

# Karyotypic Analysis of Diploid or Near Diploid Metastatic Harvey *ras* Transformed Rat Embryo Fibroblasts

Ruth J. Muschel, Ken Nakahara, Elizabeth Chu, Rudy Pozzatti, and Lance A. Liotta

Laboratories of Pathology [R. J. M., K. N., E. C., L. A. L.] and Molecular Virology [R. P.], National Cancer Institute, Bethesda, Maryland 20892

## ABSTRACT

The hypothesis of tumor progression proposed by Nowell [P. C. Nowell, *Science* (Wash. DC), 194: 23-28, 1976] states that one mechanism for the development of the metastatic phenotype could be the induction of chromosomal instability. We have developed a new experimental system for studying the induction of the metastatic phenotype using early passage fibroblasts which become metastatic in nude mice after transformation with the Harvey *ras* oncogene [R. J. Muschel *et al.*, *Am. J. Pathol.*, 121: 1-8, 1985; R. Pozzatti *et al.*, *Science* (Wash. DC), 232: 223-227, 1986]. Since the early passage fibroblasts themselves are diploid, we reasoned that this might be a system in which karyotypic change after tumor formation or metastasis might easily be evaluated. Thus, we performed cytogenetic analysis on multiple metastases and tumors which had been derived from cells transformed with the cellular Harvey *ras* oncogene and compared their karyotypes. The karyotypes of the cells isolated from 5 tumors and 14 metastases were, as far as we could determine, identical to those of the injected cells. This could easily be evaluated because of the two clones studied one was diploid; the other has a trisomy of chromosome 4 without any other detectable abnormality. These results suggest that in this system using nude mice, selection for a necessary or even advantageous chromosomal aberration does not occur during tumor formation or metastasis. Furthermore, they indicate that the presence of the *ras* gene itself does not induce chromosomal rearrangements or aneuploidy and that a cell can be both tumorigenic and metastatic yet remain diploid.

## INTRODUCTION

The Harvey *ras* oncogene is capable of transforming a variety of fibroblasts and continuous cell lines. In most cases these cells are tumorigenic. In addition we have found that in many cases, these cells give rise to metastases. After transformation with the Harvey *ras* oncogene, either the cellular or the viral oncogene, NIH-3T3 cells become metastatic in nude mice (1). When early passage rodent fibroblasts which had been transformed by the Harvey *ras* oncogene by Spandidos and Wilkie were tested, they too were metastatic in nude mice (1, 2). We also tested early passage rat embryo cells which had been transformed with the DNA of the Harvey *ras* oncogene (3). The cells transformed in this way were highly tumorigenic in nude mice. In addition, they were metastatic in nude mice using two different assays, the lung metastasis assay and the spontaneous metastasis assay. In the spontaneous metastasis assay, cells are injected s.c. and after tumor formation the animals are autopsied for metastases. In the lung metastasis assay, cells are injected i.v. and the number of lung nodules which appear are counted. The lung metastasis assay requires that cells extravasate and invade into the lung parenchyma and in that way probably requires many of the steps needed for spontaneous metastasis. The results from the lung metastasis assay have been shown to correlate closely with those from the spontaneous metastasis assay (4, 5).

Most experimental models used in the study of metastasis have utilized tumors or cell lines which are highly aneuploid,

so that the ability to analyze the effect of karyotypic abnormalities upon metastasis has been limited. Indeed, because many naturally occurring tumors and metastases are highly aneuploid as well (6, 7), it could be supposed that these karyotypic features might be obligatorily associated with tumorigenicity or metastasis. Early passage rat embryo cells are diploid yet become tumorigenic and metastatic after transformation by the Harvey *ras* gene; it was therefore of interest to study these cells cytogenetically.

## MATERIALS AND METHODS

**Cells.** 4R and 5R are described by Pozzatti *et al.* (3). Briefly, they are two independent foci of rat embryo cells (day 17 Sprague-Dawley embryo cells; Flow Laboratories, McLean, VA) which were obtained after transfection with calcium phosphate-DNA precipitates made of pEJ (8), a plasmid bearing the Harvey *ras* oncogene, and pRSVneo (9), a dominant selectable marker conferring resistance to the antibiotic G418 (GIBCO, Grand Island, NY); 48 h after transfection, G418 was applied. Morphologically transformed foci were picked and expanded. These were verified to contain the Harvey *ras* oncogene by analysis of their DNA and RNA using Southern and Northern blotting and by immunoprecipitation of Harvey *ras* M, 21,000 protein.

**Isolation of Cells from Tumors and Metastases.** Tumors were derived from these cells after  $10^5$  cells were injected s.c. into the back of 3-4-week-old female nude mice. After 2 weeks, tumors were from 1-2 cm at their greatest diameter. Animals were sacrificed and the tumors were minced and plated into Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum, penicillin (100  $\mu$ g/ml), and streptomycin (100 units/ml). The cells readily grew in tissue culture. Other animals bearing s.c. tumors were sacrificed after 4 weeks. These animals were found to have spontaneous lung metastases. Representative metastases were excised from different lobes of the lung and from several different animals to ensure that the nodules were independent. After excision the nodules were minced and placed into culture. Lung metastases were also generated via a different method,  $10^4$  cells of 4R were injected into the tail vein of 3-4-week-old female nude mice yielding experimental metastases. Some of the resultant lung metastases (approximately 10-20 developed/mouse after 3 weeks) were also placed into culture. It could easily be confirmed that these cultures were derived from the tumor cells and were not murine fibroblast contaminants because the mouse does not have any metacentric chromosomes and as can be seen in Figs. 1 and 2 these cells contain the metacentric chromosomes that would be expected in rat cells.

**Cytogenetics.** As soon as  $10^8$  cells were obtained the cells were karyotyped. The cultures were incubated in Colcemid (150 ng/ml) in medium for 1 h at 37°C. They were collected, lysed in 0.075 M KCl for 10 min at room temperature, washed three times with cold methanol:acetic acid (3:1), spread on cold wet slides, and air dried. At least 50 spreads were counted for each cell culture. Chromosomes were banded using the trypsin-Giemsa techniques as described by Sun *et al.* (10). The slides were incubated at 60°C in 0.025 M phosphate buffer, pH 6.8. They were then flooded with Giemsa-trypsin [6.5 ml of above buffer, 2.5 ml of methanol, 0.5 ml of 1% trypsin (GIBCO)] and 0.25 ml of stock Giemsa [Fisher, Pittsburg, PA]. Chromosomes were numbered using the scheme of the Committee for a standard karyotype of *Rattus norvegicus* (11). At least 5 banded spreads were obtained from every cell culture.

Received 2/20/86; revised 5/1/86; accepted 5/7/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

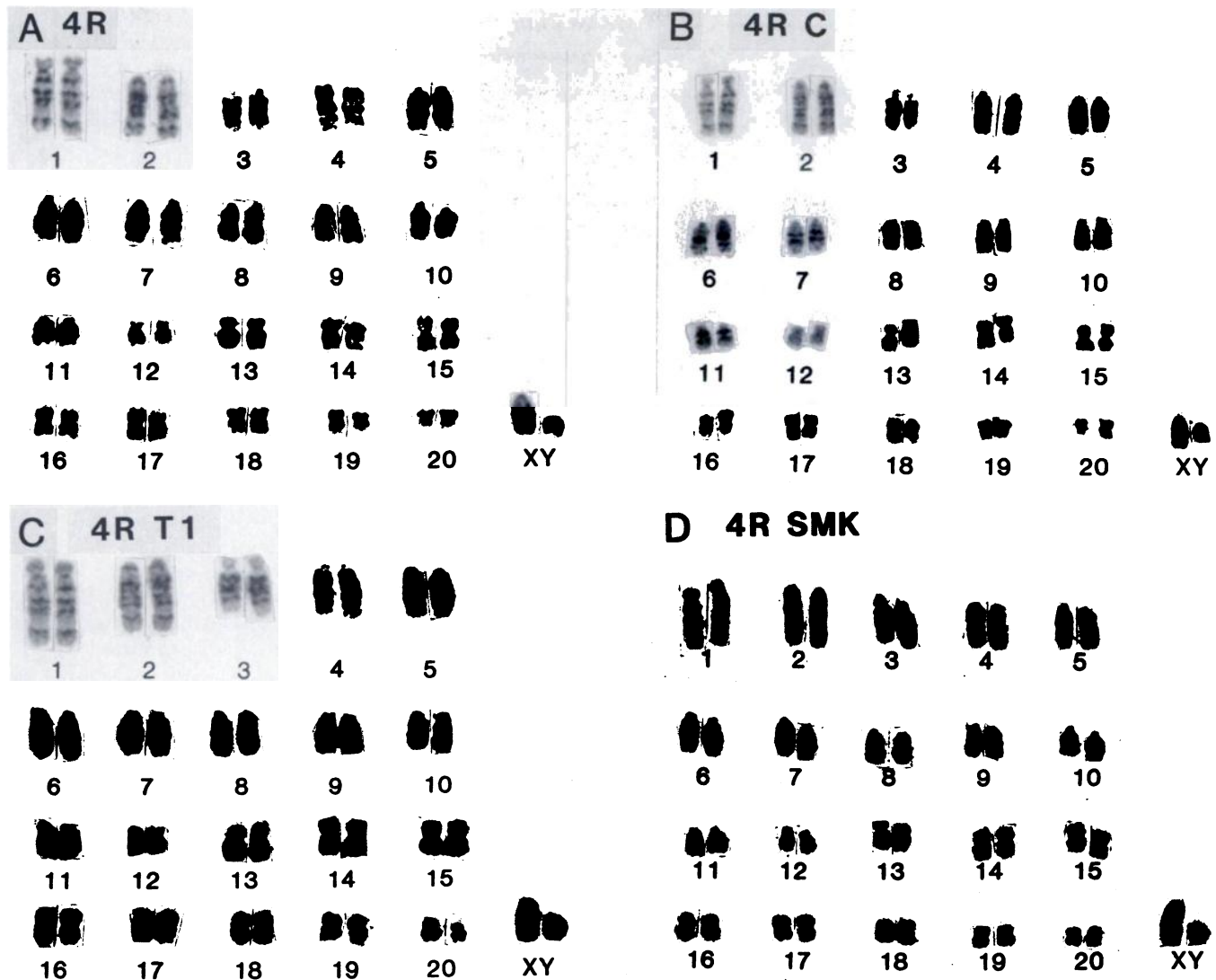


Fig. 1. Chromosome banding from parent cell 4R and sample tumors and metastases derived from 4R. Representative spreads from those karyotypes. *A*, banded spread from the original cell 4R; *B*, banded spread from one lung metastasis derived from the lung metastasis assay, 4R C; *C*, banded spread from one tumor derived from 4R, 4R T1; *D*, banded spread from one spontaneous metastasis 4R SMK.

Table 1 Chromosome numbers of cell line 4R and the tumors and metastases derived from that cell

Cells	Modal no.	% at modal no.	% of tetraploid cells	Range <sup>a</sup>
Parent cell 4R	42	74	4	40–42
Tumors				
4R T2	42	85	11	38–42
4R T3	42	94	2	40–42
Lung metastases				
4R C	42	100	0	42
4R D	42	88	5	39–43
4R E	42	92	2	40–43
Spontaneous lung metastases				
4R SML	42	90	3	41–43
4R SMK	42	74	10	41–42
4R SMM	42	80	10	40–42

<sup>a</sup> Excluding those with 80–86 chromosomes.

## RESULTS

We chose two independent foci (4R and 5R) of the rat embryo cells which had been transformed with the cellular Harvey *ras*<sub>1</sub>

oncogene for further analysis. Both of the cell lines readily formed rapidly growing tumors. Furthermore, at least 75% of the mice bearing tumors had spontaneous lung metastases within 4 weeks of the initial injection. Similarly in the lung metastasis assay, after i.v. injection of  $5 \times 10^4$  cells/mouse more than 200 nodules were usually found in the lungs of each mouse (3). Thus, these cell lines were both highly tumorigenic and metastatic in nude mice.

Karyotypic analysis was performed on each of these cell cultures at times between passages 12 and 20. The cell line 4R was found to have a modal chromosome number of 42, identical to normal rat cells (Table 1; Fig. 1). The other cell line studied extensively, 5R, was found to have a modal chromosome number of 43 (Table 2). Chromosome banding revealed that the additional chromosome was due to a trisomy of chromosome 4 (Fig. 2). The actual banding pattern of these cells is not detectably different from that of normal rat cells (Figs. 1 and 2). Thus both of these cell lines had a simple karyotype which could be readily followed in tumors and metastases. Furthermore these transformed rat cells could be easily distinguished from potential contaminant murine fibroblasts since the chromosomes of the rat and the mouse are quite different.

Table 2 Chromosome numbers of cell line 5R and the tumors and metastases derived from that cell

Cells	Modal no.	% at modal no.	% of tetraploid cells	Range <sup>a</sup>
Parent cell				
5R	43	79	0	38–47
Tumors				
5R T1	43	94	1	41–43
5R T2	43	96	2	42–43
5R T4	43	84	4	40–44
Lung metastases				
5R B	43	88	6	42–43
5R F	43	64	14	42–43
5R H	43	84	1	39–43
5R K	43	81	7	42–43
Spontaneous metastases to the lung				
5R SMC2	43	82	9	40–44
5R SMC4	43	90	10	43
5R SMA3	43	91 <sup>b</sup>	7	41–43
Spontaneous metastasis to lymph node				
5R LNB	43	86	4	41–43

<sup>a</sup> Excluding those from 80–86 chromosomes.

<sup>b</sup> 75% with 4q-, see Fig. 3 and "Results."

In order to investigate the karyotype of the tumors and of the metastases from these cell lines, 4R and 5R were injected s.c. into nude mice. Some of these mice were sacrificed at 2 weeks and the tumors which had formed were cultured *in vitro* (designated 4R T1, T2, etc.). Other mice were sacrificed at approximately 4 weeks and the spontaneous metastases which were found were also placed into culture (designated 4R SMA, etc.). Although most of the metastases were in the lungs, on one occasion an axillary lymph node metastasis was found after injection of 5R (5R LNB). The cell lines were also injected i.v. and some of the resultant lung metastases were also placed into culture (designed 4R C, D, etc.). These cell cultures derived from the tumors or the metastases of the cell lines, the diploid 4R and the near diploid 5R, were subjected to karyotypic analysis. The two tumors derived from the diploid cell line 4R also had diploid chromosome numbers. Three independent spontaneous metastases and three independent lung metastases produced after i.v. injection of nude mice were also analyzed and each was diploid and identical to the parent cell line 4R (Table 1).

The banding patterns of the chromosomes of the all of the tumors and all of the metastases isolated were also identical to those of normal rat embryo cells and of 4R. Representative spreads are shown in Fig. 1. Occasional 4R cells on one examination were found to have chromosome spreads which were strikingly abnormal with fragments of chromosomes, ring chromosomes, and double minutes. However, these have represented less than 1% of the spreads observed and were never seen in the tumors or metastases. Additionally, another population, from 0 to 11% of the total, composed of tetraploid cells is routinely seen. We have also rarely seen isolated instances of deletions or translocations but without any higher frequency in the tumors or the metastases than in the original cell line and without any apparent pattern. The metastases, however, had to have arisen from a diploid cell and not from one of these aberrant cells since they are themselves diploid.

The karyotypes of tumors and metastases derived from the cell line 5R were also studied. The parent line 5R has 43 chromosomes with a trisomy of chromosome 4. The three tumors examined also had a modal chromosome number of 43

with at least 84% of the cells having this number (Table 2). Banding indicated that there was also an additional chromosome 4 in the tumors derived from 5R (Fig. 2). Three independent cell cultures derived from lung nodules produced after i.v. injection of nude mice were also tested and again the predominant population was of cells with 43 chromosomes with a trisomy of chromosome 4. Three spontaneous metastases, two to the lung, 5R SMC2 and 5R SMC4, and 5R LNB from a lymph node were analyzed and the vast majority of the population had modal numbers of 43 with trisomy of chromosome 4.

The spontaneous metastasis 5R SMA3 was a slight exception; it also had a modal number of 43, and some of the cells had a trisomy of chromosome 4. More than one-half (75%) had retained the trisomy but had a deletion at the same point in the distal region of the q arm of one of the three chromosomes (Fig. 3). We would suggest that this culture is derived from a metastasis with the usual karyotype of 5R but that early in its growth a deletion occurred on one copy of chromosome 4 and that these two cellular populations are now mixed in a single metastasis. It should be noted that Talmadge *et al.* (12) have shown that spontaneous metastases are clonal in origin. Thus, the examination of the series of cells derived from 5R confirms the pattern seen in 4R, that there was no apparent selection for chromosomal alterations seen after formation of tumors or metastases. Again the rare aberrations described for 4R were also infrequently seen in 5R and its derivatives.

## DISCUSSION

Since the Harvey *ras* transformed rat embryo fibroblasts which we studied were diploid or near diploid, and since they were also highly tumorigenic and metastatic, we were able to study the karyotype in the tumors and the metastases, and we found that they were identical to the unselected cell lines. Since these were rat cells being tested in nude mice there was no possible confusion with murine cells because the mouse karyotype does not contain any metacentric chromosomes while the rat does. Thus, in this system there is no karyotypic selection during tumorigenesis or metastasis. In the past, because most tumors are highly aneuploid it was not possible to systematically evaluate whether karyotypic change had occurred during metastasis or tumor formation. In some isolated cases, studies on tumors and metastases from those tumors have indicated a selection for an alteration in modal chromosome number or chromosome pattern (13–16) while in others it has not (17–20). However, in such heterogeneous populations it is difficult to reach a firm conclusion. In this report we provide evidence in a more homogeneous system using cloned cell lines of transformed rat embryo cells that the karyotype of tumors and metastases is identical to the cells from which they were derived. It still remains possible that karyotypic change is a common mechanism causing tumor progression, but on the basis of these results in which 14 independent metastases were studied it is apparent that gross chromosomal alterations are not required for tumor formation or metastasis in this system. Certainly these experiments in this highly artificial system, using experimental cell lines and nude mice do not constitute a test of the Nowell hypothesis (21). They do indicate that in this system recognizing its limitations, chromosomal change was minimal and was not correlated with metastases.

Furthermore, we demonstrate that it is possible for a diploid cell to be metastatic. Indeed in this study we describe an early passage diploid cell which was transformed by the cellular

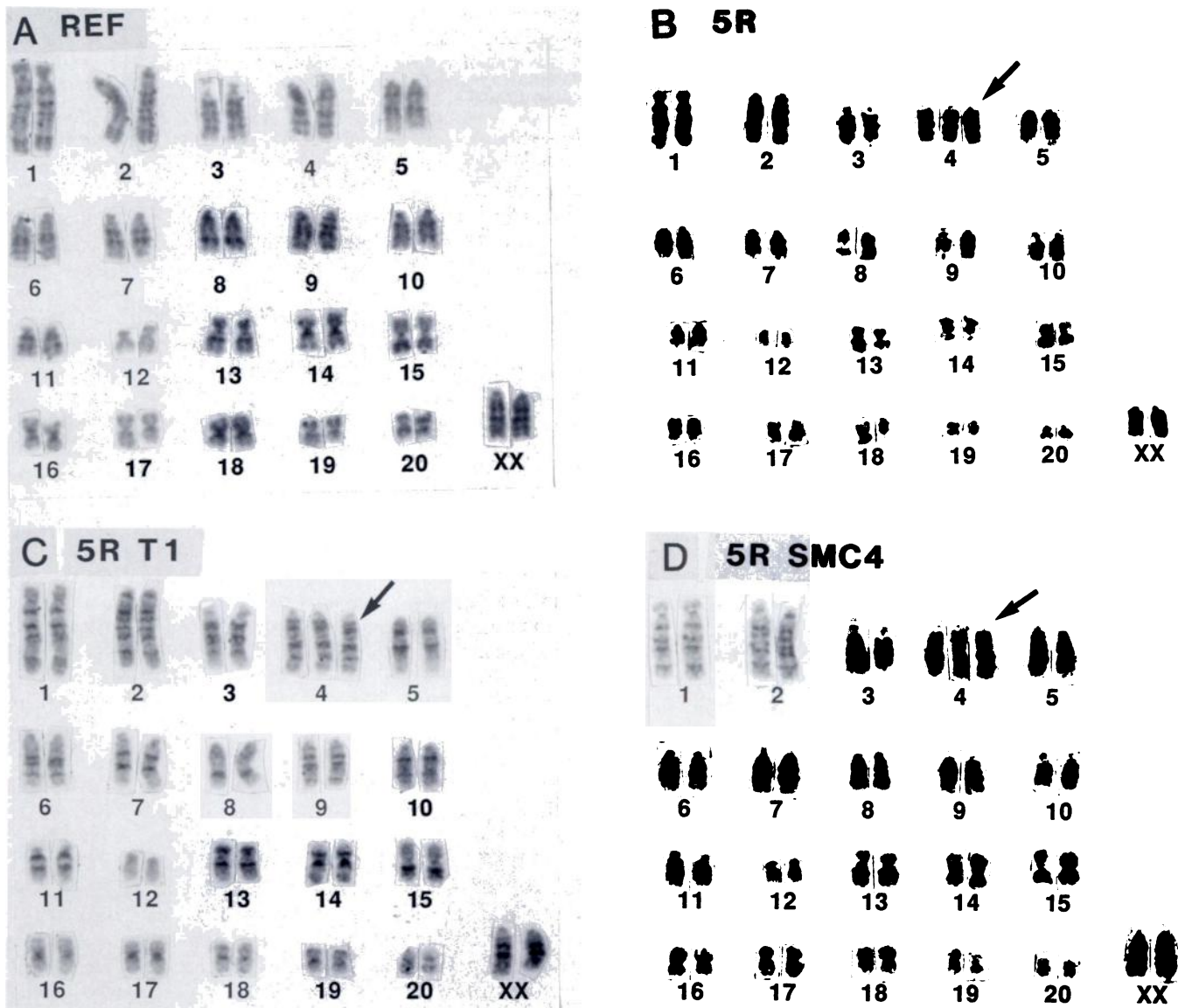


Fig. 2. Chromosome banding of rat embryo fibroblasts, the parent cell line 5R, and a sample tumor and metastasis derived from that cell line. *A*, embryo fibroblasts; a female spread although the population was a pooled population containing both sexes; *B*, 5R; *C*, 5R T1 from a tumor derived from 5R. *D*, 5R SMC4, a spontaneous metastasis from a 5R tumor. *Arrows*, trisomy of chromosome 4.



Fig. 3. Deletion of chromosome 4 found in 75% of spreads from 5R SMA3.

Harvey *ras*<sub>1</sub> gene and which is metastatic in nude mice yet has remained diploid. This is not unprecedented. Wolman *et al.* (22) have demonstrated the existence of diploid human breast carcinoma cells, sometimes even in metastases.

These results also indicate that transformation with the

Harvey *ras* oncogene does not by itself necessarily lead to gross chromosomal abnormalities. In comparison, Oshimura *et al.* (23) transformed Syrian hamster fibroblasts with a cotransfection of *v-myc* and *v-Ha-ras*. They selected for tumors but did not study metastases. Although a variety of chromosomal aberrations were seen in cells derived from the tumors, there was a consistent loss of one copy of chromosome 15 in all. By contrast they found that polyoma transformed Syrian hamster tumor cells were diploid. It should be noted that other workers have found that both bovine papilloma virus transformed and herpes virus transformed Syrian hamster cells show rearrangements in chromosome 15 (24, 25). Nonetheless, these results are in contrast to our own in which Harvey *ras* transformed rat embryo fibroblasts do not reveal any consistent karyotypic alterations and indeed one of our cell lines was diploid, even in metastases. This difference in results may be due to the *v-myc* gene or to the cell type used. Nonetheless, we can conclude that the Harvey *ras* gene itself does not invariably cause gross chromosomal alterations.

## ACKNOWLEDGMENTS

We thank Jeannette Williams for expert animal work and George Dudley for enthusiastic technical assistance.

## REFERENCES

- Muschel, R. J., Williams, J. E., Lowy, D. R., and Liotta, L. A. Harvey *ras* induction of metastatic potential depends upon oncogene activation and the type of recipient cell. *Am. J. Pathol.*, *121*: 1-8, 1985.
- Spandidos, D. A., and Wilkie, N. M. Malignant transformation of early passage rodent cells by a single mutated human oncogene. *Nature (Lond.)*, *310*: 469-475, 1984.
- Pozzatti, R., Muschel, R., Williams, J., Padmanabhan, R., Howard, B., Liotta, L., and Khoury, G. Primary rat embryo cells transformed by one or two oncogenes exhibit different metastatic potentials. *Science (Wash. DC)*, *232*: 223-227, 1986.
- Nicolson, G. L., and Poste, G. Tumor implantation and invasion at metastatic sites. *Int. Rev. Exp. Pathol.*, *25*: 77-181, 1983.
- Kripke, M. L., Gruys, E., and Fidler, I. J. Metastatic heterogeneity of cells from an ultraviolet light-induced murine fibrosarcoma of recent origin. *Cancer Res.*, *38*: 2962-2967, 1978.
- Mitelman, F., and Levan, G. Clustering of aberrations to specific chromosomes in human neoplasms. III. Incidence and geographic distribution of chromosome aberrations in 856 cases. *Hereditas*, *89*: 207-232, 1978.
- Sandberg, A. A. *The Chromosomes in Human Cancer and Leukemia*. Amsterdam: Elsevier/North-Holland Biomedical Press, 1980.
- Shih, C., and Weinberg, R. A. Isolation of a transforming sequence from a human bladder carcinoma cell line. *Cell*, *29*: 161-169, 1982.
- Gorman, C., Padmanabhan, R., and Howard, B. H. High efficiency DNA-mediated transformation of primate cells. *Science (Wash. DC)*, *221*: 551-553, 1983.
- Sun, N. C., Chu, E. H. Y., and Chang, C. C. Staining method for the banding patterns of human mitotic chromosomes. *Mamm. Chromosome News Lett.*, *1*: 26-28, 1973.
- Committee for a Standardized Karyotype of *Rattus norvegicus*. *Cytogenet. Cell Genet.*, *12*: 199-205, 1973.
- Talmadge, J. E., Wolman, S. R., and Fidler, I. J. Evidence for the clonal origin of spontaneous metastases. *Science (Wash. DC)*, *217*: 361-363, 1982.
- Kakati, S., and Sandberg, A. A. Chromosomes in solid tumors. *Virchows Arch. Abt. B Zellpathol.*, *29*: 129-137, 1978.
- Kusyk, C. J., Seski, J. C., Medlin, W. V., and Edwards, C. L. Progressive chromosome changes associated with different sites of one ovarian carcinoma. *J. Natl. Cancer Inst.*, *66*: 1021-1025, 1981.
- Lagarde, A. E., Donaghue, T. P., Dennis, J. W., and Kerbel, R. S. Genotypic and phenotypic evolution of a murine tumor during its progression *in vivo* toward metastasis. *J. Natl. Cancer Inst.*, *71*: 183-191, 1983.
- Smith, H. S., Liotta, L. A., Hancock, M. C., Wolman, S. R., and Hackett, A. J. Invasiveness and ploidy of human mammary carcinomas in short-term culture. *Proc. Natl. Acad. Sci. USA*, *82*: 1805-1809, 1985.
- Wang, N., Trend, B., Bronson, D. L., and Fraley, E. E. Non-random abnormalities in chromosome 1 in human testicular cancers. *Cancer Res.*, *40*: 796-802, 1980.
- Feder, M. K., and Gilbert, F. Clonal evolution in a human neuroblastoma. *J. Natl. Cancer Inst.*, *70*: 1051-1056, 1983.
- Balaban, G., Herlyn, M., Guerry, D., IV, Bartolo, R., Koprowski, H., Clark, W. H., and Nowell, P. C. Cytogenetics of human malignant melanoma and premalignant lesions. *Cancer Genet. Cytogenet.*, *11*: 429-439, 1984.
- Herlyn, M., Balaban, G., Bencicelli, J., Guerry, D., Hababan, R., Herlyn, D., Elder, D. E., Maul, G. G., Stepleski, Z., Nowell, P. C., Clark, W. H., and Koprowski, H. Primary melanoma cells of the vertical growth phase: similarities to metastatic cells. *J. Natl. Cancer Inst.*, *74*: 283-289, 1985.
- Nowell, P. C. The clonal evolution of tumor cell populations. *Science (Wash. DC)*, *194*: 23-28, 1976.
- Wolman, S. R., Smith, H. S., Stampfer, M., and Hackett, A. J. Growth of diploid cells from breast cancers. *Cancer Genet. Cytogenet.*, *16*: 49-64, 1985.
- Oshimura, M., Gilmer, T. M., and Barrett, J. C. Nonrandom loss of chromosome 15 in Syrian hamster tumours induced by v-Ha-*ras* plus v-*myc* oncogenes. *Nature (Lond.)*, *316*: 636-639, 1985.
- Pratt, N. R., Lowther, G. W., Rees, R. C., Teale, D. M., and Potter, C. W. Non-random chromosome changes in a herpes-virus-transformed Syrian hamster cell line and its metastatic derivatives. *Int. J. Cancer*, *34*: 849-853, 1984.
- Gamperl, R., Amtmann, E., and Pfister, H. Chromosomal changes in bovine papillomavirus type 1-induced Syrian hamster tumors. *Cancer Genet. Cytogenet.*, *12*: 151-162, 1984.