RAPID COMMUNICATION Jpn. J. Cancer Res. 82, 371–375, April 1991

Chromosomal Insertion and Amplification of Human Papillomavirus 16 DNA Sequences in a Cell Line of Argyrophil Small Cell Carcinoma of the Uterine Cervix

Tada-aki Hori,[1] Hiroshi Ichimura,[2] Masako Minamihisamatsu,[3] Ei-ichi Takahashi,[1] Masatake Yamauchi,[1] Yuko Hama,[1] Osamu Kurimura,[2] Masato Yamasaki[4] and Takashi Kurimura[5]

[1]Division of Genetics and [3]Division of Radiation Hazard, National Institute of Radiological Sciences, 4-9-1 Anagawa, Chiba 260, [2]Institute of Clinical Research, Kure National Hospital, 3-1 Aoyama, Kure 737, [4]Department of Obstetrics and Gynecology, Osaka Rosai Hospital, 1179-3 Agasone, Sakai 591 and [5]Department of Pathology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita 565

The chromosomal location of human papillomavirus (HPV) 16 DNA sequences integrated in a cell line derived from argyrophil small cell carcinoma of the uterine cervix was determined by means of fluorescence *in situ* hybridization (FISH). The HPV 16 DNA sequences were integrated near a fragile site and the location of the c-*myc* oncogene at 8q24.1. Amplification of the integrated viral sequences resulted in an abnormally banded region. The amplified HPV 16 DNA sequences were also detected in every interphase nucleus by FISH.

Key words: Cervial carcinoma — Human papillomavirus (HPV 16) — Fluorescence *in situ* hybridization

Recent investigations have implied a close association of certain types of human papillomavirus (HPV), in particular HPV 16 and 18, and cervical carcinoma.[1,2] It has been suggested that genomic insertion of HPV DNA sequences may play a key role in the etiology of cervical carcinoma.[3–6] However, the mechanism by which genomic insertion of HPV might facilitate malignant transformation of the cells is not clear. Several studies have indicated that there are no specific sites for viral integration but the possibility of integration into a specific chromosomal domain could not be excluded.[7–9] Argyophil small cell carcinoma of uterine cervix (ASCC)[10] is a rare form of cervical carcinoma which is characterized clinically by rapid metastasis and poor prognosis.[11] It has been demonstrated in ASCC that HPV 16 DNA sequences were integrated in a multiple-copy form in original tumor tissue and an ASCC tumor cell line (YIK-1) in nude mice, as well as in an *in vitro* ASCC cell line (TC-YIK) established from YIK-1.[12,13] Southern blot analyses on these cells have indicated that the viral DNA sequences were integrated into at least three sites of the host cell genome. In the present study, we performed fluorescence *in situ* hybridization (FISH) experiments to determine whether the multiple copies of HPV 16 DNA sequences present in the TC-YIK cells are integrated at a specific site or at dispersed sites in the genome.

Chromosome preparations were made from logarithmically growing cells of the TC-YIK cell line. Metaphase cells were harvested after a short exposure (30 min) to colcemid (0.05 μg/ml). After a brief (10 min) hypotonic treatment with 0.075 *M* KCl solution, cells were fixed with methanol/acetic acid mixture (3:1, v/v) and chromosome spreads were made by a conventional air-drying method. Some sets of slides were subjected to G-banding (GTG) and Q-banding (QFQ) and photographed for karyotype analysis. Some of them were destained and used for FISH. The TC-YIK cell line used in the present study was hyperdiploid and had a modal chromosome number of 51 with a range of 49–53.[13] Fig. 1 shows an example of a Q-banded karyotype which had the following chromosome constitution: 50,XX, −4, +6, +7, −8, −9, −9, +20,dup(2)(p13-p25), dup(11)(q13-q25),t(17;19)(q21;p13), + der(4)t(4;?)(q13;?), +8q$^+$, + der(9)t(9;?)(q13;?), + der(9)t(9;?)(q13;?), + mar. The most characteristic chromosome change seen in the cell line was an abnormally long chromosome 8 (8q$^+$) which was observed in every cell. On the basis of Q-banding analysis, the 8q$^+$ chromosome appeared to contain a homogeneously staining region (HSR) at the 8q24 region. However, upon G-banding, this region exhibited some evidence of indistinct aderrant bands as shown in the insert of Fig. 1. Thus, this seemed more consistent with a designation of an abnormally banded region (ABR).[14]

For FISH experiments, pBR322 plasmid clone containing a subgenomic 7.2 kb sequence of HPV 16[1] obtained from the Japanese Cancer Resources Bank

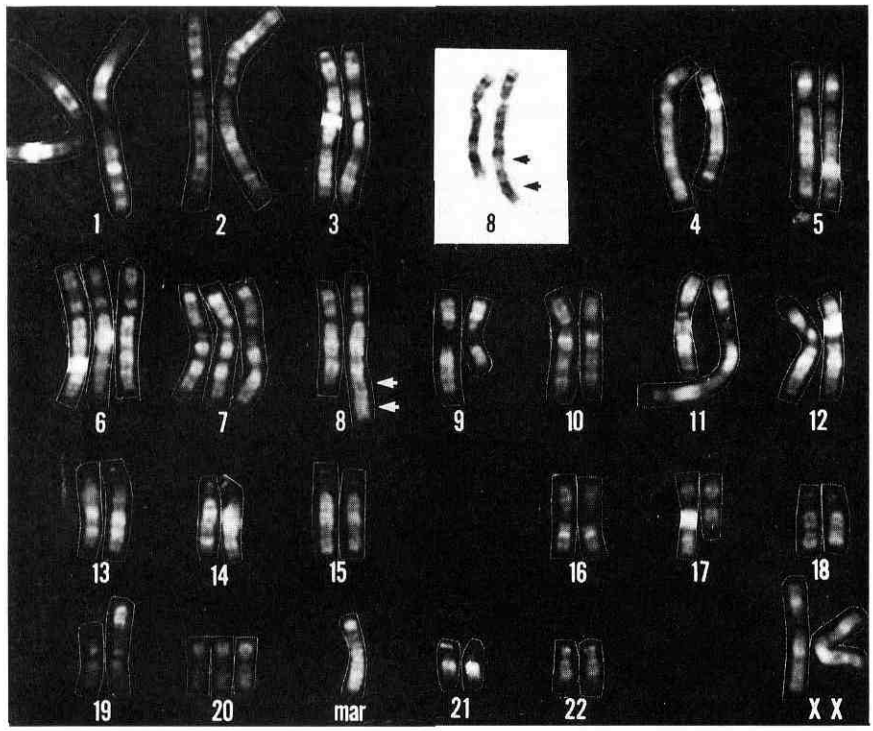

Fig. 1. A representative Q-banded karyotype of TC-YIK cell line. The insert shows G-banded chromosome 8. Arrows indicate the abnormally banded region (ABR).

(Tokyo) was used as a DNA probe. The DNA probe was labeled by nick-translation using biotin 16-dUTP (Boehringer), and *in situ* hybridization and detection were carried out according to the protocol described previously.[15, 16] In brief, hybridization was carried out for 18 h at 37°C in a mixture which consisted of 0.5–10 μg/ml biotinylated DNA probe, 50% formamide, 2 mg/ml BSA, 10% dextran sulfate, and 2×SSC. For biotin detection, the slides were incubated with 3 μg/ml FITC-avidin DCS (Vector) in 4×SSC (0.6 *M* NaCl/0.06 *M* sodium citrate) containing 1% BSA at 37°C for 45 min. The chromosomal DNA was counterstained with 1 μg/ml propidium iodide (PI, Sigma) in antibleaching medium. Both FITC-signals of the hybridized HPV 16 DNA probe and PI-stained chromosomal DNA were excited at a wavelength of near 450–490 nm (Nikon fluorescence microscope FX-2-FL; filter combination B-2E); the former fluoresces greenish-yellow and the latter red.

For a first inspection, we hybridized 5, 10 and 50 ng of biotinylated DNA probe of the HPV 16 per slide of non-banded metaphase spreads. Without exception, all metaphases exhibited strong hybridization signals localized on a particular chromosome. Even in the case of 5 ng of DNA probe, the intensity of hybridization was very high and appeared to be as strong as in the case of centromeric signals hybridized with alphoid sequences known to be highly repeated. Thus, the integrated HPV DNA sequences must be amplified to a great extent at the particular chromosomal region. This is in agreement with our previous result that the TC-YIK cell contains approximately 1000 copies of HPV 16 DNA per diploid cell.[13] To determine the location of the integrated HPV 16, the prephotographed G- and Q-banded metaphase spreads were subjected to FISH. Representative results are shown in Fig. 2a–d. The hybridization signals were localized at the ABR on chromosome 8. The location was proximal to the band 8q24.2. This site appears to coincide with the location of the c-*myc* oncogene at 8q24.1-qter.[17] More recently, c-*myc* was further localized to 8q24.12-q24.13 by high-resolution FISH on R-bands combined with fragile site expression at 8q24.1.[18] It seems likely that after integration of HPV 16 DNA into the 8q24.1 region, the sequence was amplified and formed the ABR. As can be seen in Fig. 2a and 2c, although the hybridization signals were localized at the ABR, they were not distributed evenly in the region and could be detected as segments. This is consistent with our previous results from Southern blot analysis, suggesting that the viral sequences were integrated into at least three sites of the host cell genome.[13] This segmental pattern of hybridiza-

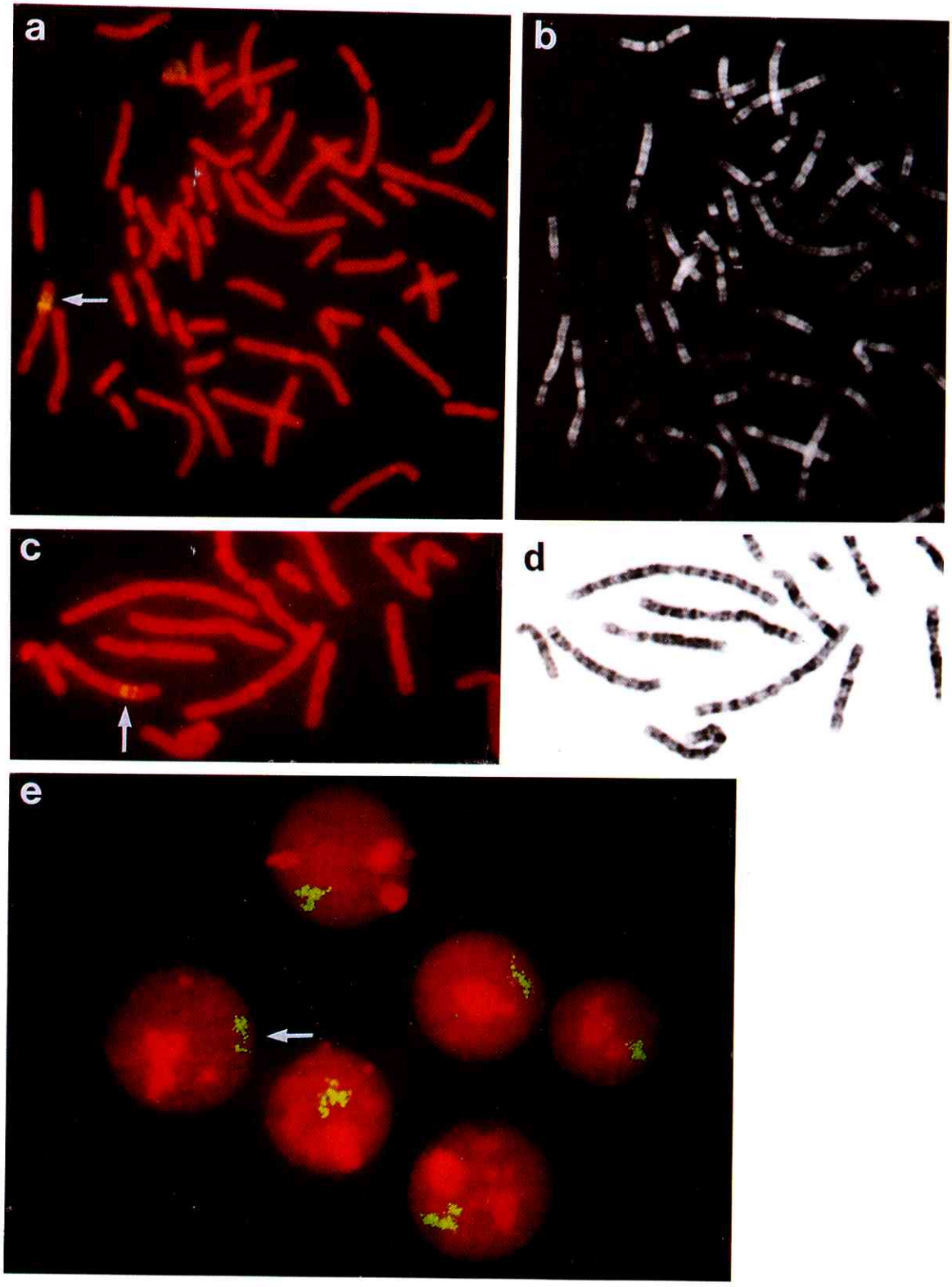

Fig. 2. HPV 16 DNA sequences integrated in ABR of $8q^+$ chromosome (a–d) and interphase nuclei (e) detected by fluorescence *in situ* hybridization (FISH). b (Q-band) and d (G-band) were taken from the same metaphases shown in a and b, respectively. The arrow indicates the integration site.

tion signals might be due to the consequences of complex amplification processes via recurrent chromosomal duplications after a single site integration. Alternatively, independent amplifications might have occurred after multiple integrations into a limited region of chromosome 8. Another possible mechanism in which recurrent replication and recombination of viral DNAs occur before integration may not be ruled out. A preliminary FISH experiment involving chromosome painting with a chromosome 8-specific DNA library (obtained from ATCC) revealed that the ABR contained smaller amounts of human genomic DNA sequences including repetitive DNA sequences like the *Alu*-sequence than did other regions of chromosome 8 (data not shown). Thus, the great majority of DNA sequences in the ABR appeared to be of HPV origin, and contained smaller amounts of host genomic DNA.

There have been several reports on chromosomal locations of integrated HPV DNA sequences in cervical carcinomas.[7-9] Popescu *et al.*[7] have reported that HeLa, a cervical carcinoma cell line, has multiple copies of integrated HPV 18 DNA which are localized on four chromosomal sites at 8q23-q24, 9q31-q34, p11-p13 on an abnormal chromosome 5 and q12-q13 on an abnormal chromosome 22. Ambros and Karlic[9] have also assigned HPV 18 integration in HeLa cells to 8q24, to an abnormal chromosome 22 and to a marker chromosome. Durst *et al.*[8] have mapped the HPV 18 integration site within 40 kb 5′ of the c-*myc* gene, by using cellular sequences flanking an integrated HPV 18 DNA chromosome 8 in HeLa cells. A consistent finding in these studies on HeLa cells was that the HPV 18 DNA sequence was inserted into an 8q24 region. The present result provides further evidence for the association of HPV DNA integration, HPV 16 in this case, and a specific chromosomal domain at 8q24. In the present case of TC-YIK cells from ASCC, amplification of the integrated HPV 16 DNA sequences resulted in the formation of ABR. Similar evidence for ABR representing the site of amplification has been obtained in the case of c-*myc* gene amplification at 8q24 in a human promyelocytic leukemia cell line, HL60,[14] and in the case of fowl adenovirus DNA-transformed rat cells.[19] With regard to chromosomal instability, the 8q24 region of the human chromosome is especially interesting, because of the presence of both common and rare types of fragile sites.[20] It has been suggested that fragile sites may represent chromosomal hot spots for recombination, duplication and deletion, and viral integration and/or modification.[21,22] In addition to the presence of fragile sites and the c-*myc* oncogene, this region includes c-*myc* amplicons, breakpoints involved in Burkitt lymphoma, a region homologous to the rodent chromosomal site of murine leukemia virus integration, and an insertion/deletion site associated with human adult T-cell leukemia virus (HTLV-1).[23] Various studies on HPV 16 have demonstrated that the long control region and the E6 and E7 open reading frames (ORFs) of the integrated HPV 16 genome are preferentially retained, and the E2 ORF seems to be the hot spot for viral integration.[3-7] Thus, it has been suggested that these regions code for *trans*-acting factors that activate cellular genes functioning in the cellular transformation process. Additionally, inserted HPV sequences could act as *cis*-acting mutagens which activate nearby cellular oncogenes. Further molecular cloning and mapping studies of the HPV integration site at the ABR in the ASCC and studies on the expression of nearby genes, including the c-*myc* gene should provide clues for understanding the role of HPV in cervical carcinoma.

As can be seen in Fig. 2e, every interphase nucleus examined showed clear hybridization signals. The amplified HPV 16 DNA sequences are dispersed but occupy a discrete domain in the nuclei. Similar to the hybridization pattern seen in the metaphase chromosomes, discontinuity of the signals can be detected in the interphase nuclei. Various detection systems have been used to determine the rate of occurrence of HPV in cervical carcinoma. They include Southern blot hybridization, isotopic *in situ* hybridization[24] modified filter *in situ* hybridization[25] and polymerase chain reaction techniques.[25,26] The *in situ* hybridization method has the advantage that individual cells or foci of cells in biopsy samples or cell preparations of smear samples can be examined. The method used in the present study is a rather simple and rapid technique which does not require the use of radioactive materials. The present FISH method may be useful for a quick examination of amplification of integrated viral DNA sequences. For the detection of a single viral DNA sequence integrated in a host genome, one can employ a sensitive FISH method[16] for mapping a single-copy gene (applicable for an insert size of larger than 1 kb).

(Received December 14, 1990/Accepted January 31, 1991)

REFERENCES

1) Durst, M. Gissmann, L., Ikenberg, H. and zur Hausen, H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. USA*, **80**, 3812–3815 (1983).
2) Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, A.,

Scheurlen, W. and zur Hausen, H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J.*, **3,** 1151–1157 (1984).

3) Tsunokawa, Y., Takebe, N., Kasamatsu, T., Terada, M. and Sugimura, T. Transforming activity of human papillomavirus type 16 DNA sequences in a cervical cancer. *Proc. Natl. Acad. Sci. USA,* **83,** 2200–2203 (1986).
4) Shirasawa, H., Tomita, Y., Sekiya, S., Takamizawa, H. and Simizu, B. Integration and transcription of humman papillomavirus type 16 and 18 sequences in cell lines derived from servical carcinomas. *J. Gen. Virol.,* **68,** 583–591 (1987).
5) Takebe, N., Tsunokawa, Y., Nozawa, S., Terade, M. and Sugimura, T. Conservation of E6 and E7 regions of human papillomavirus types 16 and 18 present in cervical cancers. *Biochem. Biophys. Res. Commun.,* **143,** 837–844 (1987).
6) Choo, K. B., Lee, H. H., Pan, C. C., Wu, S. M., Liew, L. N., Cheung, W. F. and Han, S. H. Sequence duplication and internal deletion in the the integrated human papillomavirus type 16 genome cloned from a cervical carcinoma. *J. Virol.,* **62,** 1659–1666 (1988).
7) Popescu, N. C., DiPaolo, J. A. and Amsbaugh, S. C. Integration sites of human papillomavirus 18 DNA sequences on HeLa cell chromosomes. *Cytogenet. Cell. Genet.,* **44,** 58–62 (1987).
8) Durst, M., Croce, C. M., Gissmann, L., Schwarz, E. and Huebner, K. Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. *Proc. Natl. Acad. Sci. USA,* **84,** 1070–1074 (1987).
9) Ambros, P. F. and Karlic, H. I. Chromosomal insertion of human papillomavirus 18 sequences in HeLa cells detected by nonisotopic *in situ* hybridization and reflection contrast microscopy. *Hum. Genet.,* **77,** 251–254 (1987).
10) Albores-Saavedra, J., Poucell, S. and Rodriguez-Martines, H. A. Primary carcinoid of the uterine cervix. *Sobr. Pathol.,* **10,** 185–193 (1972).
11) Yamasaki, M., Tateishi, R., Hongo, J., Ozaki, Y., Inoue, M. and Ueda, G. Argyrophil small cell carcinomas of the uterine cervix. *Int. J. Gynecol. Pathol.,* **3,** 146–152 (1984).
12) Ichimura, H., Yamasaki, M., Yamane, T., Shimizu, H., Katsumoto, T., Kurimura, O. and Kurimura, T. Heterotransplantation of argyrophil small cell carcinoma of the uterine cervix integrating HPV16 DNA into nude mice. *Jpn. J. Cancer Res.,* **79,** 1255–1258 (1988).
13) Ichimura, H., Yamasaki, M., Tamura, I., Katsumoto, T., Sawada, M., Kurimura, O., Furuyama, J. and Kurimura, T. Establishment and characterization of a new cell line TC-YIK originating from argyrophil small cell carcinoma of the uterine cervix integrating HPV16 DNA. *Cancer* (1990), in press.
14) Nowell, P., Finan, J., Dalla-Favera, R., Gallo, R. C., ar-Rushdi, A., Ramanczuk, H., Selden, J. R., Emanuel, B. S., Rovera, G. and Croce, C. M. Association of amplified oncogene c-*myc* with an abnormally banded chromosome 8 in a human leukemia cell line. *Nature,* **306,** 494–497 (1983).
15) Hori, T., Takahashi, E., Ayusawa, D., Takeishi, K., Kaneda, S. and Seno, T. Regional assignment of the human thymidylate synthase (TS) gene to chromosome band 18p11.32 by nonisotopic *in situ* hybridization. *Hum. Genet.,* **85,** 576–580 (1990).
16) Takahashi, E., Hori, T., O'Connell, P., Leppert, M. and White, R. R-Banding and nonisotopic *in situ* hybridization: precise localization of the human type II collagen gene (COL2A1). *Hum. Genet.,* **86,** 14–16 (1990).
17) Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R. C. and Croce, C. M. Human c-*myc* onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc. Natl. Acad. Sci. USA,* **79,** 7824–7827 (1982).
18) Takahashi, E., Hori, T., O'Connell, P., Leppert, M. and White, R. Mapping of the MYC gene to band 8q24.12-q24.13 by R-banding and distal to fra (8) (q 24.11), FRA8E, by fluorescence in situ hybridization. *Cytogenet. Cell Genet.* (1991), in press.
19) Ishibashi, M., Yosida, H. and Yasue, H. Preferential clustering of viral DNA sequences at or near the site of chromosomal rearrangement in fowl adenovirus type 1 DNA-transformed cell lines. *J. Virol.,* **61,** 151–158 (1987).
20) Takahashi, E., Hori, T. and Murata, M. Population cytogenetics of rare fragile sites in Japan. *Hum. Genet.,* **78,** 121–126 (1988).
21) Shabtai, F., Orlin, J., Hart, J., Halbrecht, I., Klar, D. and Halbrecht, I. Familial fragile site found at the cancer breakpoint (1)(q32): inducibility by distamycin A, concomitance with fragile (16)(q22). *Hum. Genet.,* **73,** 232–234 (1986).
22) Hori, T., Takahashi, E. and Murata, M. Nature of distamycin A-inducible fragile sites. *Cancer Genet. Cytogenet.,* **34,** 189–194 (1988).
23) Mengle-Gaw, L. and Rabbitts, T. H. A human chromosome 8 region with abnormalities in B cell, HTLV-1 T cell and c-*myc* amplified tumours. *EMBO J.,* **6,** 1959–1965 (1987).
24) Ostrow, R. S., Manias, D. A., Clark, B. A., Okagaki, T., Twiggs, L.B. and Faras, A. J. Detection of Human papillomavirus DNA in invasive carcinomas of the cervix by *in situ* hybridization. *Cancer Res.,* **47,** 649–653 (1987).
25) Melchers, W., van den Brule, A., Walboomers, J., de Bruin, M., Herbrink, P., Meijer, C., Lindeman, J. and Quint, W. Increased detection rate of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared to modified FISH and southern-blot analysis. *J. Med. Virol.,* **27,** 329–335 (1989).
26) Shimada, M., Fukushima, M., Mukai, H., Kato, I., Nishikawa, A. and Fujinaga, K. Amplification and specific detection of transforming gene region of human papillomavirus 16, 18 and 33 in cervical carcinoma by means of the polymerase chain reaction. *Jpn. J. Cancer Res.,* **81,** 1–5 (1990).