.

transformants and some secondary transformants were further subcloned by limiting dilution to a single cell.

Growth in Soft Agar. For testing anchorage-independent growth of the cells and cloning of the cells, 0.3% agar containing DMEM with 5% calf serum underlayed by 1.5% agar was used. Cell aggregates of more than 100 cells, formed upon culture of the cells in soft agar for 2 weeks at 37°C, were scored as colonies.

Tumorigenicity. NIH 3T3 cells or transformed cells were collected, washed with phosphate-buffered saline, and suspended in serum-free medium. Then 1×10^6 cells in 0.1 to 0.2 ml of serum-free medium were injected subcutaneously into nude mice. The mice were examined every 3 to 4 days for tumors. In each experiment, NIH 3T3 cells and a1-1 cells were also inoculated into mice as negative and positive controls, respectively.

Southern Blot Hybridization. Cellular DNAs were digested with restriction endonuclease for 3 to 12 hr under the conditions recommended by the suppliers except that 3-fold excess of the enzyme was used. Samples of 15 μg of the DNA digests were subjected to electrophoresis in 0.8–1.2% agarose gels. The DNAs were transferred to nitrocellulose filters (22), and the filters were baked at 80°C *in vacuo* for 2 to 4 hr.

For detection of *Alu* sequences, human-specific repetitive DNA sequences (23), hybridization was performed at 69°C for 36–40 hr in 6× NaCl/Cit (1× NaCl/Cit is 0.15 M sodium chloride/0.015 M sodium citrate) containing 5× Denhardt's solution (20), 100 μg of denatured salmon testis DNA per ml, 0.1% $NaDodSO_4$, and $2–5 \times 10^6$ cpm of ^{32}P-labeled human 0.3-kilobase-pair (kbp) *Alu* sequence probe per ml prepared from BLUR8 (23).

For hybridization with probes other than BLUR8 under high-stringency conditions, hybridization was performed at 42°C for 16–24 hr in 50% formamide containing a hybridization solution of 7× NaCl/Cit/5× Denhardt's solution/denatured salmon testis DNA at 100 μg/ml/0.1% $NaDodSO_4$/10% dextran sulfate/^{32}P-labeled probe at $2–5 \times 10^6$ cpm/ml. For hybridization under low-stringency conditions, hybridization was performed at 37°C in 30% formamide containing hybridization solution. The probe was labeled with [α-^{32}P]dCTP or [α-^{32}P]TTP by nick translation to a specific activity of more than 2×10^8 cpm/μg of DNA. After hybridization, the filters were washed and exposed at −70°C to Kodak XRP-5 film with an intensifying screen for 1–3 days.

Molecular Cloning. Genomic DNA of a second-cycle transformant, T361-2nd-1, was partially digested with *Eco*RI, fractionated in a sucrose gradient to obtain 10- to 20-kbp fragments and cloned in phage λ Charon 4A. The library was screened with ^{32}P-labeled nick-translated *Alu* sequences as a probe. Hybridization was performed under the same conditions as for Southern blot hybridization of *Alu* sequences. From overlapping λ phage inserts, partial maps of the transforming gene were determined. To identify sequences expressed as mRNA, restriction fragments from λ phage inserts, or their subcloned sequences, were used as probes for blot-hybridization analyses of cytoplasmic poly(A)$^+$ RNA from the secondary transformant T361-2nd-1. RNA was extracted from the cytoplasmic fraction of T361-2nd-1 by the CsCl/guanidium isothiocyanate method, and poly(A)$^+$ RNA was obtained by passing the fraction through an oligo(dT)-cellulose column (20). Samples of 10 μg of poly(A)$^+$ RNA were electrophoresed on 1.0% formaldehyde agarose gel, blotted onto nitrocellulose filter, and hybridized under the same conditions as those for Southern blot hybridization using 50% formamide (20).

RESULTS

Transforming Activities of DNAs from Human Stomach Cancer. DNAs were isolated from 21 stomach cancer tissues, 16 lymph node metastases of stomach cancers, and 21 noncancerous regions of stomach mucosae, obtained from 26 patients at the time of surgery. High molecular weight DNA samples were used to transfect NIH 3T3 cells by the calcium phosphate coprecipitation technique, and foci of morphologically transformed cells were scored.

Three samples of DNAs gave positive transformed foci (Table 1). Two samples of DNAs, samples no. 361 and 363, were isolated from the primary stomach cancer of the pylorus, diagnosed as a moderately differentiated tubular adenocarcinoma, and from noncancerous mucosa of the stomach body near greater curvature, respectively, of patient K.S. Careful histological examination of serial sections of tissues adjacent to this portion of the stomach showed no signs of the presence of malignant cells. Moreover, no intestinal metaplasia was detected in this portion of the stomach. The patient had not received cancer chemotherapy prior to gastrectomy, and there was no family history of high incidence of malignant disease. The other sample of DNA, no. 51, was prepared from a lymph node metastasis of a mucinous adenocarcinoma of the stomach in patient I.S. The primary transformants induced by DNA samples no. 361, 363, and 51 were designated T361, T363, and T51 cells, respectively. The other DNA samples, including DNAs from a lymph node metastasis in patient K.S. and a noncancerous mucosa of the stomach in patient I.S., did not induce morphological transformation. The primary stomach cancer tissue of patient I.S. was not available for transfection assay.

Characterization of Transformants. Morphologically, the transformants were refractile and showed a criss-cross and piled-up arrangement of cells, but they appeared less malignant than a1-1 cells or NTF-7 cells. To determine whether human DNA sequences were present in these transformants, we prepared DNAs from the primary transformants and examined them for the presence of the human *Alu* family of highly repeated DNA sequences by Southern blot hybridization with BLUR8 (Fig. 1*A*). All of the primary transformants contained conspicuous amounts of human repeated sequences. The primary transformants grew well in 0.3% soft agar and were highly tumorigenic when injected subcutaneously into nude mice; on inoculation of 1×10^6 cells subcutaneously, a visible tumor was formed within 2 weeks, and the host mice died within 4 to 5 weeks. DNAs were also extracted from tumors grown in nude mice. These DNAs contained human-specific *Alu* sequences, although in much smaller amounts, probably reflecting the fact that tumors grown in nude mice consisted of both NIH 3T3 transformants and supporting tissues of nude mouse origin (Fig. 1*A*).

DNAs from primary transformants—T361, T363, and T51 cells—were used to transfect NIH 3T3 cells. The transforming activities of the DNAs were in the range of 0.02–0.04 foci per μg of DNA, which were 3- to 5-fold higher than those of the original DNA samples. Six secondary transformants obtained with DNAs from T361 cells, three transformants induced by transfection of DNAs from T363 cells, and four secondary transformants obtained by transfection of DNAs from T51 cells were analyzed further. One of the secondary transformants induced by no. 361 DNA was designated

Table 1. Transforming activity of DNAs from human stomach cancers and noncancerous stomach mucosae

	No. of samples	No. of positive samples*
Stomach cancer		
Primary	21	1†
Lymph node metastasis	16	1
Noncancerous stomach mucosa	21	1†

*Each sample was tested at least four times.

†DNA from the same patient, K.S.

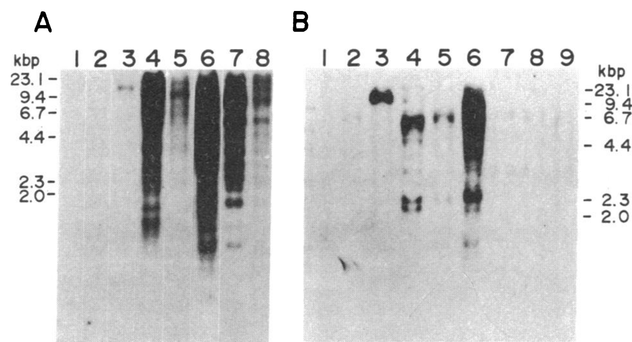

FIG. 1. Presence of human *Alu* sequences in the NIH 3T3 primary transformants. Samples of 15 μg of high molecular weight DNA were digested with *Eco*RI and subjected to electrophoresis and Southern blot hybridization with the ^{32}P-labeled human repetitive sequence DNA fragment in BLUR8. (*A*) Analysis of DNA samples from primary transformants. Lanes: 1, NIH 3T3 cells; 2, NTF-7 cells; 3, a1-1 cells; 4, T361 cells; 5, tumor induced by injection of T361 cells into a nude mouse; 6, T363 cells; 7, T51 cells; 8, tumor induced by injection of T51 cells into a nude mouse. (*B*) Analysis of DNA samples from secondary transformants. Lanes: 1, NIH 3T3 cells; 2, NTF-7 cells; 3, a1-1 cells; 4, T361-2nd-1 cells; 5–9, secondary transformants induced by transfection of T361 cellular DNA.

T361-2nd-1 cells and used for cloning the transforming gene. All of these secondary transformants grew well in soft agar, forming colonies, and they were highly tumorigenic. Secondary transformants induced by no. 361 DNA all had human *Eco*RI fragments containing *Alu* sequences; fragments of 7.2 and 4.9 kbp were detected in all six secondary transformants (Fig. 1*B*). Three secondary transformants gave additional bands containing *Alu* sequences. The three secondary transformants induced by no. 363 DNA and four secondary transformants induced by no. 51 DNA also contained *Eco*RI fragments with different sizes that hybridized with *Alu* sequences (data not shown).

Molecular Cloning of Portions of the Transforming Gene. We used *Alu* family sequences to isolate portions of the transforming gene from a phage λ Charon 4A library constructed by using *Eco*RI-digested DNA from a secondary transformant, T361-2nd-1 cells. Partial restriction maps were constructed from overlapping λ phage inserts (Fig. 2). The inserts or their subcloned DNA fragments were used as

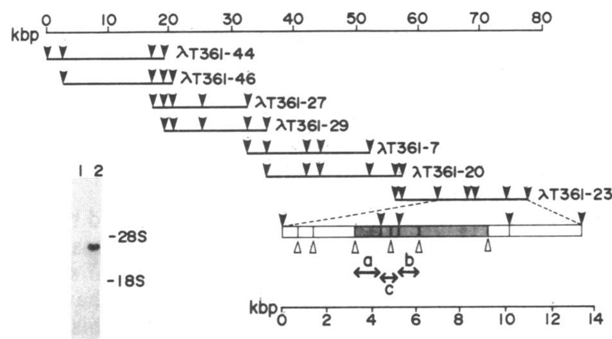

FIG. 2. Restriction maps of part of the transforming sequences. A portion of the transforming gene from T361-2nd-1 cells was cloned in phage λ Charon 4A, and DNA fragments hybridized with poly(A)$^+$ RNA from T361-2nd-1 cells were identified (shaded area). ▼, *Eco*RI site; △, *Sst* I site. Fragments **a**, **b**, and **c** were respectively a 1.1-kbp *Sst* I–*Eco*RI fragment, 0.78-kbp *Eco*RI–*Sst* I fragment, and a 0.79-kbp *Eco*RI–*Eco*RI fragment of λ T361-23 and were used as probes for Southern blot hybridization in Fig. 3. A 3.3-kbp *Sst* I–*Sst* I fragment contained the *Alu* sequence. (*Inset*) RNA blot hybridization with probe **a**. Lanes: 1, poly(A)$^+$ RNA from NIH 3T3 cells; 2, poly(A)$^+$ RNA from T361-2nd-1 cells.

probes for hybridization analyses of cytoplasmic poly(A)$^+$ RNA from T361-2nd-1 cells. DNA fragments of 1.1, 3.3, 0.78, and 0.79 kbp hybridized with 3.3-kilobase poly(A)$^+$ RNA from T361-2nd-1 cells and were identified as DNA fragments containing an exon of the transforming gene (Fig. 2).

Presence of Common Transforming Gene in All of the Transformants. A 1.1-kbp *Sst* I–*Eco*RI fragment, a 0.78-kbp *Eco*RI–*Sst* I fragment, and a 0.79-kbp *Eco*RI–*Eco*RI fragment were isolated, designated probes **a**, **b**, and **c**, respectively (Fig. 2), and used for examination of the presence of these sequences in the transformants. Southern blot hybridization analysis with probe **a** of *Eco*RI-digested DNA from Epstein–Barr virus (EBV)-transformed lymphocytes from patient K.S. (364EBV DNA) showed a strong band at 2.8 kbp with faint bands at 7.7 and 4.7 kbp. DNA from NIH 3T3 cells contained 6.7- and 4.3-kbp fragments weakly hybridized to probe **a** (Fig. 3*A*). With probe **a**, Southern blot hybridization patterns of *Eco*RI-digested DNA samples no. 361 and 363, and DNAs from noncancerous tissues from a different patient were the same as those of *Eco*RI-digested 364EBV DNA (data not shown). The primary transformant, T361 cells, and the secondary transformants obtained by transfection of DNA from T361 cells contained a 4.5-kbp fragment in addition to the mouse-type fragments of 6.7 and 4.3 kbp. The other primary transformants, T363 and T51, contained fragments of 2.8 and 2.2 kbp in addition to the mouse-type fragments.

Studies with probe **b** showed that all of the primary and secondary transformants originally induced by the three samples of DNA, nos. 361, 363, and 51, contained common sequences with rearrangement in addition to the mouse sequences. A sample of DNA, 364EBV, contained an *Eco*RI fragment of 6.0 kbp, while NIH 3T3 cells contained a 2.0-kbp fragment (Fig. 3*B*). The primary transformants T361, T363, and T51 contained *Eco*RI fragments of 4.9, 2.9 and 2.6 kbp, respectively, in addition to a 2.0-kbp mouse fragment. Five of six secondary transformants obtained by transfection of DNA from T361 cells contained a 4.9-kbp fragment like T361 cells; one secondary transformant contained a 4.3-kbp fragment in addition to the 2.0-kbp mouse fragment (Fig. 3*B*).

Results with probe **c** showed that all of the primary transformants, T361, T363, and T51 cells, and all of the secondary transformants contained an *Eco*RI fragment of 0.79 kbp and a 1.9-kbp mouse fragment, which were weakly homologous to probe **c** fragment (Fig. 3*C* and *D*). A sample of DNA, 364EBV, contained a 0.79-kbp *Eco*RI fragment.

Hybridization with Known Oncogenes. Since most of the human transforming genes so far identified belong to the *ras* gene family, the primary transformants, T361, T363, and T51 cells, were examined for the presence of c-Ha-*ras*, c-Ki-*ras*, or N-*ras* of human origin by Southern blot hybridization. The results showed these transformants did not contain c-Ha-*ras*, c-Ki-*ras*, or N-*ras* of human origin (data not shown).

Southern blot hybridization under the conditions used for *ras* probes showed that the transforming genes in these transformants did not have homology with *met*, v-*raf* (*RAF*),‡ v-*erbB* (*ERBB*),‡ v-*abl* (*ABL*),‡ v-*mos* (*MOS*),‡ v-*fos* (*FOS*),‡ c-*myc* (*MYC*),‡ N-*myc* (*NMYC*),‡ and v-*myb* (*MYB*)‡ (data not shown).

Plasmids or phage DNAs containing the oncogene inserts were analyzed by Southern blot hybridization with 3.2-kbp cDNA as a probe under low-stringency conditions. The cDNA used was one of four cDNA clones, prepared from poly(A)$^+$ RNA from T361-2nd-1 cells, containing sequences hybridized to probes **a**, **b**, and **c** and had transforming activity when it was inserted into an expression vector (unpublished data). The cDNA did not hybridize with B-*lym*, v-*erbA*, c-*erbB*-2 (*ERBB2*),‡ v-*ets* (*ETS1*),‡ v-*fes*/*fps* (*FES*),‡ v-*fgr*, v-*fms* (*FMS*),‡ v-*rel* (*REL*),‡ v-*ros*, v-*sis* (*SIS*),‡ v-*src* (*SRC*),‡ and v-*yes* (*YES*)‡.

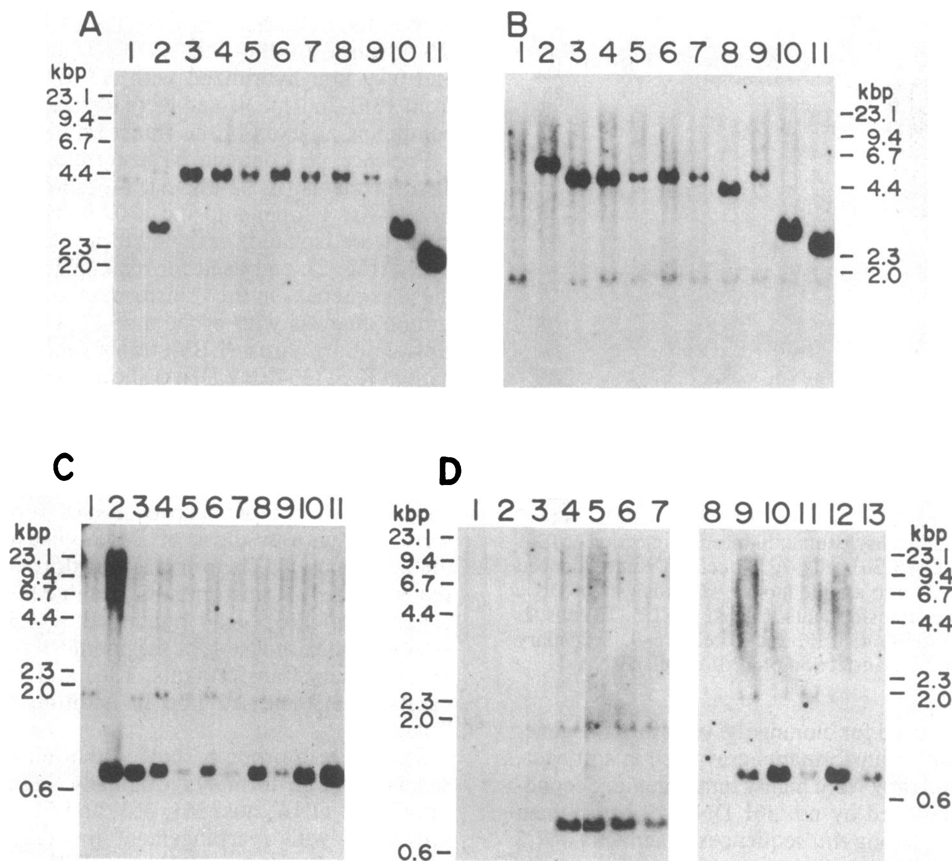

FIG. 3. Presence of common DNA sequences in the transformants. After digestion with *Eco*RI, Southern blot hybridization analysis of DNAs was performed with probe **a** (*A*), **b** (*B*), and **c** (*C* and *D*). Probes **a**, **b**, and **c**, are illustrated in the legend to Fig. 2. (*A–C*) Sources of DNA samples in lanes: 1, NIH 3T3 cells; 2, EBV-transformed lymphoblasts from patient K.S. (364EBV); 3, T361; 4, T361-2nd-1; 5–9, secondary transformants induced by DNA no. 361; 10, T363; 11, T51. (*D*) Sources of DNA samples in lanes: 1 and 8, NIH 3T3 cells; 2, NTF-7 cells; 3, a1-1 cells; 4 and 9, T361-2nd-1; 5–7, secondary transformants induced by DNA from T363; 10–13, secondary transformants induced by DNA from T51.

DISCUSSION

We demonstrated here that two of 37 DNA samples extracted from surgically removed human stomach cancers, including 21 primary tumors and 16 lymph node metastases, and one of 21 DNA samples purified from noncancerous portions of stomach mucosae contained dominant gene(s) capable of transforming NIH 3T3 cells upon transfection. The percentage of positive samples was much lower than those reported in previous studies (5, 6, 24, 25). The transformation efficiencies were also about one-fifth of those obtained with other human tumors. Since most of the transforming genes detected by transfection assay with NIH 3T3 cells are members of the *ras* gene family with a point mutation (1–4), it is very likely that a point mutational activation of the *ras* gene is not frequently involved in development of stomach cancers.

We cloned the portion of the transforming gene from a secondary transformant, T361-2nd-1, induced by DNA from a stomach cancer of patient K.S. and identified the sequences expressed as mRNA in the transformant. Hybridization experiments showed that the transforming gene was not related to any previously identified 24 oncogenes tested, and we suggest the gene to be designated *hst*. These sequences were present not only in all of the transformants induced by DNA from the stomach cancer of patient K.S. but also in all of the transformants induced by DNAs from the noncancerous portion of stomach mucosae of patient K.S. and the metastasis of stomach cancer in a lymph node of patient I.S., although there were rearrangements in the sequences corresponding to DNA fragments hybridized to probes **a** and **b**. There were no rearrangements in the sequences homologous to probe **c**. We concluded that the same human transforming gene, *hst*, was responsible for acquisition of transforming activity by these three samples of DNAs. Probably acquisition of transforming activity by these samples of DNA was not a fortuitous event, since only 3 of 58 samples of DNA tested with a total number of more than 230 transfection assays gave transformed foci upon transfection into NIH 3T3 cells, and all of the transformants contained a common sequence.

One of 21 samples purified from noncancerous portions of the stomach mucosa also had transforming activity upon transfection into NIH 3T3 cells. This DNA sample with transforming activity was isolated from a noncancerous portion of the stomach mucosa of patient K.S. A DNA sample of stomach cancer of the pylorus from this patient showed transforming activity.

The following three possible mechanisms should be taken into consideration to explain the presence of transforming activity in DNA samples from noncancerous stomach mucosa. First, the transforming gene *hst* may have been activated in noncancerous stomach mucosa of this patient K.S., but other genetic changes were required for development of tumors. It is now well established that the carcinogenic process consists of multiple steps, and at least two cooperating oncogenes are required to convert a normal cell to a malignant cell (26–29). It should be noted, however, that the family history of patient K.S. showed no evidence of a high incidence of malignant disease, and the patient had not received chemotherapy before gastrectomy. Second, this sequence may have acquired activity to transform NIH 3T3 cells by rearrangement or mutation at the time of transfection, and the transforming activities of the other two samples

of DNA may also have been due to changes of this sequence at the time of transfection. It is known that transfected genes can be rearranged (13, 21), and it was recently shown that a transforming gene designated *ret* was formed as a result of rearrangement during transfection (10). Third, the transforming sequences that we have reported here may be regulatory sequences that enhance expression of a transforming gene in NIH 3T3 cells.

We thank Dr. M. Wigler for helpful advice and discussion and for providing NIH 3T3 cells and a1-1 cells. We are grateful to Dr. M. Kitaoka for providing surgical specimens and to Drs. Y. Shimosato and T. Hirota for pathological diagnoses. We also thank Drs. S. A. Aaronson, J. M. Bishop, M. A. Baluda, P. Deininger, H. Duesberg, R. W. Ellis, H. Hanafusa, P. Leder, U. R. Rapp, H. M. Temin, S. R. Tronick, N. Tsuchida, and T. Yamamoto for providing recombinant DNA clones. This study was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control, Japan. Masanori Taira and Teruhiko Yoshida received Research Resident Fellowships from the Foundation for Promotion of Cancer Research, Japan.

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