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CLONING OF Not I-CLEAVED GENOMIC DNA FRAGMENTS APPEARING AS SPOTS IN 2D GEL ELECTROPHORESIS

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Summary: RLGS (Restriction Landmark Genomic Scanning) is a simple and rapid scanning of genomic DNA in two-dimensional electrophoresis. Human genomic DNA is first cleaved by NotI, and the cleaved ends are radio-labeled and cleaved further by EcoRV, followed by size-fractionation by first dimensional electrophoresis. The sample is then cleaved *in situ* by the second enzyme HinfI and resolved by the second dimensional electrophoresis. Nearly 2,000 spots emerge with spot intensities reflecting the copy number in the genome. Because of the resolving power and capacity to scan the entire genome, RLGS has been used to monitor genomic aberrations and imprinting. Here, we report a means of cloning the DNA in spots. The DNA was eluted and ligated to biotinylated NotI and HinfI likers followed by affinity separation using streptavidin. The ligated fragment was recovered by EcoRV cleavage, the target sequence of which was located in the NotI linker and amplified by PCR using a primer pair, the sequences of which lie in the linkers. The products were then cloned into a vector for further tests. An amplified spot in stomach cancer genomic DNA and a dwindling spot in liver cancer genomic DNA were taken as examples for cloning.

Genomic aberrations, such as deletion, amplification or translocation, often occur in neoplastic tissues. Understanding the cause of these aberrations and their effect on growth control of the cell is regarded as important subjects of cancer research. Many intensive studies have been performed to analyze individual aberrations among genes

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or genetic sites, but so far, comprehensive surveys of the entire genome have been scant. Restriction landmark genomic scanning (RLGS) has been developed to circumvent this difficulty(1). This is a two-dimensional electrophoresis of genomic DNA cleaved first by a restriction enzyme NotI as the landmark site, followed by end-labelling with ^{32}[P]. The sample is digested with EcoRV to produce smaller fragments and then resolved by the first dimensional gel electrophoresis. The separated DNAs are then cleaved with HinfI in the gel and size fractionated again during the second dimension. NotI, of which there are about 4,000 cleavage sites in the mammalian genome, and about 85% of which lie in the so-called CpG islands lying upstream of the regulatory regions of genes(2,3) is used to produce landmarks. In RLGS analysis, 2000 spots, or 1000 NotI sites(2000/2)covering about 1/4 of all the NotI sites can be scanned in one gel run(1,4)

We compared genomic DNA from hepatocellular carcinomas (HCC) and its normal counterpart by this means, and showed that five spots are recurrently intensified, while some 60 spots recurrently dwindle in HCC samples(4). DNA from a gastric cancer cell line also displayed intensified as well as dwindled spots, although the global view of the spot changes differed from that of HCC (Kim et al., manuscript in preparation). These changes reflect gain or loss of genomic regions or a change in the methylation status in the genome. To better understand these genomic aberrations, it is necessary to clone the DNA from the spot of interest. Here, we describe the means of cloning the spot DNA in the RLGS gel.

RESULTS AND DISCUSSION

Cloning the DNA in an RLGS spot. Details of the RLGS analysis have been reported elsewhere(1, 4). For cloning, genomic DNA (10μg) from a human placenta was cleaved by NotI, and admixed with a one-tenth amount of a NotI-cleaved sample, the ends of which were radiolabeled with ^{32}[P]. The mixture was then cleaved by EcoRV and run on the first dimensional electrophoresis. It was then cleaved in the gel by HinfI, and resolved by the second dimensional electrophoresis. Thereafter, the fresh wet gel was analysed directly using the image analyser BAS2000 (Fuji Inc., Tokyo) that can detect radiolabelled DNA at the attomolar level after overnight exposure. In our standard RLGS, there are about 2,800 spots, among which 2,000 are clearly separated (1,4). An example of the radioactive spot image obtained by the BAS2000 with wet fresh gel is

Fig.1. The RLGS profile in a fresh 2D gel that is used for cloning.
A small amount of human genomic DNA, cleaved by NotI, and ^{32}[P]-end-labeled, was admixed with ten times the amount of unlabeled human placental genomic DNA digested with NotI. The mixture was then digested with enzymes and analyzed by RLGS (1, 4). After the run, the fresh wet gel was placed overnight in Fuji BAS2000 image analysis system. Spots with arrows are the radioactive markers admixed with a dye used to match the exposed pattern to the fresh wet gel.

shown in Fig.1. These spots represent ^{32}[P] end- labelled fragments of NotI-NotI, NotI-EcoRV or NotI-HinfI. Because there are large numbers of unlabelled HinfI-HinfI, HinfI-EcoRV, and EcoRV-EcoRV fragments in the same gel, a spot identified by the NotI end-labelled fragment must be freed, prior to cloning, from unlabelled background fragments of which the concentration can be about 1,500 times that of the spot of interest.

The selective cloning strategy is shown in Fig.2. The DNA in the spot area was extracted from the fresh gel by crushing and soaking (5). Under our conditions, only DNA from fresh gels could be efficiently cloned. The DNA was placed in a 5μl reaction volume, treated with 8U of DNA ligase in the presence of 10pmol each of biotinylated NotI linker and HinfI linkers (Fig3). The former linker contained biotinylated A, a primer sequence I, a NotI cohesive terminus sequence, and an EcoRV target sequence. The latter linker contained a primer II seqneuce and a HinfI cohesive terminus sequence in which N is an equal mixture of A, T, G, C. The DNA was incubated in the

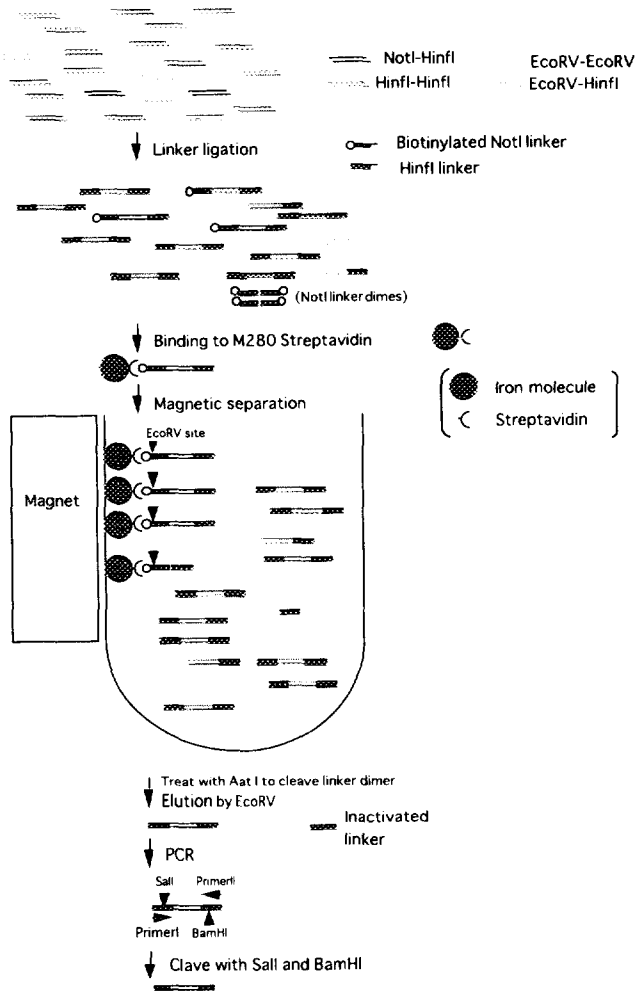

Fig.2. The strategy used to clone the DNA fragment in the RLGS spot having a NotI-terminus.
This strategy applies only for cloning the majority of fragments having NotI and HinfI ends. Other fragments, such as those having NotI-NotI ends or NotI-EcoRV ends, should appear as minority components in the 2-dimensional gel, but they lie on the "arc" of the electropherogram, since HinfI does not affect their size. To clone these poorly separated minority components, modification of the RFLP protocol is necessary. For details, see text. When the DNA is extracted from a spot, it may consist of the target NotI-HinfI, along with HinfI-HinfI, HinfI-EcoRV and EcoRV-EcoRV fragments, the latter of which is carried over from the "arc". Altogether, these "background" fragments that do not have a NotI terminus add up to 1500 fold the concentration of the target DNA. The DNA mixture eluted from a spot was ligated to a biotinylated NotI and a HinfI linker. Fragments ligated to the NotI linker were collected by admixing with iron-streptavidin, followed by magnetic separation. Only the target DNA ligated to the NotI linker and the NotI linker dimers remain on the magnet. The fragments were eluted by cleavage with EcoRV, then amplified by PCR using primers I and II whose sequences lie in the linkers (see text). Finally, the amplified DNA is cleaved by SalI and BamHI whose target sequences lie within linkers I and II, respectively, and cloned in p Bluescript having corresponding termini.

presence of lmM ATP and appropriate buffer (50mM TrisCl pH7.6, 10mM $MgCl_2$, 10mM DTT, 50mg/ml BSA) for 16 hours, and added to 10μl of iron-coated streptavidin (Dynabeads M280 Streptoavidin, Dynal Inc.), followed by three washes with high salt

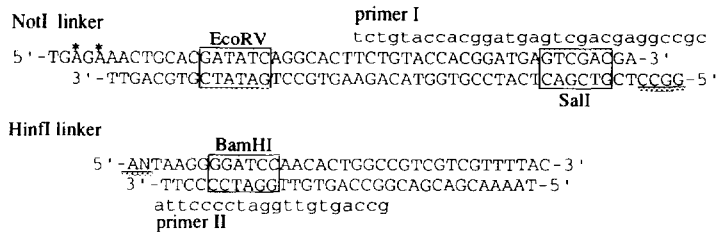

Fig.3. The biotinylated NotI and the HinfI linker used for selective cloning.

buffer (50mM TrisCl pH7.5, 100mM NaCl and 10mM $MgCl_2$). After the magnetic separation, the DNA fragments were eluted by digestion with EcoRV. Dimers of biotinylated NotI linkers that are formed in the reaction and that hinder the subsequent PCR reaction were eliminated by cleaving the mixture by AatI, that acts at *AGGCCT* produced at regenerated sites, prior to EcoRV digestion. An aliquot (1µl) of the reaction mixture was then diluted with 50µl reaction buffer (100mM TrisCl pH8.3, 50mM KCl, 15mM $MgCl_2$, 0.1% gelatin) containing 2.5U of Taq polymerase (Cetus), and the PCR reaction proceeded in the presence of primers I and II (Fig 3) for 35 cycles (94^0C, 30sec;

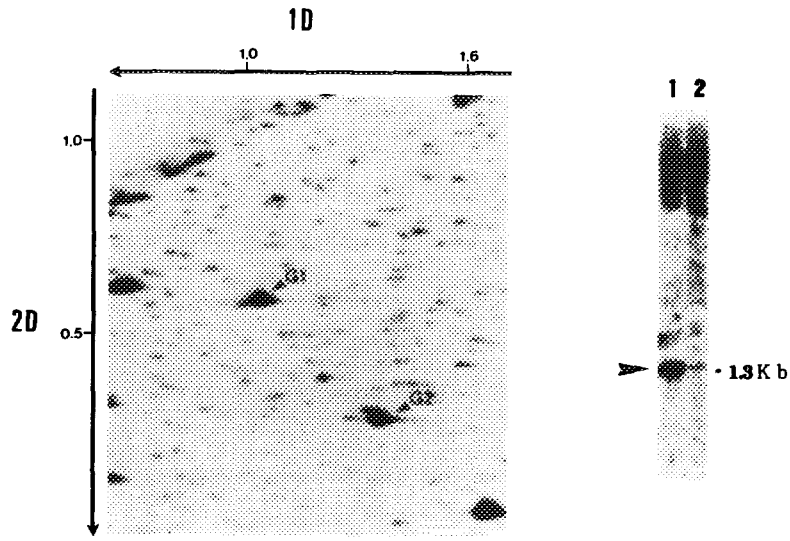

Fig.4. Cloning an intensified spot in an RLGS gel
Left: Expanded view of a region of an electropherogram carrying two intensified spots. The genomic DNA was prepared from the human gastric cancer cell line HuGC-Oohira (6). This profile was compared with that obtained with normal gastric mucosal DNA (data not shown). Seven spots were intensified in the HuGC-Oohira DNA, and two of them (G1 and G2) are indicated by arrowheads.
Right: Southern blotting profiles using the cloned G2 spot DNA as a probe. Lanes 1 and 2 contain, respectively, 5µg of genomic DNA from the HuGC-Oohira cell line and from non-malignant region of human gastric mucosa, both digested with NotI and EcoRV prior to the run. A 1.3Kb fragment is amplified in lane 1.

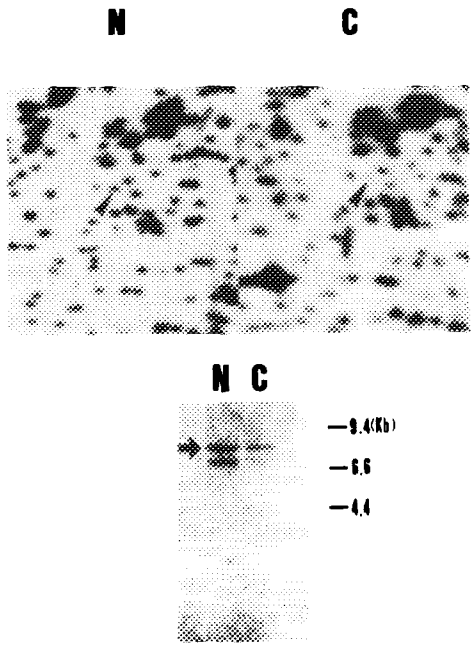

Fig.5. Cloning a dwindling spot in an RLGS gel.
Upper panel: Expanded view of a region of an electropherogram carrying the dwindling spots (arrowheads). DNA from an HCC (C) and its normal counterpart (N) were used. The intensity of this spot was about 0.5 in the HCC DNA. Compared with normal. Lower panel: Southern blot profiles of the genomic DNAs from the same HCC (C) and its normal counterpart (N), using this spot DNA as a probe. The conditions for DNA preparation and electrophoresis were as described in the legend to Fig.4. Each lane contains 5μg of genomic DNA digested with TaqI. Bands at 7.6 and 6.8kb appeared as heterozygotic markers in the DNA from this patient. An arrow shows the unchanged 7.6kb band, whereas the 6.8kb band is missing in the HCC DNA.

55°C, 20sec; 72°C, 1min). The products were digested with SalI and BamHI, of which their target sequences are in the NotI linker/primer I and HinfI linker primer II sequences, respectively, purified through a 5% polyacrylamid a gel, then cloned into SalI- and BanHI- cleaved pBluescript IIKS+ (Stratagene, La Jolla).

Cloning an intensified or a dwindling spot. G2, one of seven intensified spots in a gastric cancer cell line HuGC-Oohira(6), was cloned as described above. The left panel of Fig.4 shows a partial view of the RLGS profile of the genomic DNA from HuGC cell line, wherein intensified spots Gl and G2 are marked. The size of the cloned G2 DNA was 450bp, as predicted from the spot position in the gel. To determine whether the intensity increase of this spot is due to augmentation of the DNA copy number in the genome, the cloned DNA was used as a probe for the genomic Southern blot using NotI and EcoRV digested genomic DNAs. The results shown in Fig. 4b confirmed that a

1.3Kb DNA fragment that yields signal with this probe was indeed amplified in the HuGC cell line. Sequencing revealed that this was a NotI-HinfI fragment covering part of exon I of the c-myc oncogene, from 2,348 to 2,803 of GenBank accession No.J00120. Another spot, G1, was similarly cloned and identified as another NotI-HinfI fragment of the same gene (data not shown).

In our previous studies, the intensity of a number of spots decreased to half in HCC, compared with the normal counterpart (4). Because RLGS uses end-labelling of the NotI termini (1,4), the decrease in intensity most likely reflects a loss of one allele. One of these spots, No.59 (4) was selected for cloning. The local profiles of spots in HCC and its normal counterpart are shown in Fig.5, upper panel.

A 440bp DNA fragment was cloned. It was then radiolabelled and used as a probe in Southern blots of genomic DNA from HCC and its normal counterpart. As shown in the lower panel of Fig.5, 7.6 and 6.8kb TaqI fragments emerged as heterozygous bands in the DNA from non-malignant tissue, whereas the 6.8kb fragment was absent in the HCC DNA. This observation is in accordance with the loss of heterozygosity.

The use of NotI enzyme whose restriction sites often lie in CpG islands immediately upstream of genes is advantageous for further studies, and spots of interest can now be cloned. One application of this will be in studies of spots that recurrently undergo intensity changes in cancer samples. In addition to NotI, the selection of other landmark restriction enzymes will allow different parts of the genome to be surveyed. Studies along these lines are now in progress.

Some of the regions having NotI terminus in RLGS have been cloned by Hirotsune et al.(7) However, their method relied upon purification of NotI-cleaved DNA in large quantities prior to electrophoresis, whereas we cloned a very small amount of DNA from the spots.

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REFERENCES

1. Hatada, I., Hayashizaki, Y., Hirotsune, S., Komatsubara, H., and Mukai, T. (1991) A genomic scanning method for higher organisms using restriction sites as landmark. Proc. Natl. Acad. Sci. USA., 88: 9523-9527.

2. Larsen, F., Gundersen, G., Lopez, R. and Prtdz, Z. (1992) CpG islands as gene markers in the human genome. Genomics, 13: 1095-1107.
3. Lindsay, S. and Bird, A. (1987) Use of restriction enzyme to detect potential gene sequences in mammalian DNA. Nature (Lond.), 327: 336-338.
4. Nagai, H., Ponglikitmongkol, M., Mita, E., Ohmachi, Y., Yoshikawa, H., Saeki, R., Yumoto, Y., Nakanishi, T. and Matsubara, K. (1994) Aberration of genomic DNA in association with human hepatocellular carcinomas detected by 2-D gel analysis. Cancer Res., 54: 1545-1550.
5. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning., second edition, Cold Spring Harbor Laboratory Press.
6. Baba, M., Hasegawa, H., Nakayabu, M., Shimizu, N., Tamaki, S., Kaito, M., Shima, T., Suzuki, S. and Kamada, N. The establishment and characteristics of a gastric cancer cell line (HUGC-OOHIRA) producing high levels of G-CSF, GM-CSF and IL-6; The presence of autocrine growth control of G-CSF. (1995) Am. J. Hematology, in press.
7. Hirotsune, S., Shibata, H., Okazaki, Y., Sugino, Y., Imoto, H., Sasaki, N., Hirose, K., Okuizumi, H., et. al., (1993) Molecular cloning of polymorphic markers on RLGS gel using the spot target cloning method. Biochem. Biophys. Res. Comm. 194: 1406-1412, 1993.