RAPID COMMUNICATION Jpn. J. Cancer Res. 83, 1248–1252, December 1992

Physical Ordering of Three Polymorphic DNA Markers Spanning the Regions Containing a Tumor Suppressor Gene of Renal Cell Carcinoma by Three-color Fluorescent *in situ* Hybridization

Johji Inazawa,[1] Takeshi Ariyama and Tatsuo Abe

Department of Hygiene, Kyoto Prefectural University of Medicine, Kajii-cho, Hirokoji-Kawaramachi, Kamigyo-ku, Kyoto 602

Fluorescent *in situ* hybridization (FISH) is a powerful technique for gene mapping and multi-color FISH allows us to determine directly the order of two or more probes on both metaphase and interphase chromosomes. We report the physical ordering of three DNA markers by three-color FISH using two fluorochrome dyes, fluorescein isothiocyanate (FITC; green) and rhodamine (red). The third color was visualized as a pseudo-color (yellow) generated by optical interference with FITC and rhodamine. Using this system we could rapidly determine the order of three polymorphic DNA markers located on the 3p23-p21.2 bands which span a tumor suppressor gene for renal cell carcinoma, lung carcinoma, and several other types of tumors.

Key words: Three-color FISH — Physical ordering — RFLP marker — Renal cell carcinoma

The construction of a high-resolution map of a target chromosome region is an essential step for the positional cloning of a putative tumor suppressor gene(s) and an unknown genetic disease locus. In this process, cytogenetic mapping by fluorescent *in situ* hybridization (FISH[2]) and linkage mapping with polymorphic DNA markers have been employed.[1,2] Based on FISH combined with replication R-banding, high-density cytogenetic maps have been constructed.[3,4] Further, FISH with two different haptenized probes, so-called two-color FISH, has provided a new ordering system of closer loci within a limited region on both metaphase and interphase chromosomes.[5–10] In addition, Ried *et al.*[11] recently reported simultaneous visualization of up to seven different centromere probes using a digital imaging camera and computer software for pseudocoloring and margining images of fluorescent signals. Herein we report the physical ordering of DNA markers by three-color FISH with a conventional epifluorescence microscope to detect simultaneously three different DNA loci on metaphase and interphase chromosomes. We employed a pseudocolor (yellow), as the third color, generated by optical interference with two different fluorochromes of FITC (green) and rhodamine (red) as described by Nederlof *et al.*[12] This ordering system allowed us to determine rapidly and precisely the order of three polymorphic DNA loci on bands 3p23-p21.2, which span a tumor suppressor gene of renal cell carcinoma,[13–17] lung carcinoma,[18–22] and several other types of tumors.[23,24]

Metaphase chromosomes were prepared by the thymidine synchronization/BrdU release technique as previously reported.[25] The slides were prepared by conventional methods, and fixed at 70°C for 5 h. Just before hybridization *in situ*, the slides were denatured in 70% formamide/2×SSC (0.6 *M* NaCl/0.06 *M* sodium citrate) at 75°C for 2 min, immersed in 70% ethanol at −20°C, and dehydrated through an ethanol series.

A human chromosome 3 specific cosmid DNA library (CI3) was constructed from a human-mouse hybrid cell line, and a genetic linkage map of chromosome 3 using 41 new RFLP markers was constructed.[1,26] Among them, three markers, cCI3-245, 515, and 382 have been chosen as probes in the present study, because they are located within the region on the short arm of chromosome 3 spanning a putative tumor suppressor gene(s).[17,22]

The cCI3-245 and 515 (0.5 μg of each) were labeled with biotin-16-dUTP (Boehringer), and the cCI3-515 and 382 (0.5 μg of each) with digoxigenin-11-dUTP (Boehringer) by nick translation. Labeled probes were precipitated with sonicated salmon sperm DNA and *E. coli* tRNA, and were dissolved in 20 μl of formamide. Bio- and dig-labeled 515 probe solutions were mixed in a ratio of 7:3, and the mixture was used as the bio/dig-515 dual probe instead of a double-labeled probe. In a hybridization with three different probes, solutions of bio-245, dig-382 and bio/dig-515 were mixed in a ratio of 1.5:2.5:6.0 (v/v). To eliminate background noise due to repetitive sequences of *Alu* and L1, 0.6 μl of sonicated human

[1] To whom correspondence should be addressed.

[2] Abbreviations: FISH, fluorescent *in situ* hybridization; BrdU, 5-bromo-2′-deoxyuridine; RFLP, restriction fragment length polymorphism; DAPI, 4′-6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.

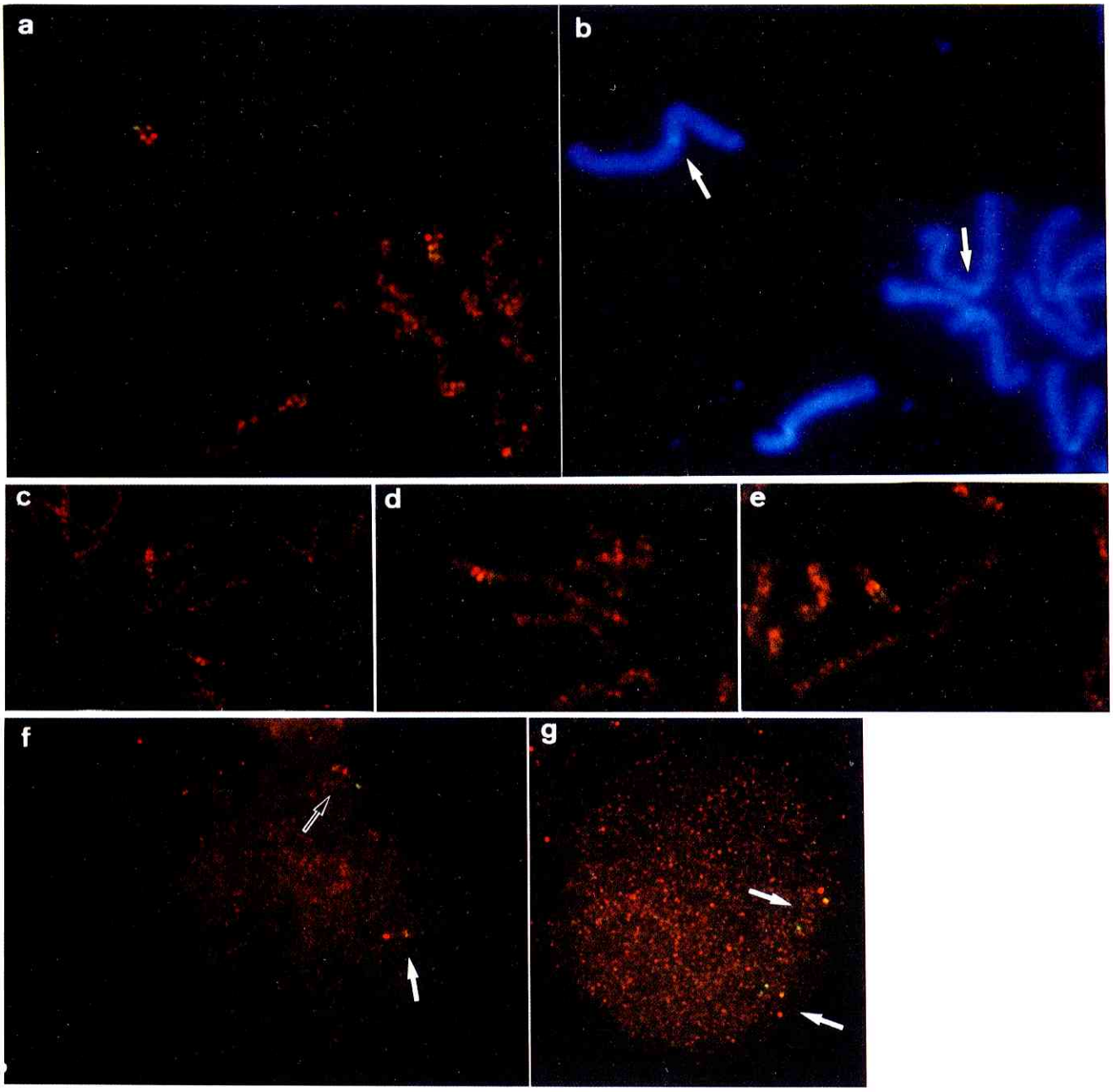

Fig. 1. Three-color FISH on both metaphase and interphase nuclei with three polymorphic markers of cCI3-245, 515 and 382. The 245 and 382 were singly labeled with biotin (bio) and digoxigenin (dig), respectively. The 515 was independently labeled with bio and dig, and the bio-515 and dig-515 were mixed, and used as a dual labeled bio/dig-515 probe. Triple hybridization sites were detected with FITC-avidin (green) and anti-dig rhodamine (red), and simultaneously visualized through a double band-pass filter (Omega Optical). This system visualized the bio/dig-515 signals as a discernible pseudo-color (yellow). (a): A representative partial metaphase with three twin-spots on both sister chromatids of homologous chromosomes 3. Three-color signals were arranged in the order, 3cen-green(382)-yellow(515)-red(245)-pter. (b): The same is shown with DAPI. Arrows indicate chromosomes 3. (c, d, e): Three sets of partial metaphase spreads. The distance of 515(yellow)-382(green) was physically longer than that of 245(red)-515(yellow) in 33 among 35 chromosomes 3 examined, as shown in these figures. (f, g): Simultaneous detection of three color signals on interphase unclei. The three color signals show different pattern arrangements; (f) aligned patterns with the true order of red-yellow-green (closed arrow) and with a false order (open arrow). (g) arrows indicate non-aligned patterns.

placental DNA (10 mg/ml) was added to 9.4 μl of the triple-probe solution. The final mixture was denatured at 70°C for 5 min and mixed with an equal volume of 4× SSC with 20% dextran sulfate.

The hybridization mixture was placed on denatured slides, covered with Parafilm, and incubated in a humid box at 37°C for 16–18 h. After being washed in 50% formamide/2×SSC, 2×SSC, and 1×SSC (37°C, 15 min, each), the slides were treated with 4% Block Ace™ (Dainippon Pharmaceutical Co., Ltd.) at 42°C for 15 min to block fluorochrome background noise. They were then incubated in 4×SSC with 1% Block Ace™ containing avidin-FITC (5 μg/ml) (Boehringer) and anti-digoxigenin rhodamine (1 μg/ml) (Boehringer) at 37°C for 40 min, and then washed for 10 min in each of 4× SSC, 4×SSC/0.05% Triton X-100, and 4×SSC. Then, the slides were counterstained with DAPI (1 μg/ml) and mounted in an anti-fade solution containing 1% DABCO (1,4-diazabicyclo[2,2,2]octane) (Sigma).

Microscopy was performed with a Nikon Y2F-EDF2 epifluorescence microscope. Both metaphase and interphase chromosomes stained with DAPI were screened through Nikon UV-2A filter (exciter, 400–440 nm; barrier, 470 nm). To estimate the signal conditions for each probe, FITC and rhodamine signals were observed with filters of B-2E (exciter, 450–490 nm; barrier, 520–560 nm) and G-2A (exciter, 510–560 nm; barrier, 590) respectively. Signals of the three probes were simultaneously visualized through a double band-pass filter (excitation centers, 490 nm and 560 nm; emission centers, 530 nm and 650 nm) (Omega Optical). The microphotographs were taken using Fujichrome 400D film exposed at 800 ASA. The photoslides were projected onto a screen, and the distances between the centers of fluorescent signals were measured (mm).

In the triple hybridization experiment, two or three twin-spot signals could be detected on both the short arms of homologous chromosome 3 in all 100 metaphase cells examined. Among them, 58 metaphase cells showed a simultaneous delineation of three-color signals with a linear arrangement of centromere-green-yellow-red-telomere along one or both of the short arms (Fig. 1a, c–e). This result clearly demonstrates that the order of the three sequences is 3cen-382-515-245-pter.

From these 58 cells, we selected 35 in which the straight short arm of chromosome 3 had complete three-color twin-spot signals, and measured the distance between the centers of the signals along one side of a chromatid. The signals sometimes appeared as an oblong spot rather than a twin-spot (Fig. 1a, c–e) and in these cases, the measuring point was set at the center of the transverse diameter of the signal. The distance between the yellow (cCI3-515) and green (cCI3-382) signals was longer than that between the red (cCI3-245) and yellow (cCI3-515) signals in 33 chromosomes (Fig. 1c–f) with statistical significance by the sign test.

To test the validity of the interphase ordering of the three clones used in the present study, we examined the arrangement of signals in 34 interphase nuclei. Nuclei showing two separate sets of chromatin domain with complete three-color signals were selected to avoid confusion with signals between the chromatin domains of both homologous chromosomes 3. Among the 68 cases, 37 showed a non-aligned pattern (Fig. 1g), and 31 showed an aligned pattern with the order green-yellow-red in 18, but with other order patterns in 13 cases (Fig. 1f). Thus, the correct order of the three sequences in interphase nuclei could not be determined by this approach.

In general, for simultaneous visualization of three target sequences by FISH, three different haptenized probes and three independent signal detection systems are required.[12, 27–29] In these systems, FITC (green), rhodamine/Texas Red (red), and AMCA (blue) have been used most commonly as the three fluorochromes,[12, 27–29] although these three fluorescent signals cannot be visualized and micro-photographed simultaneously through the same filter. On the other hand, the three-color FISH used by us can simultaneously show three different color signals through a double band-pass filter, permitting the rapid and precise ordering of three different sequences on metaphase chromosomes without a filter change.

The physical order of cCI3-245, 515, and 382 determined by our three-color FISH agrees with the result of linkage analysis.[1, 26] Recently, Takahashi *et al.* constructed a high-resolution cytogenetic map of chromosome 3 with 291 cCI3 DNA markers by direct R-banding FISH,[4] and reported the loci of these three clones as cCI3-245 (3p23), cCI3-515 (3p21.33-3p22.3), and cCI3-382 (3p21.2-21.31). Our data also agree with the order of the three sequences estimated indirectly according to this cytogenetic map. Further, a comparison of the 245-515 and the 515-382 distances showed the existence of a statistically significant difference between the physical lengths (the sign test). Thus, our three-color FISH system allows us to compare each distance among the three sequences and to order the sequences on metaphase chromosomes.

Since the chromatin domains of interphase nuclei are decondensed and extended, interphase mapping by FISH has come into use to order closer sequences.[5–7, 9, 10] We evaluated the feasibility of ordering the three sequences in interphase nuclei using the present three-color FISH. As described above, however, suitable alignment of the three signals was present in 31 of 68 cases, but further 13 cases revealed a false order (Fig. 1f). The physical distance of the entire chromosome 3 has been estimated to

be 214 Mb, and the 3p23-p21.2 bands spanning the three probes used here represent about 29.5 Mb according to the relative length of the ISCN ideogram (ISCN 1985).[30] Thus, these three sequences are assumed to be scattered over a distance of about 10 Mb, suggesting that the interphase ordering system is applicable for sequences that are even closer as previously reported.[7]

In a previous interphase ordering system by two-color FISH, a laborious "pairwise analysis" was required,[5-7, 9, 10] because one probe was detected with one color among two others detected with another color. However, our three-color FISH technique can simply and simultaneously detect three sequences with three discernible colors in both metaphase and interphase nuclei. Therefore, this system may permit the rapid and precise ordering of three or more close sequences in interphase nuclei.

We are grateful to Dr. Yamakawa for providing cCI3-245, -515 and -382 cosmid probes, and also to Dr. Takahashi for his kind contribution through discussions. This work was supported in part by Grants-in-Aid from the Special Coordination Funds for Promoting Science and Technology, Science and Technology Agency, and from the Ministry of Education, Science and Culuture, Japan.

(Received June 11, 1992/Accepted September 14, 1992)

REFERENCES

1) Yamakawa, K., Morita, R., Takahashi, E., Hori, T., Lathrop, M. and Nakamura, Y. A genetic linkage map of 41 restriction fragment length polymorphism markers for human chromosome 3. *Genomics,* **11,** 565–572 (1991).
2) Tokino, T., Takahashi, E., Mori, M., Tanigami, A., Glaser, T., Park, J. W., Jones, C., Hori, T. and Nakamura, Y. Isolation and mapping of 62 new RFLP markers on chromosome 11. *Am. J. Hum. Genet.,* **48,** 258–268 (1991).
3) Hori, T., Takahashi, E., Tanigami, A., Tokino, T. and Nakamura, Y. A high resolution cytogenetic map of 168 cosmid DNA markers for human chromosome 11. *Genomics,* **13,** 129–133 (1992).
4) Takahashi, E., Yamakawa, K., Nakamura, Y. and Hori, T. A high-resolution map of human chromosome 3: localization of 291 new cosmid markers by direct R-banding fluorescence *in situ* hybridization. *Genomics,* **13,** 1047–1055 (1992).
5) Trask, B., Pinkel, D. and van den Engh, G. The proximity of DNA sequences in interphase cell nuclei is correlated to genomic distance and permits ordering of cosmids spanning 250 kilobase pairs. *Genomics,* **5,** 710–717 (1989).
6) Trask, B., Massa, H., Kenwrick, S. and Gitscier, J. Mapping of human chromosome Xq28 by two-color fluorescence *in situ* hybridization of DNA sequences to interphase cell nuclei. *Am. J. Hum. Genet.,* **48,** 1–15 (1991).
7) Slim, R., Weissenbach, J., Nguyen, V. C., Danglot, G. and Bernheim, A. Relative order determination of four Yp cosmids on metaphase and interphase chromosome by two-color competitive *in situ* hybridization. *Hum. Genet.,* **88,** 21–26 (1991).
8) Lebo, R. V., Lynch, E. D., Bird, T. D., Golbs, M. S., Barker, D. F., O'Conell, P. and Chance, P. F. Multicolor *in situ* hybridization and linkage analysis order Charcot-Marie-Tooth Type 1 (CMT1A) gene-region markers. *Am. J. Hum. Genet.,* **50,** 42–55 (1991).
9) Brandriff, B., Gordon, L. and Trask, B. A new system for high-resolution DNA sequence mapping in interphase pronuclei. *Genomics,* **10,** 75–82 (1991).
10) Trask, B. J., Massa, H. F. and Burmeister, M. Fluorescent *in situ* hybridization establishes the order cen-DXS28(C7)-DXS67(B24)-DXS68(L1)-tel in human chromosome Xp-21.3. *Genomics,* **13,** 455–457 (1992).
11) Ried, T., Baldini, A., Rand, T. C. and Ward, D. C. Simultaneous visualization of seven different DNA probes by *in situ* hybridization using combinatorial fluorescence and digital imaging microscopy. *Proc. Natl. Acad. Sci. USA,* **89,** 1388–1392 (1992).
12) Nederlof, P. M., van den Filter, S., Wiegant, J., Raap, A. K., Tanke, H. J., Ploem, J. S. and van der Ploeg, M. Multiple fluorescent *in situ* hybridization. *Cytometry,* **11,** 126–131 (1990).
13) Kovacs, G., Erlandsson, R., Boldog, F., Ingvarsson, S., Muller-Brechin, R., Klein, J. and Sumegi, J. Consistent chromosome 3p deletion and loss of heterozygosity in renal cell carcinoma. *Proc. Natl. Acad. Sci. USA,* **85,** 1571–1575 (1988).
14) Bergerheim, U., Nordenskjold, M. and Collins, V. P. Deletion mapping in human renal cell carcinoma. *Cancer Res.,* **49,** 1390–1396 (1989).
15) Zbar, B., Brauch, H., Talmadge, C. and Linehan, M. Loss of alleles of loci on the short arm of chromosome 3 in renal cell carcinoma. *Nature,* **327,** 721–724 (1987).
16) van der Hout, A. H., Kok, K., van der Berg, A., Osterhuis, J. W., Carritt, B. and Buys, C. H. C. M. Direct molecular analysis of a deletion of 3p in tumor from patients with sporadic renal cell carcinoma. *Cancer Genet. Cytogenet.,* **32,** 281–285 (1988).
17) Yamakawa, K., Morita, R., Takahashi, E., Hori, T., Ishikawa, J. and Nakamura, Y. A detailed deletion mapping of the short arm of chromosome 3 in sporadic renal cell carcinoma. *Cancer Res.,* **51,** 4707–4711 (1991).
18) Kok, K., Osinga, J., Carritt, B., Davis, M. B., van der Hout, A. H., vab der Veen, A. Y., Lnadsvater, R. M., de Leji, L. F. M. H., Berendsen, H. H., Postmus, P. E., Poppema, S. and Buys, C. H. C. M. Deletion of a DNA sequence at the chromosomal region 3p21 in all major

types of lung cancers. *Nature,* **330,** 578–581 (1987).
19) Naylor, S., Johnson, B., Minna, J. and Sakaguchi, A. Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. *Nature,* **329,** 451–454 (1987).
20) Brauch, H., Johnson, B., Hovis, J., Yano, T., Gazdar, A., Pettengill, O. S., Graziano, S., Sorenson, G. D., Poiesz, B. J., Minna, J., Linehan, M. and Zbar, B. Molecular analysis of the short arm of chromosome 3 in small-cell and non-small-cell carcinomas of the lung. *N. Engl. J. Med.,* **317,** 1109–1113 (1987).
21) Brauch, H., Tory, K., Kotler, F., Gazder, A., Pettengill, O., Johnson, B., Grazianao, S., Winton, T., Buys, C., Sorenson, G., Poiesz, B., Minna, J. and Zbar, B. Molecular mapping of deletion sites in the short arm of chromosome 3 in human lung cancer. *Genes Chromosomes Cancer,* **1,** 240–246 (1990).
22) Yokoyama, S., Yamakawa, K., Tsuchiya, E., Murata, M., Sakiyama, S. and Nakamura, Y. Deletion mapping on the short arm of chromosome 3 in squamous cell carcinoma and adenocarcinoma of the lung. *Cancer Res.,* **52,** 873–877 (1992).
23) Yokota, J., Tsukada, Y., Nakajima, T., Gotoh, M., Shimosato, Y., Mori, N., Tsunokawa, Y., Sugimura, T. and Terada, M. Loss of heterozygosity on the short arm of chromosome 3 in carcinoma of the uterine cervix. *Cancer Res.,* **49,** 3598–3601 (1989).
24) Seizinger, B. R., Klinger, H. P., Junien, C., Nakamura, Y., LeBeau, M., Cavenee, W., Emanuel, B., Ponder, B., Naylor, S., Mitelman, F., Louis, D., Menon, A., Newsham, I., Decker, J., Kaelbling, M., Henry, I. and Deimling, A. V. Report of the committee on chromosome and gene loss in human neoplasia. Eleventh International Workshop on Human Gene Mapping. *Cytogenet. Cell Genet.,* **58,** 3–4, 1080–1096 (1991).
25) Inazawa, J., Fukunaga, R., Seto, Y., Nakagawa, H., Misawa, S., Abe, T. and Nagata, S. Assignment of the human granulocyte colony-stimulating factor receptor gene (CSF3R) to chromosome 1 at region p35-p34.3. *Genomics,* **10,** 1075–1078 (1991).
26) Yamakawa, K., Takahashi, E., Saito, H., Sato, T., Oshimura, M., Hori, T. and Nakamura, Y. Isolation and mapping of 75 new DNA markers on human chromosome 3. *Genomics,* **9,** 536–543 (1991).
27) Nederlof, P. M., Robinson, R., Abuknesha, R., Wiegrant, J., Hopman, A. H. N., Tanke, H. J. and Raap, A. K. Three-color fluorescent *in situ* hybridization for the simultaneous detection of multiple nucleic acid sequences. *Cytometry,* **10,** 20–27 (1989).
28) Wiegant, J., Ried, T., Nederlof, P. M., van der Ploeg, M., Tanke, H. J. and Raap, A. K. *In situ* hybridization with fluoresceinated DNA. *Nucleic Acids Res.,* **19,** 3237–3241 (1991).
29) Ried, T., Lengauer, C., Cremer, T., Wiegant, J., Raap, A. K., van der Ploeg, M., Groitl, P. and Lipp, M. Specific metaphase and interphase detection of the breakpoint region in 8q24 of Burkitt lymphoma cells by triple-color fluorescence *in situ* hybridization. *Gene Chromosomes Cancer,* **4,** 68–74 (1992).
30) ISCN (International System for Chromosome Nomenclature), Harden, D. G. and Klinger, H. P. (ed.). "An International System for Human Cytogenetic Nomenclature" (1985). Karger, Basel.