Volume 229, number 2, 333–339 FEB 05660 March 1988

Integration and loss of a single *v-Ki-ras* gene affects tumorigenic potential of human osteosarcoma cells

Guido Carloni[+°], Anne-Marie Venuat*, Leela Daya-Grosjean[†], Pierre Nardeux[°], Johng S. Rhim[△] and Bruno Azzarone

[+]*Istituto di Medicina Sperimentale, CNR, Rome, Italy,* [°]*Laboratoire de Génétique Cellulaire, IRSC,* **INSERM U 66, IGR,* [†]*Laboratoire de Mutagénèse Moléculaire, IRSC, Villejuif, France,* [△]*Laboratory of Cellular and Molecular Biology, NCI, NIH, Bethesda, MD, USA* and *INSERM U 268, ICIG, Hôpital Paul Brousse, Villejuif, France*

Received 2 December 1987; revised version received 22 January 1988

The human osteosarcoma cell line Te85 clone F-5 is not tumorigenic in vivo. Its transformation with Kirsten murine sarcoma virus (KiMSV) (KHOS) confers full malignant properties and stable non-tumorigenic revertants of this KHOS cell line have been obtained. Here we show that integration and expression of a single copy of the KiMSV proviral DNA, which is totally lost in the HOS 240S revertant, is responsible for the acquisition of tumorigenicity. Cytogenetic analysis and the absence of a residual LTR copy in the revertant cellular genome suggest that the loss of KiMSV provirus is caused either by chromosomal segregation or by recombination not involving the LTR. In addition analysis of the expression of ras proteins revealed no changes in the pattern of *c-ras* products and the expression of *v-ras* only in the KHOS cells. All these data suggest that Te85 and HOS 240S cell lines could represent a human alternative recipient system to rodent cells in studies with oncogenes.

ras oncogene; Tumorigenicity; Reversion; (Human osteosarcoma cell)

1. INTRODUCTION

As a rule human normal cells are resistant to the action of *ras* oncogenes [1], which indeed fully transform only established cell lines [2]. Moreover, at least in murine cells, the number and the type of biological properties affected seem to depend on the number of integrated *ras* gene copies [3]. Since it has been postulated that cellular oncogenes have an activity very similar to their viral counterparts [4], we decided to carry out a molecular analysis on the acquisition and reversion of the neoplastic phenotype in human cells transformed by a retrovirus, the Kirsten murine sarcoma virus (KiMSV), in order to better understand how these cells are affected by *ras* oncogenes [5]. In this respect, a very useful human system is the established non-tumorigenic human osteosarcoma cell line Te85, which after transformation (KHOS) by KiMSV becomes tumorigenic in vivo [6]. KHOS cells also acquire the ability to penetrate and to proliferate within normal tissues in vitro and lose the capacity to organize a provisional fibrin matrix [7]. Flat revertants HOS 240S have been obtained which have lost all the malignant properties of the parental non-producer KiMSV transformed osteosarcoma cell line (KHOS) [8]. In this report we investigate the mechanisms involved in the process of acquisition and loss of the malignant phenotype in the HOS cell system by analyzing: (i) the number of integrated KiMSV proviral copies, (ii) the expression of v- and c-ras proteins, and (iii) the cytogenetic modifications.

Correspondence address: B. Azzarone, INSERM U 268, ICIG, Hôpital Paul Brousse, Villejuif, France

2. MATERIALS AND METHODS

2.1. *Cells and culture conditions*

The HOS cells used in this study were: a human osteosarcoma line, designated Te85 clone F-5, a non-virus-producer

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

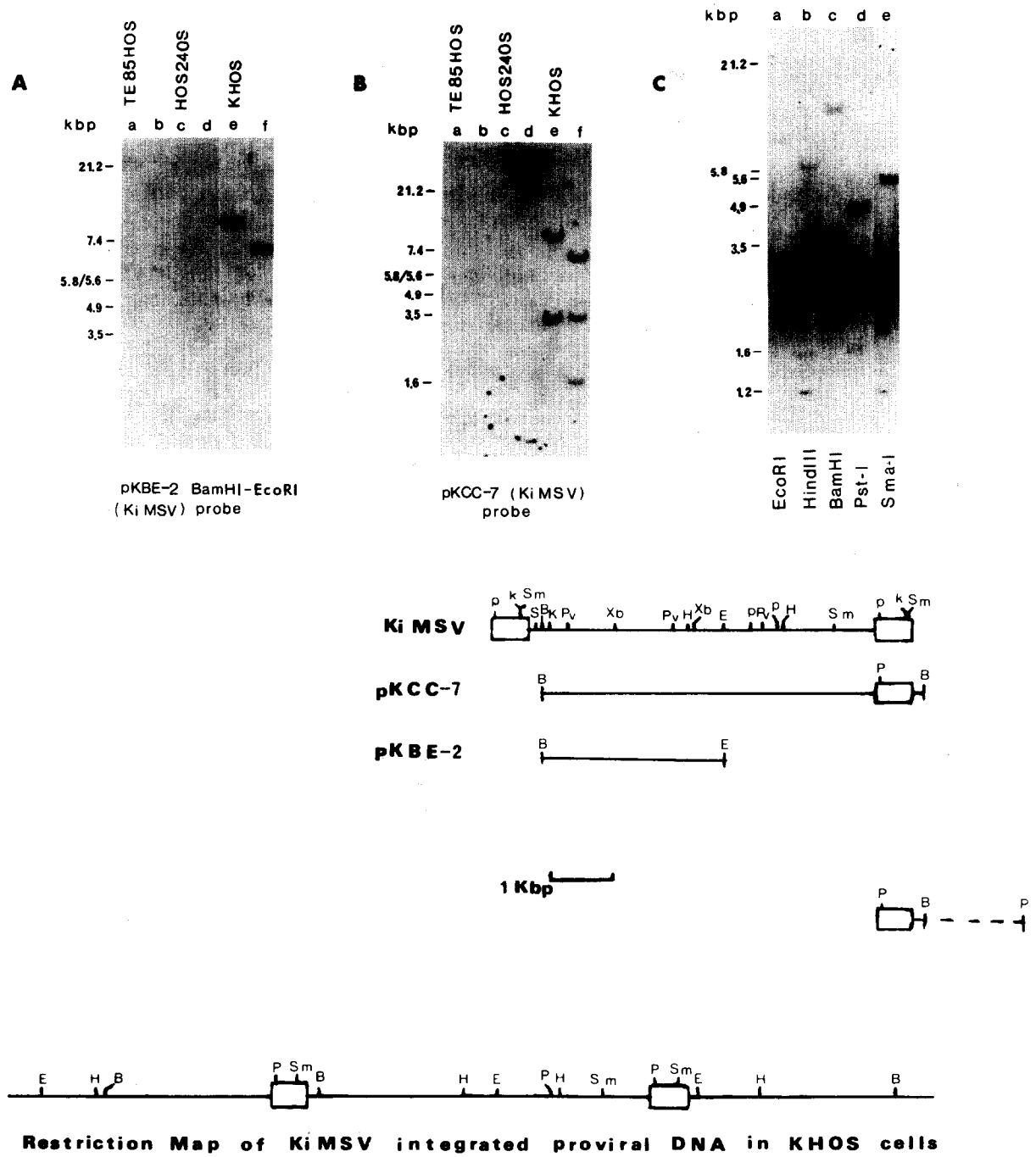


Fig.1. KiMSV proviral DNA in KHOS and HOS 240S revertant cells. Panels A and B show the state of KiMSV proviral DNA in HOS cell lines. Southern genomic blots after digestion with the restriction enzymes *Eco*RI (lanes a,c,e) and *Hin*dIII (lanes b,d,f), using pKBE-2 (A) and pKCC-7 (B) viral probes: DNA fragments were separated on 0.8% agarose gel and transferred to nitrocellulose paper before hybridization. Panel C shows the restriction pattern of KiMSV integrated provirus in KHOS cell line. Below it is shown the restriction map of the integrated KiMSV provirus with reference to the linear KiMSV proviral map and the probe described in section 2. Markers obtained after digestion of λ phage with *Eco*RI are indicated (21.2, 7.4, 5.8, 5.6, 4.9 and 3.5 kb). Restriction site abbreviations: P, *Pst*I; K, *Kpn*I; Sm, *Sma*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

tumorigenic line (KHOS) derived from transformed foci induced by KiMSV [7], and the non-tumorigenic revertant HOS 240S of the KHOS [8,9]. Growth and maintenance medium consisted of Eagle's minimal essential medium (EMEM), supplemented with 10% calf serum and 16 μg gentamycin per ml.

2.2. *DNA extraction and hybridization*

Total cell DNA was isolated from transformed cells by a modification of the procedure described by Gross-Bellard et al. [9]. Restriction enzyme digestions were under the conditions specified by the suppliers, New England Biolabs (Beverly, MA, USA) and Boehringer (Mannheim, FRG). The following probes labeled by nick-translation were used: the pKBE-2 clone, which is a 3.1 kb long *Bam*HI-*Eco*RI viral fragment [10], covers the 5'-half of the KiMSV genome containing the *v-ras* regions but not the 5'-LTR. The pKCC-7 clone [11] which is representative of the total in vivo synthesized KiMSV DNA [12] including the 3'-LTR but where the 5'-LTR is missing. The pKCC-7 is a generous gift from Dr J.D. Norton. The 3'-LTR probe was a *Pst*I fragment (about 1.8 kb in length) which was obtained from pKCC-7 after separation from a 0.8% agarose melting gel. Southern transfer and hybridization were by standard procedures. Stringent hybridization conditions were used: 50% (v/v) deionized formamide, 5 × SCC, 1 × Denhardt's, 24 h at 42°C.

2.3. *^{32}P,[^{35}S]methionine labelings, immunoprecipitation and SDS-polyacrylamide gel electrophoresis of p21*

Cells were labeled with ^{32}P or with 100 μCi/ml of [^{35}S]methionine (1000 Ci/mmol, NEN). The procedures for immunoprecipitation and SDS-polyacrylamide gel electrophoresis of p21 protein were performed as described [13,14].

2.4. *Cytogenetic studies*

A standard procedure for air-dried preparation was used for chromosome number evaluation and analysis of morphological abnormalities. Some slides were treated by heating denaturation at 86°C to obtain RHG banding following the Dutrillaux technique [15].

3. RESULTS

3.1. *State of KiMSV provirus in transformed KHOS and revertant HOS 240S cell lines*

It has been observed that the revertant HOS 240S no longer possessed a rescuable MSV genome [7]. Here we show that (fig.1A) only KHOS DNA gave restriction fragments which were detected by the 5'-end specific probe, pKBE-2, namely one 8 kb *Eco*RI fragment (lane a) and a 6.4 kb *Hin*dIII fragment (lane f). No hybridization could be detected in the Te85 clone F-5 (lanes a,b) or the HOS 240S (lanes c,d) DNAs. When the KiMSV complete pKCC-7 probe was used (fig.1B), 2 specific *Eco*RI bands of 8 kb and 3.2 kb (lane e) and 3 specific bands of 6.4, 3.3 and 1.6 kb, respectively (lane f), were detected only in the KHOS DNA. No hybridization was detected with the Te85 clone F-5 (lanes a,b) nor HOS 240S (lanes c,d) DNAs. Since we used stringent hybridization conditions (see above), *c-Ki-ras* genes are of course not detected (fig.1A,B, lanes a–d) by the two viral probes used. This indicates that, in the absence of cross-hybridization with *c-ras* endogenous genes (fig.1A,B, lanes e,d), the revertant HOS 240S cell line appears to have lost the totality of the proviral DNA sequences. Since the *Eco*RI 8 kb and the *Hin*dIII 6.4 kb bands were detected with both the pKBE-2, 5'-end specific KiMSV probe and the

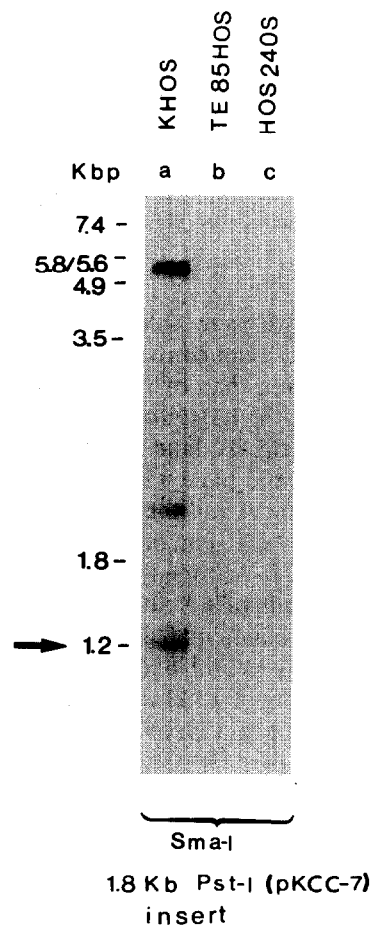


Fig.2. Southern blot of genomic DNA from HOS cell lines using the 1.8 kb probe specific for the KiMSV LTR. DNA fragments were separated on a 1.2% agarose gel after digestion with *Sma*I. Markers obtained after digestion of λ phage with *Eco*RI are indicated (7.4, 5.8, 5.6, 4.9 and 3.5 kb) and 1.2 kb fragments DNA obtained after *Sma*I digestion of pKCC-7 probe.

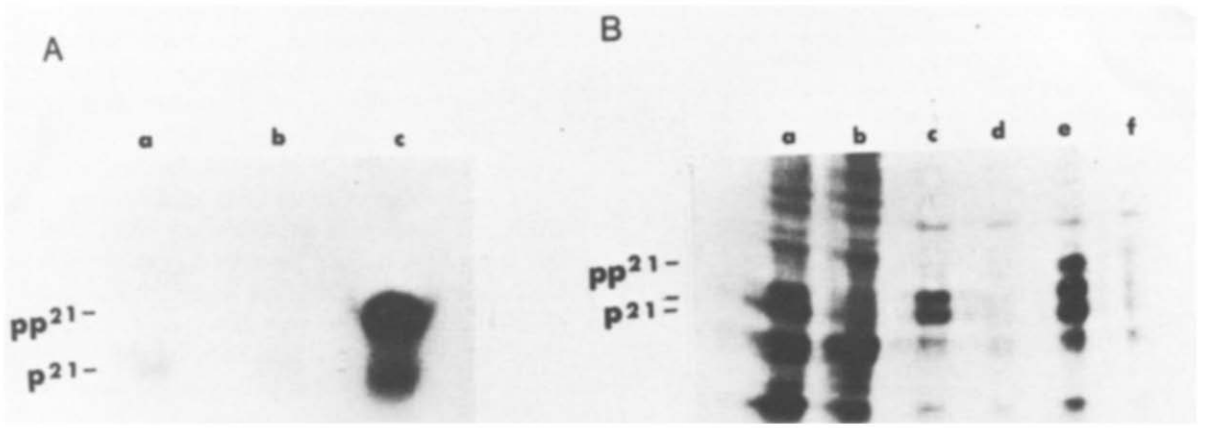


Fig.3. Analysis of phosphorylated p21 protein (A) and specific immunoprecipitation of ras proteins in human osteosarcoma cells (B). (A) Human osteosarcoma cells: Te85 clone F-5 (lane a), revertant, HOS 240S cells (lane b), KiMSV transformed osteosarcoma cells, KHOS (lane c) were labeled with ^{32}P and immunoprecipitated with Mab 259, recognizing all the *ras* coded proteins. Phosphorylated p21 protein (pp21) is only seen in the cells transformed by KiMSV which express the viral coded Ki-ras p21 protein. (B) [^{35}S]Methionine (1000 Ci/mmol, NEN) labeled extracts of human osteosarcoma cells, Te85 clone F-5 (lanes a,b); HOS 240S cells (lanes c,d); KHOS cells (lanes e,f) were analyzed by immunoprecipitation using the wide spectrum, Mab 259 and electrophoresis in SDS-polyacrylamide gels (lanes a,c,e). Immunoprecipitates in lanes (b,d,f) were obtained with Mab 238 which recognizes all the p21 proteins except the *v-Ki-ras* and *N-ras* coded proteins.

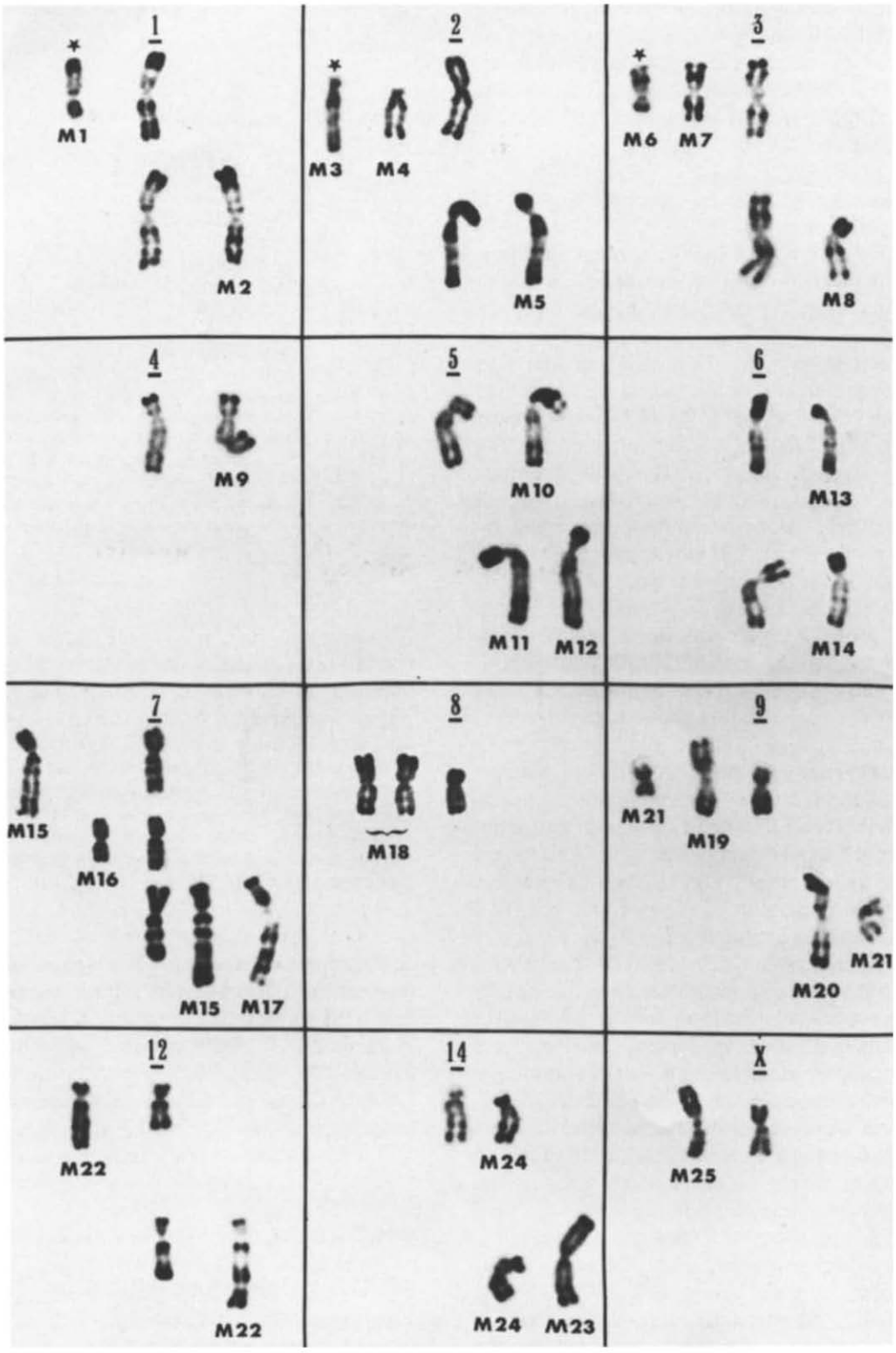
pKCC-7 probe we were able to locate this large fragment at the 5′-end of the viral genome and to obtain a restriction map of the integrated KiMSV proviral DNA in the KHOS cell line. Therefore the 1.6 kb *Hin*dIII (fig.1B, lane f) band appears to be an internal genomic band while the *Eco*RI 3.2 kb band (fig.1B, lane e) and the *Hin*dIII 3.3 kb band can be placed at the 3′-end of the KiMSV provirus.

Using the pKCC-7 probe (fig.1C) we observed 2 internal *Pst*I bands of 4.8 and 1.6 kb (lane d) and 2 internal *Sma*I bands of 1.2 and 5.06 kb (lane e) and of 2 *Bam*HI hybrid bands of 12 and 3.8 kb (lane c). The larger 12 kb, strongly hybridizing band is composed of a large part of the total proviral DNA and some flanking cellular DNA at the 3′-end. The 3.8 kb band obtained with *Bam*HI is much fainter as most of this fragment at the 5′-LTR is composed mainly of cell DNA. In conclusion, the restriction patterns shown after digestion with *Eco*RI (fig.1A,B, lanes a,c,e) and with *Hin*dIII (lanes b,d,f) and the restriction map of the KiMSV integrated proviral DNA (fig.1C), are compatible with the presence of a single unrearranged integrated KiMSV provirus complete with both the LTRs in the KHOS cell line.

3.2. *Evidence for loss of LTR sequences in the revertant HOS 240S cells*

The evidence against the presence of any residual LTR copy in the HOS 240S cell line is given in fig.2. In the KHOS genomic DNA, digestion with *Sma*I gives two viral specific bands of 5.06 and 1.2 kb, respectively, using a specific *Pst*I fragment of 1.8 kb containing 750 bp of the 3′-LTR of the pKCC-7 plasmid (fig.1). The 1.2 kb band specific of the Kirsten genome plus the first 410 bp of the 3′-LTR is absent in the HOS 240S DNA (fig.2, lane c). An additional hybrid band of 2.4 kb, probably corresponding to part of the 5′-LTR sequences upstream of the *Sma*I restric-

Fig.4. Representation of all the identified chromosomal markers. In each panel the normal chromosome is presented in the central file. On its left are markers of the KHOS cell line and on its right markers of the HOS 240S cell line. For each chromosome involved in rearrangements, the chromosome on the same lines, normal or abnormal, belong to the same mitosis except for those labeled with a star. The frequency and terminology of each marker are described in table 1.


1
M1
M2
2
M3
M4
M5
3
M6
M7
M8
4
M9
5
M10
M11
M12
6
M13
M14
7
M15
M16
M15
M17
8
M18
9
M21
M19
M20
M21
12
M22
M22
14
M24
M24
M23
X
M25

tion site in a cellular flanking region, is also found in the KHOS DNA *Sma*I digest (lane a). This 2.4 kb band is not detected in the HOS 240S revertant cell line DNA. These results confirm that the revertant HOS 240S cell line lost all the proviral DNA including both the LTRs.

3.3. *Expression of v- and c-ras proteins in HOS cell lines*

The phosphorylated v-Ki-ras protein (pp21) is found in the KHOS cells (fig.3A, lane c) but not in the parental (fig.3A, lane a) or in the revertant (fig.3A, lane b) cell lines. Immunoprecipitation was performed with the wide spectrum Mab 259 which recognizes all the *ras* coded p21 proteins. Expression of *c-ras* coded products was checked using the Mab 259 (fig.3B, lanes a,c,e) and the Mab 238 (fig.3B, lanes b,d,f) which does not recognize v-Ki-ras, and N-ras proteins. All cell lines show p21 protein immunoprecipitated by Mab 259 (lanes a,c,e). The slower moving band in the p21 doublet is probably the pp21. As no p21 is immunoprecipitated in these cell lines by the Mab 238 and only KHOS cells have been virally transformed, one can deduce that the p21 expressed in the HOS cells is probably coded by the *N-ras* gene.

3.4. *Cytogenetics analysis of the differences between KHOS and revertant cells*

The cytogenetic analysis revealed a modal number of 49 and 47 chromosomes for KHOS and HOS 240S cells, respectively, demonstrating a loss of 2 chromosomes in the revertant cell line associated with an increased chromosome fragility (0.83 abnormalities/mitose in HOS 240S cells with respect to 0.46/mitose in the tumorigenic KHOS cells). The R banding [15] showed the presence in both cell lines of about 20 chromosomal markers resulting from chromosomic rearrangements, some of which could be identified and detailed in table 1 and fig.4. We also observed a monosomy for the chromosome no.15 in the KHOS cells and its nullosomy in the HOS 240S cell line. In the KHOS cells there was also a tetrasomy of the long arms for chromosome no.8 (two normal no.8 plus two t (8q, 14q); however, only the two normal chromosomes no.8 were observed in HOS 240S cell line). Finally, chromosome regions, bearing the different *c-ras* genes, are not involved in the observed rearrangements. The appearance in the revertant cell line of a significant increase in the rate of chromosome fragility and rearrangements is probably the consequence of the selection methodology employed to obtain morphologically flat revertants from KHOS cells (exposure to high temperatures) [9].

Table 1

Identified chromosomal markers in the KHOS and HOS 240S human osteosarcoma cell lines

Name[b]	Terminology	Frequency (%)[a]	
		KHOS	HOS 240S
M1(M2)	der(1)t(1,?)(q11,?)	*80*	0
M2	inv(1)(q12 q31)	13.3	100
M3	del(2)(p12)	*66.6*	0
M4	del(2)(p11)	*20*	0
M5	der(2)t(2,?)(p11,?)	0	*75*
M6(M13)	del(3)(q22)	*46.6*	0
M7	i(3p)	46.6	6.25
M8	der(3)t(3,22)(p11,p12)	0	*37.5*
M9	der(4)t(4,?)(p32,?)	0	*100*
M10	der(5)t(5,?)(p12,?)	0	*37.5*
M11	t(5q22q)	0	*6.25*
M12	der(5)t(5,?)(p12,?)	0	*62.5*
M13	del(6)(p22)	0	*43.75*
M14	t(6q,?)	0	*75*
M15(M1)	der(7)t(1,7)(q12,q31)	100	100
M16	i(7p)	*26.6*	0
M17	t(7p,?)	0	*31.25*
M18	t(8q14q)	*93.3*	0
M19	t(3q9q)	*100*	0
M20	t(9,9)(p21,q12)	0	*100*
M21	t(9p,?)	26.6	56.25
M22(M3)	der(12)t(12,?)(q34,?)	100	100
M23	t(14q14q) or i(14q)	26.6	50
M24	der(14)t(14,?)(p12,?)	0	*93.75*
M25	der(X)t(X,?)(p21,?)	*60*	0

[a] Frequency of identified markers established from 15 and 16 RHG-banded karyotypes of respectively KHOS and HOS 240S cell lines, following the technique of Dutrillaux [17]. Italicized values indicate the specific markers of each cell line

[b] In parentheses are indicated the already described markers of the parental cell line TE35 [20]

4. DISCUSSION

In this study, the presence of a unique integrated provirus in the KHOS cells and its total absence in the revertant HOS 240S cells reveals that the acquisition of the pleiotropic properties, linked to the tumorigenic phenotype [8], is associated with

the continuous expression of a single-copy *v-Ki-ras* oncogene. Loss of KiMSV provirus in HOS 240S revertant does not involve an LTR to LTR recombination since no single residual LTR can be found. Cytogenic analysis suggests that the loss of provirus could be mediated by a partial or total loss of chromosomic material, since HOS 240S cells exhibit an increased chromosome fragility and decreased chromosome number in comparison with KHOS cells. Transforming potential of human oncogenes has been mainly assessed on rodent cell systems. A recent report proposed HOS Te85 cells as a suitable alternative to 3T3 murine cells in this type of studies [16]. Here we propose that the revertant HOS 240S cell line represents an additional and perhaps better candidate. These cells are indeed completely devoid of any viral information, do not present changes in the expression of c-ras proteins and display an increased retransformation efficiency by KiMSV when compared with the parental HOS Te85 clone F-5 [8].

Moreover, in preliminary experiments with the pEJ-neo recombinant vector, which contains the activated human *H-ras* oncogene and a selectable neomycin marker [17], we found that the HOS 240S cells are significantly more susceptible to transformation than the parental Te85 cell line (24 colonies versus 4 colonies/μg DNA per 5×10^5 plated cells).

Acknowledgements: This paper is dedicated to the memory of Yves Malpiece who died on the 25th October 1985. We thank Drs J.D. Norton, M. Hill, B. Dutrillaux for their criticism and Dr R. Cassingena for his support. One of us (G.C.) had fellowships from ARC EMBO and CNR/CNRS. This work was supported by the CNRS (ATP No.959056) and ARC (Contrat no.1222 and 3055).

REFERENCES

[1] Sager, R., Tanaka, K., Lau, C.C. and Anizovicz, A. (1983) Proc. Natl. Acad. Sci. USA 80, 7601–7605.
[2] Marshal, C.J., Vonsden, K., Hall, A., Malcom, S., Newbold, R.F., Paterson, H. and Weiss, R.A. (1984) in: Cancer Cells (Van de Woude, G.F. et al. eds) vol.2, pp.441–445, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[3] Sistohen, L., Kiski, O.J.A., Ulmann, I., Höltta, E., Wikgren, B. and Alitalo, K. (1987) Exp. Cell Res. 168, 518–530.
[4] Varmus, H.E. (1984) Annu. Rev. Genet. 18, 553–612.
[5] Tabin, C.J., Bradley, S.M., Bargmann, C.L., Weinberg, R.A., Papageorge, A.G., Scolnick, E.M., Dhar, R., Lowy, D.R. and Chang, E.H. (1982) Nature 300, 143–149.
[6] Rhim, J.S., Cho, H.J.Y., Vernon, M.L., Arstein, P., Hubner, R.G., Gilden, R.V. and Nelson-Rees, W.A. (1975) Int. J. Cancer 16, 840–849.
[7] Azzarone, B., Carloni, G., Marel, M., Varnier, O. and Macieira-Coehlo, A. (1985) in: Retroviruses and Human Pathology (Gallo, R.C., Stehelin, D. and Varnier, O. eds) pp.473–483, Humana, Clifton, NJ.
[8] Cho, H.Y., Cutchins, E.C., Rhim, J.S. and Hubner, R.J. (1976) Science 194, 951–953.
[9] Gross-Bellard, M., Oudet, P. and Chambon, P. (1973) Eur. J. Biochem. 36, 32–38.
[10] Ellis, R.W., Defeo, D., Shih, T.Y., Gonda, M.A., Young, H.A., Tsuchida, N., Lowy, D.R. and Scolnick, E.M. (1981) Nature 292, 506–511.
[11] Norton, J.D. and Avery, R.J. (1982) Biochem. Biophys. Res. Commun. 108, 1631–1637.
[12] Norton, J.D., Connor, J. and Avery, R.G. (1984) Nucleic Acids Res. 17, 6839–6852.
[13] Furth, M.F., Davis, L.J., Fleudelys, B. and Scolnick, E.M. (1982) J. Virol. 43, 294–304.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Dutrillaux, B. (1973) Nobel Symp. 25, 38–42.
[16] Tainsky, M.A., Shamansky, F.L., Blair, D. and Van de Woude (1987) Mol. Cell. Biol. 7, 1280–1284.
[17] Southern, P. and Berjer, P.J. (1982) Mol. Appl. Genet. 1, 327–341.