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MOLECULAR CLONING OF THE 25 KBP REGION UPSTREAM OF EXON 0 OF THE HUMAN KI-*RAS* ONCOGENE AND ITS CONSERVATION IN TRANSFORMED MOUSE NIH 3T3 CELLS

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Human sequences associated with the Ki-*ras* oncogene of the mammary tumour cell line, H-466B have been cloned from a tertiary NIH3T3 mouse transfectant. These sequences are located 5' upstream of exon 0 of the Ki-*ras* oncogene, span over 25 kbp of DNA and are conserved in half of the primary transfectants obtained with the Ki-*ras* gene of different types of tumours. No gross alterations were observed in the sequences upstream of the Ki-*ras* gene. The partial or total deletion of these sequences in the other half of primary transformants argues that they are not absolutely required for the transforming activity of the Ki-*ras* oncogene. The even distribution of the human-mouse junction points in primary transformed mouse cells suggests the absence of a specific region of recombination in the 5' flanking region of Ki-*ras*.

Alterations in *ras* genes have been detected in human tumours by their capacity to transform NIH 3T3 cells by transfection. Transforming *ras* genes and their wild type counterparts are distinguished by point mutations which lead to amino acid substitutions in the p21 *ras* protein at amino acid 12, 13 or 61 (1). Among the *ras* genes, the c-Kirsten(Ki)-*ras* has been found to be the most frequently activated oncogene in solid tumours. The frequency of activation can reach 40 % in colon cancers (2,3) and 95% in exocrine pancreatic carcinomas (4). In addition, there is evidence that an increased level of normal Ki-*ras* gene expression can lead to cellular transformation (5). Despite the relevance of the Ki-*ras* gene in human malignancies, little is known about the regulation of its expression. This study is hindered because of 1) the relatively low level of Ki-*ras* expression in most tissues; 2) the complexity of the gene whose homology with its viral counterpart extends over 40 kbp and consists of a 5' untranslated exon (exon 0) plus five exons, the last two of which represent alternative 3' coding exons (6,7).

In the course of our transfection experiments with the DNA of the human mammary tumour cell line, H-466B, we observed the conservation, during 3 successive rounds of transfection in the mouse transfected cells, of human sequences which did not belong to the Ki-*ras* gene, previously found to be responsible for the transforming capacity of H-466B DNA (8). To determine the nature of these sequences, which could be involved in the

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transforming process, these sequences were cloned from a tertiary transformant obtained after transfection with H-466B DNA. Most of the conserved sequences were found to belong to a 25 kbp segment of DNA located upstream of exon 0 of the Ki-*ras* oncogene. Analysis of several transformants revealed that half of them retained the 25 kbp of sequences upstream of exon 0, whereas in the other half the human-mouse DNA junction is distributed along the 25 kbp region. These results indicate that although these sequences are frequently present in transformed NIH 3T3 cells, they are not absolutely necessary for the transforming activity of the Ki-*ras* oncogene.

MATERIALS AND METHODS

Tumour and cell lines

Cell lines, except H-466B, were obtained from the American Type Cell Culture Collection (ATCC) and propagated in culture as prescribed by the supplier. BTPM1 is a primary breast tumour which carries an aspartic acid substitution at position 12 (Prosperi and Goubin; unpublished).

DNA transfection assay

DNA transfection assay was carried out by the calcium phosphate precipitation method as described (8).

Molecular cloning

High molecular weight DNA extracted from a tertiary transformant of H-466B cells was partially digested by Mbo I (Biolabs) and size fractionated in a 5-20% NaCl gradient for 4 h at 40,000 rpm in a Beckman SW41 rotor (9). Fractions containing DNA fragments ranging from approximately 8 to 20 kbp were pooled and used to construct libraries in λ 47.1 or λ 2001. Positive phage plaques were isolated by the method of Benton and Davis (10), using a nick-translated Alu probe (Blur-8) (11). Suitable fragments were subcloned in pUC19 plasmid DNA.

DNA blotting analysis

DNA samples (10 μg) were digested with restriction endonucleases, subjected to horizontal agarose gel electrophoresis and filter-blot transferred by the method of Southern. Hybridizations were carried out at 42°C in 50% formamide, 0.75 M NaCl, 0.075 M Na citrate, 10% dextran sulfate with nick-translated DNA fragments (1.5 x 10^6 cpm/ml) for 20 h, and washed under stringent conditions.

RESULTS

Molecular cloning of sequences located upstream of the exon 0 of the Ki-*ras* oncogene

The Ki-*ras* oncogene of H-466B mammary tumour was previously shown to transform NIH 3T3 cells with a transforming activity ranging from 0.05 focus/μg for tumour cell DNA to 0.3 focus/μg of DNA from transformed NIH3T3 cells, and the activating lesion to result from two adjacent mutations located at codon 12 of the Ki-*ras* oncogene (8). Comparison of the restriction patterns obtained after hybridization with an Alu probe revealed the presence of additional Alu fragments not belonging to the Ki-*ras* locus in secondary and tertiary transformants (not shown). These sequences were estimated to represent approximately 30 to 35 kbp of DNA. In order to characterize the human sequences associated with the Ki-*ras* oncogene, a genomic library was constructed from the tertiary transformant NIH(H-466B)-1:3. DNA was partially digested with MboI restriction endonuclease and cloned into the BamH1 restriction sites of bacteriophage λ 47.1 (clones λ NH) and λ 2001 (clones λ M). A

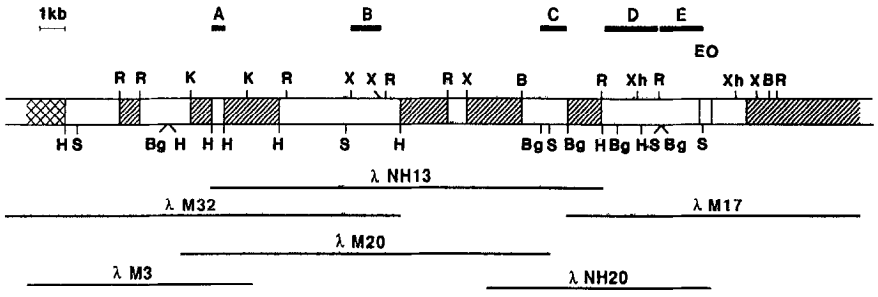

Figure 1: **Restriction map of upstream sequences of the transforming Ki-*ras* gene .** A restriction map of sequences upstream of exon 0 was established from the restriction maps of DNA fragments cloned from a tertiary transformant of H-466B mammary tumour cells in overlapping phage vectors. Clones designated λ NH13, 20 represents MboI partial digested DNA fragments inserted in λ 47.1 and λ M3, 17, 20 and 32 inserted in the λ 2001 vectors. The bars at the top of the figure indicate the restriction fragments subcloned in pUC19 ; A, a 0.8 kbp HindIII-HindIII fragment of λ M32; B, a 1.5 kbp XbaI-XbaI fragment of λ M32; C, a 1.1 kbp BglII-BglII fragment of λ NH13; D and E respectively a 2.2 kbp EcoRI-EcoRI and a 1.8 kbp SacI-SacI fragment of λ M17. Restriction endonuclease cleavage sites: B, BamHI; Bg, BglII; R, EcoRI; H, HindIII; K, KpnI; S, SacI; X, XbaI; Xh, XhoI. ▭, location of exon 0 of the Ki-*ras* oncogene hybridizing with the v-Ki-*ras* oncogene (clone HiHi3); ▨, location of DNA fragments that contain Alu repeat sequences; ▩, location of cloned restriction fragments hybridizing with mouse DNA.

set of lambda phage clones containing Alu sequences was isolated. Clones devoid of Ki-*ras* sequences, with the exception of λ M17 and λ NH20 which carried exon 0, were further characterized by restriction mapping analysis. Appropriate fragments (A-E, fig1) free of human repetitive sequences were subcloned and used as probes to orientate overlapping clones. Probe C, derived from clone λ NH13, hybridized to clone λ NH20 carrying exon 0 indicating that the 19 kbp of human sequences cloned in λ NH20 and λ NH13 were located upstream of exon 0. Probe A isolated from λ M32 hybridized with the 5' end of the cloned region of λ NH13. Since λ M32 contained Alu fragments not present in λ NH13 and λ NH20, it carried additional human sequences also located upstream of exon 0. To locate the junction between human and mouse sequences, the clones were probed with total mouse DNA. Hybridization was detected in clones λ M3 and λ M32, upstream of the leftmost HindIII site indicating that recombination between human and mouse DNA took place at approximately 25 kbp upstream of exon 0 in cells transformed by H-466B DNA. Therefore most of the human sequences which persisted in tertiary transformants are located upstream of exon 0 of the Ki-*ras* oncogene. The complete restriction map of the 25 kbp of this region is presented fig. 1.

A series of DNA blotting analyses was performed to verify that the restriction map of the clones matched the cellular sequences of H-466B and H-466B transformed mouse cells. Hybridization with probe B, C, D and E revealed identical genomic restriction fragments in DNA of H-466B, NIH(H-466B)-1:3 when digested by EcoRI or SacI (fig.2) and additional endonucleases (not shown). Comparison of these restriction patterns with those of human placental DNA also failed to reveal significant differences. These results ruled out that gross alterations in sequences upstream of the Ki-*ras* oncogene, either occurred during the

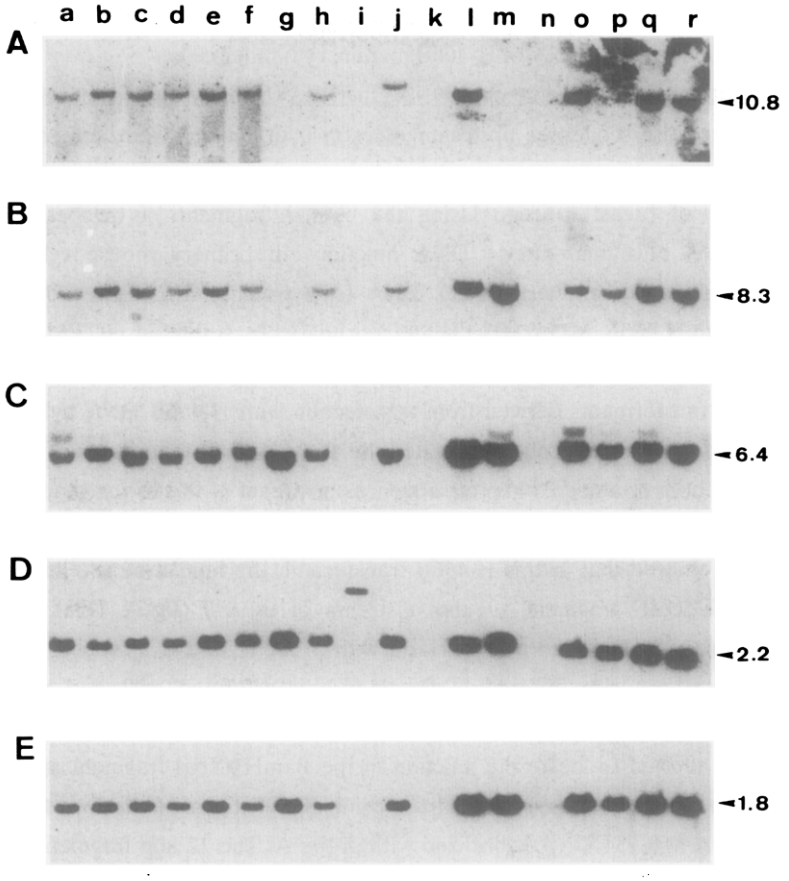

Figure 2: **Southern blot analysis of human tumour and transformed mouse DNAs with probes A-E.** DNA samples (10 μg) were digested with SacI(A,B,E) or EcoRI(C,D),electrophorezed in 0.7% agarose gels, and analyzed by blot hybridization with ^{32}P-labeled DNA fragments (A-E). Human DNA was from placenta (lane a), SW480 (lane b), BTPM1 (lane c), MDA MB 134 (lane d), Calu-1 (lane e), H-466B (lane f); DNA of primary transformants derived from transfection by BTPM1 (lane g), SW480 (lane h), MDA MB 134 (lane i,j), Calu-1 (lane l-n), H-466B (lane o-q) DNA; NIH3T3 mouse DNA (lane k); DNA from a H-466B quaternary transfectant (lane r). Molecular weights of the relevant hybridizing fragments are indicated in kbp on the right of the figure.

transfection or were involved in the activation of the Ki-*ras* oncogene of H-466B mammary tumour cells.

Probe A was used to localize more precisely the recombination point between human and mouse DNA in the tertiary transformant. No difference in the size of the restriction fragments was observed when DNA of H-466B, NIH(H-466B)-1:3, human placenta was digested by SacI (fig.2) or BglII, EcoRI, KpnI (not shown). This result, together with the presence of mouse repetitive sequences in the leftmost HindIII fragment, indicated that recombination with mouse DNA took place upstream near the leftmost SacI site. Blot analysis of a quaternary focus obtained by transfection with NIH(H-466B)-1:3 DNA generated the same 10.8 kbp SacI restriction fragment, observed after digestion of H-466B or human placental DNA when probed with fragment A (fig.2). Thus, the 25 kbp of

sequences located upstream of exon 0 can be retained during 4 successive rounds of transfection with the DNA of H-466B mammary tumour cells.

Localization of the human mouse DNA junction in primary transformants

Since the sequences upstream of exon 0 originated from a mammary tumour, we examined whether the conservation of these sequences was a property of the Ki-*ras* oncogene of breast cancers. Using the cloned fragments as probes, we analyzed the distribution of human-mouse DNA junctions in primary mouse cells transformed by different human mammary DNAs. DNA from primary foci derived by transfection with DNA from H-466B, MDA-MB-134 and a primary breast tumour (BTPM1) were digested by EcoRI or SacI and analyzed by Southern blot with probes A to E (fig.2). DNA from 3 primary transformants derived from transfection with H-466B DNA hybridized with probe B. Hybridization with probe A revealed the 10.8 kbp SacI fragment in 2 of them, indicating that they both retained 25 kbp of sequences upstream of the Ki-*ras* gene. In contrast, DNA from 1 of these H-466B primary transfectants failed to hybridize with probe A. Precise mapping showed that in this H-466B transfectant, the human-mouse junction is located in the KpnI-EcoRI fragment 19 kbp upstream of exon 0 (fig.3). DNA from the primary transformant BTPM1 did not hybridize with probe A and B. Probe C revealed an EcoRI fragment whose size differed in the mouse transformed cells and the BTPM1 DNA, indicating that recombination took place inside this restriction fragment (fig.2). Accurate mapping allowed to locate the junction in the BamHI-XbaI fragment approximately 9 kbp upstream of exon 0 (fig.3). One of the primary transformants derived from a transfection with MDA MB 134 DNA hybridized with probe A. The 12 kbp fragment differed from the 10.8 kbp fragment observed in the tumour cell indicating that this transformant retained approximately 21 kbp of sequences upstream of exon 0 of the Ki-*ras* oncogene. In contrast, in an other primary transformant derived from MDA MB 134, no hybridization was detected with probes A to D. A large SacI fragment, not visible on fig.2E, different from the 1.8 kbp fragment was revealed with probe E after SacI digestion of MDA MB 134 DNA, thus mapping the junction with mouse DNA within the SacI fragment in the vicinity of exon 0 (fig.3). These results suggest that the sequences upstream of exon 0 are not absolutely required for the transforming activity of the Ki-*ras* oncogene of mammary tumour cells.

To extend these results to other types of tumours, we also determined the Ki-*ras* 5' boundary in 3 primary transformants derived from the lung tumour cell line Calu-1 and 1

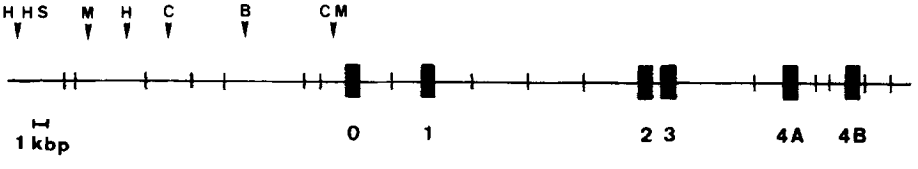

Figure 3: **Localization of the human mouse junction in Ki-*ras* NIH 3T3 mouse transformed cells.** A simplified restriction map was drawn using the previously reported structure of the human c-Ki-*ras*-2 oncogene and the 25 kbp upstream sequences. ▮, location of exon 0 to 4B. ▼ indicates the human mouse junction of primary transformants of H-466B (H), primary transformants of Calu-1 (C), MDA MB 134 (M), SW480 (S), a primary breast tumour BTPM1 (B) DNA.

primary transformant derived from the colon carcinoma cell line, SW480 (fig.2). We found that junctions between human and mouse DNA can occur in the vicinity of exon 0 (Calu-1), at 16 kbp (Calu-1) or as far as 25 kbp of exon 0 (Calu1 and SW480) (fig.3). Taken together, these results showed that approximately half of the primary mouse cells transformed by DNA of different tumour types could retain as much as 25 kbp of DNA located upstream of the Ki-*ras* oncogene. The even distribution of the human-mouse junction sites upstream of the Ki-*ras* oncogene strongly suggests the absence of a preferential region of recombination between mouse and human sequences (fig.3).

DISCUSSION

The observation that additional sequences, outside the previously characterized Ki-*ras* oncogene, were conserved during 3 rounds of transfection in H-466B cells prompted us to investigate the nature of these sequences and to examine whether they are required for the transforming activity of the Ki-*ras* oncogene, particularly in breast tumours. We therefore cloned 25 kbp of human DNA from a tertiary NIH 3T3 transformant derived from DNA of the mammary tumour, H-466B. These sequences are located upstream of exon 0 of the Ki-*ras* oncogene. A rough estimate of the size of the conserved sequences, based on the molecular weight of Alu bands suggested the persistence in the H-466B tertiary transfectant of approximately 30-35 kbp of human DNA. Thus, 5 to 10 kbp of human sequences located downstream of the 3' end could possibly have persisted in this transfectant. Characterization of these sequences was unsuccessful because of the impossibility to obtain λ clones containing them.

In contrast, isolation of cloned fragments of the upstream region devoid of repetitive sequences allowed us to demonstrate the absence of gross alterations relative to the structure of these sequences in human placenta. These results suggest that other events, in addition to mutations at codon 12, such as large structural alterations in the 5' flanking region of the Ki-*ras* gene, are not required to confer a transforming activity to the Ki-*ras* oncogene of H-466B cells.

Blot analysis of 3 primary transformants of H-466B cells showed that the retention of the 25 kbp of sequences is not a characteristic of the transformation by this tumour cell line, since in one of the primary transformants, breakage of these sequences occurred at 19 kbp of exon 0. Analysis of primary transformants derived from transfection with other breast carcinoma revealed that the recombination between human and mouse DNA is distributed along the 25 kbp region. Thus it is likely that retention of the upstream region of Ki-*ras* is not a property of breast tumours. The similar results obtained with the DNA extracted from the lung tumour Calu-1 strongly suggests the absence of a preferential region of recombination between mouse and human sequences.

The conservation of the sequences upstream of the Ki-*ras* gene in half of the transfectants raised a paradox. Since no special care was taken to avoid mechanical shearing during DNA extraction, the average size of the transfecting DNA was approximately 50-60 kbp. Considering the size of the Ki-*ras* locus (45 kbp) and the additional 25 kbp found in half

of the primary transformants, the size of the transforming locus (70 kbp) is above the average size of the transfecting DNA. This suggests that longer molecules could exhibit a higher transforming activity. Alternatively, sequences located as far as 25 kbp from exon 0 could be involved in the transforming activity of the Ki-*ras* gene.

The promoter region of the human Ki-*ras* gene has been located immediately upstream of exon 0 (15). This region of approximately 200 bp consists of multiple elements and lacks TATA and CCAAT boxes. The presence of multiple sites for the initiation of the transcription and Sp1 binding motifs is characteristic of the promoter region of house-keeping and growth related genes. Using S1 mapping analysis, Yamamoto et Perucho (15) have found, in addition to the protected fragments generated by transcription at the major sites of initiation, a minor set of protected fragments in human cells, which could originate in sequences upstream of the promoter region. Nucleotide sequence analysis of the region encompassing exon 0 and sequences upstream revealed several putative splicing acceptor sites and an open reading frame. These observations lead to the suggestion that sequences of exon 0 might not correspond to the first but the second (or other) exon. However, the minor set of protected fragments was not detected in mouse cells transformed by Ki-*ras*. This could indicate that sequences upstream of the gene are required for proper expression in human cells but do not contribute to the transforming activity of mouse cells. Alternatively, if these sequences indeed participate to the transforming activity of the Ki-*ras* gene, their truncation could be overcome by additional activations occurring frequently during transfection (16) which would supply for the functions carried by upstream sequences. Thus, rearrangements or mutations can have modified the level of expression and/or the transforming activity of the Ki-*ras* gene in transformed mouse cells exhibiting partial or total deletion of upstream sequences. Because of the large size of the Ki-*ras* gene, recombination with mouse sequences is not unlikely. The transforming activity of the Ki-*ras* oncogene could be therefore regulated by mouse sequences which would have replaced sequences located farther upstream. Activating mutations are illustrated by the observation of Cohen and Levinson (17), who reported that a point mutation located in the last intron of the Ha-*ras* oncogene increased its expression and its transforming activity. Such alterations of the Ki-*ras* gene could explain the mixed pattern of conservation of the upstream sequences in our transformants.

Upstream sequences could also contain a different gene in the opposite orientation of the Ki-*ras* gene as described for the *cs1* gene, flanking the *ras2* gene of Drosophila (18). The promoter region of the *ras2* gene was found to be potentially bidirectional, like the promoter of the mouse Ki-*ras* gene (14), and could be used to promote transcription of the *cs1* gene (18). It is therefore conceivable that the human Ki-*ras* promoter can regulate transcription in opposite directions. However, in the absence of experimental evidence, we can only speculate about the nature of the sequences upstream of the Ki-*ras* gene. The synthesis of cDNA probes by primer extension at exon 0 and its use on cloned DNA of the upstream region should allow to discriminate between all these possibilities, and better understand the complex regulation of the Ki-*ras* oncogene.

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