DIFFERENCES IN *c-myc* AND *pvt-1* AMPLIFICATION IN SEWA SARCOMA SUBLINES SELECTED FOR ADHERENT OR NON-ADHERENT GROWTH

Janos MINAROVITS, Michael STEINITZ, Ferenc BOLDOG, Stephan IMREH, Zvi WIRSCHUBSKY, Sigurdur INGVARSSON,
Mona HEDENSKOG, Susanna MINAROVITS-KORMUTA and George KLEIN

Department of Tumor Biology, Karolinska Institute, S-104 01 Stockholm, Sweden.

Conversion of solid sarcomas and carcinomas into ascites tumors depends on the *in vivo* selection of phenotypically altered tumor cell variants that can grow in the dissociated form. Once selected, they retain this property even after prolonged s.c. growth as solid tumors. From an s.c.-passaged subline of an ascites-converted murine sarcoma (SEWA-AS12), we were able to separate cells adapted to the ascites form of growth from cells that can only grow in the solid form on the basis of their differential adherence to plastic. Both *c-myc* and *pvt-1* were amplified approximately 63- to 77-fold in the non-adherent subline (SEWA-AS12-NA), but only 5- to 8-fold in the adherent subline (SEWA-AS12-ADH). This suggests that *c-myc* and/or *pvt-1* amplification may provide a selective advantage to cells that can grow in the dissociated form.

Populations of neoplastic cells show considerable karyotypic heterogeneity (Nowell, 1986). Many tumors can readily give rise to sublines that differ from the original "wild type" with regard to phenotypic traits such as drug sensitivity, metastatic propensity or the ability to grow in the ascites form (Law, 1956; Klein, 1953; Price *et al.*, 1986). Ascites conversion depends on the ability of tumor cells to grow in dissociated form in the peritoneal fluid. Selection of such variants can be achieved by serial intraperitoneal passage of peritoneal exudates (Klein and Klein, 1951). Ascites-converted variants were found to retain their property of growing as free cells even after reversion to and serial passage in the solid form.

Our present study was initiated with 2 distinct but interrelated purposes. Since ascites convertibility is associated with reduced adhesiveness and increased negative surface charge (Klein, 1955; Purdom *et al.*, 1958) we wished to examine whether ascites-convertible and non-convertible variants could be separated from the same tumor-cell population, on the basis of differences in adherence to plastic. We chose the polyoma-virus-induced SEWA sarcoma which had been gradually converted to the ascites form by serial peritoneal exudate passage (Sjögren *et al.*, 1961), using a subline derived by s.c. inoculation of the ascites fluid and subsequent serial passage in the solid form. We expected that solid tumor growth would favor the growth of any ascites-inconvertible revertants and we wished to examine whether such revertants could be isolated by plastic adherence. This turned out to be the case.

Our choice of the SEWA tumor was also motivated by another consideration. In the ascites form of this tumor Levan *et al.* (1976) have identified double minute chromosomes. These were not present in serially propagated *in vitro* cultures, however. Schwab *et al.* (1985) have shown that *c-myc* is amplified in the SEWA ascites tumor. The relationship between the double minutes and the amplified *c-myc* copies is not known.

We wished to determine whether the level of *myc* amplification differs among sublines selected *in vitro* for adherent versus non-adherent growth. We have found that both *c-myc* and the linked *pvt-1* locus were amplified in the non-adherent SEWA subline (SEWA-AS12-NA). The adherent subline (SEWA-AS12-ADH) was less prone to growth in the ascites form and had a much lower *c-myc* and *pvt-1* copy number than the non-adherent variant.

MATERIAL AND METHODS

Cells

The SEWA sarcoma was induced by polyoma virus in an A.SW mouse (Sjögren *et al.*, 1961). Originally it was an osteosarcoma but its differentiation markers were lost after a few passages. It was converted into the ascites form by serial transfer of peritoneal fluid and was subsequently maintained by i.p. inoculation in syngeneic mice through an unknown number of passages. Over the last 2 decades it was kept in frozen storage. The present study was initiated with 2 sublines derived from the frozen ascites tumor. SEWA-AA7 was passaged i.p. 7 times before use. SEWA-AS12 underwent 8 i.p. passages followed by 12 s.c. passages as a solid tumor. Both sublines grew readily as ascites tumors after i.p. inoculation.

For the present studies SEWA-AS12 was cut into small pieces, digested with trypsin and incubated in 5% heat-inactivated fetal calf serum (FCS)-supplemented RPMI-1640 medium (RPMI) in plastic tissue culture flasks. SEWA-AA7 cells were obtained by needle aspiration of peritoneal fluid, centrifuged and resuspended in 5% FCS/RPMI. SEWA-AA7 cultures contained only floating, non-adherent cells, while SEWA-AS12 grew as a mixed culture that contained both attached and floating tumor cells, including floating spheroids. The 2 subpopulations were subcultured separately. Following transfer of the non-adherent cells into a new flask, adherent cells were rinsed with PBS, incubated with 0.05% trypsin/0.1% EDTA and resuspended in 5% FCS/RPMI.

Adherence assay

Cells (10^5) were seeded into plastic Petri dishes. After incubation, unattached cells were removed and adherent cells were harvested with 0.05% trypsin/0.1% EDTA and counted in a hemocytometer.

Determination of population doubling time

Tumor cells were plated at a density of 10^4 cells/16-mm well (Costar, Cambridge, MA) in FCS/RPMI. Cultures were incubated at 37°C and triplicate samples were terminated daily after plating; the cells were harvested and counted in a hemocytometer. Mean cell counts were plotted against time on semilog graph paper and growth rates were established from the steepest part of the curve.

Cytogenetic analysis

Chromosome preparations were made repeatedly over a 4-month period according to a standard air-drying procedure (Rothfels and Siminovich, 1958). Twenty consecutive well-spread mitoses were scored on each Giemsa-stained slide for chromosome counts, double minute (DM) and premature chromosome condensation (PCC) analysis; 2,000 cells were scored for polymicronucleation. Trypsin Giemsa G-banding for chro-

mosome marker analysis was slightly modified from the method of Wang and Fedoroff (1972).

Tumorigenicity tests

Cultured SEWA cells were harvested, washed in PBS, counted and inoculated s.c. or i.p. into 6- to 8-week-old syngeneic A.SW mice of either sex. Solid tumors developing at the site of inoculation were measured with calipers twice weekly. Accumulation of ascites was recorded by visual inspection and confirmed by growth curve measurements on free tumor cells (Klein and Revesz, 1953). Briefly, the total number of cells in peritoneal washings was counted in a hemocytometer and the total number of tumor cells was calculated from differential counts of the large basophilic tumor cells in methanol-fixed, Giemsa-stained smears of peritoneal fluid.

DNA extraction and Southern blotting

High-molecular-weight DNA was isolated by SDS-proteinase K lysis, organic extraction and NaCl-ethanol precipitation (Guzella *et al.*, 1979). Ten micrograms of DNA were digested with the restriction enzyme EcoRI, electrophoresed in 0.8% agarose and transferred to a Hybond N membrane, essentially as described by Southern (1975). Hybridizations were performed by standard procedures under high stringency conditions. The following probes were used: a 2.2-kb cDNA clone (pM *c-myc*) of the mouse *c-myc* mRNA (Stanton *et al.*, 1983), a cloned 2.2-kb XbaI fragment of the murine *pvt-1* locus (Graham *et al.*, 1985), a cloned Hind III fragment (pTS 6) of the murine *mlvi-2* region (a gift of Dr. P. Tsichlis) and a murine *int-1* cDNA probe (Rijsewijk *et al.*, 1986). The probes were labelled with dCTP³² as described by Feinberg and Vogelstein (1984), using an oligolabelling kit (Pharmacia, Uppsala, Sweden).

Pulsed field gel electrophoresis (PFGE)

The isolation of DNA for PFGE and digestion with restriction enzymes were described previously (Boldog *et al.*, 1989). The DNA fragments were separated in 1% agarose using the GeneLine system (Beckman, Carlsbad, CA). The pulse time was 60 sec and the running buffer consisted of 10 mM TRIS, 0.5 mM EDTA (free acid), 0.025% glacial acetic acid. The electrophoresis was performed for 18 hr at 15°C with 150 mA. *Sacharomyces cerevisiae* (X2180-1B strain) chromosomes were used as size markers. After electrophoresis and ethidium bromide staining the DNA was denatured and transferred to GeneScreen Plus membrane (Dupont-NEN, Boston MA) using 0.4 N NaOH. After neutralization in $2 \times \text{SSC}$ 0.2 M Tris HCl pH 7.5, the membranes were dried at room temperature. Hybridizations were performed in 0.5 M phosphate buffer, pH 7.5, containing $5 \times$ Denhardt's solution, 1% SDS, 10% dextran sulfate and 250 µg/ml denatured salmon sperm DNA at 65°C. The filters were washed in $2 \times \text{SSC}$, 0.1% SDS at room temperature (2×15 min) followed by 2 washings in $0.2 \times \text{SSC}$, 0.1% SDS at room temperature (15 min each) and 2 more washings in the latter solution at 65°C (30 min each). Fuji films were used for autoradiography. The probes were removed from the filters by washing in $0.1 \times \text{SSC}$, 0.1% SDS for 2×30 min at 80°C. Complete removal of label was assessed by autoradiography before hybridization with a new probe.

RESULTS

Phenotypic heterogeneity of SEWA subpopulations

Serial subculturing of plastic-adherent and non-adherent SEWA-AS12 cells led to the isolation of a stable adherent subline, designated SEWA-AS12-ADH, and a suspension line with floating spheroids and free cells designated SEWA-AS12-NA (Fig. 1). A third *in vitro* subline, derived directly from the ascites tumor and designated SEWA-AA7-NA, grew

FIGURE 1 – Photomicrographs of SEWA-AS12-derived sublines. SEWA-AS12-ADH cells (a) and SEWA-AS12-NA cells (b) were stained with Giemsa.

only in the form of multicellular spheroids and floating free cells.

Figure 2 shows the attachment kinetics of the 3 sublines to plastic. The majority of SEWA-AS12-ADH cells adhered to the plastic. Only a minor fraction of the cells of SEWA-

FIGURE 2 – Attachment of SEWA sublines to plastic; 10^5 cells were seeded into plastic Petri dishes and the unattached and attached cells were removed at the time points indicated. The data represent the average of 3 dishes.

AS12-NA and SEWA-AA7-NA adhered, even after prolonged incubation.

The population doubling times of the sublines in medium containing different concentrations of FCS are shown in Table I. The growth kinetics of the 2 non-adherent sublines were very similar to each other. SEWA-AS12-ADH grew somewhat faster in higher serum concentrations but more slowly in 0.3% FCS than the non-adherent sublines.

Cytogenetic analysis

Chromosome counts varied within a wide range with a mean number of 81 (SEWA-AA7-NA and SEWA-AS12-NA) or 79 (SEWA-AS12-ADH) (Table II). All of the sublines shared 2 Robertsonian marker chromosomes: Rb(1;5) and Rb(5;5) (Table II, Fig. 3a, b). The 2 markers were rarely found together, and then often in cells containing more than 100 chromosomes.

Ten to twenty percent of the analyzed SEWA cells contained 2–15 pairs of DMs. The frequency of mitoses with higher numbers of DMs varied between the sublines. DMs (Fig. 3c) were particularly frequent in the SEWA-AA7-NA subline (65%), less so in SEWA-AS12-NA (25%) and absent from SEWA-AS12-ADH (Table II). HSRs were not found in our G-banded preparations.

A high frequency of polychromatinization and micronuclear PCC was a characteristic feature of the non-adherent sublines (Table II, Fig. 3c,d).

Relationship between failure to adhere to plastic and ability to grow in ascites form

We measured the growth of cells in the solid compared to the ascites form by inoculating syngeneic A.SW mice s.c. or i.p. As shown in Table III and Figure 4, the adherent subline (SEWA-AS12-ADH) regularly formed solid tumors (with one exception) at the site of s.c. or i.p. inoculation. The non-adherent SEWA-AS12-NA grew in the solid form if inoculated s.c., but formed ascites tumors after i.p. inoculation. SEWA-AA7-NA grew in the ascites form but grew at the site of s.c. inoculation, only with low efficiency.

Amplification of *c-myc* and *pvt-1* sequences

Previous studies have shown that *c-myc* is amplified in sublines harboring double minutes, homogeneously staining chromosomal regions or C-bandless chromosomes (Schwab *et al.*, 1985; Martinsson *et al.*, 1988). The selected non-adherent and adherent sublines were tested for amplification of *c-myc* and the linked *pvt-1* locus, located at least 72 kb distal to *c-myc* (Graham *et al.*, 1985; Banerjee *et al.*, 1985). The same filter was hybridized to a *c-myc*-specific probe and, after stripping, to a probe representative of the *pvt-1* region. Following further stripping, the filter was hybridized with an *int-1*-specific probe (*int-1* is located on chromosome 15 distal to *c-myc* and *pvt-1*; Nusse *et al.*, 1984; Adolph *et al.*, 1987, 1988). Representative Southern blots are shown in Figure 5a–c. Table IV summarizes the amplification data, calculated from densitometric measurements. Like *int-1*, the *mlvi-2* region, located proximally to *c-myc* (Adolph *et al.*, 1988), was not amplified in the SEWA sublines (data not shown).

TABLE I – POPULATION DOUBLING TIMES OF 3 SEWA SUBLINES IN DIFFERENT CONCENTRATIONS OF FCS

SEWA subline	FCS concentration		
	5%	1.2%	0.3%
SEWA-AS12-ADH	12 ¹	18	33.6
SEWA-AA7-NA	16.8	21.6	31.8
SEWA-AS12-NA	16.8	21.6	31.8

¹Population doubling time, in hours.

TABLE II – CYTOGENETIC ANALYSIS OF THE 3 SEWA SUBLINES¹

SEWA subline	AS12-ADH	AA7-NA	AS12-NA
Mean chromosome number	79.4	81.1	80.7
(range)	(50–164)	(48–over 400)	(49–over 400)
Rb(1;5) marker frequency	40	25	40
Rb(5;5) marker frequency	45	35	45
Metaphases with 2–15 DMs	18	12.5	12.5
Metaphases with more than 15 DMs	0	65	25
Polymicronucleation frequency	0.016	10.3	13.9
PCC frequency	2.5	17.5	12.5

¹All values except chromosome numbers designate percentages.

C-myc and *pvt-1* hybridize to the same amplified 725-kb fragment of SEWA-AS12-NA DNA

In order to determine whether the amplified *c-myc* and *pvt-1* sequences are organized into distinct amplification units (amplicons) or share common amplicons, SEWA-AS12-NA cells were embedded in agarose blocks and their DNA was subjected to long-range restriction enzyme mapping after digestion with SacII, using mouse *c-myc*- and *pvt-1*-specific probes. As shown in Figure 6, both probes hybridized to a 725-kb SacII fragment. In addition, the *c-myc*-specific probe also hybridized to 3 smaller amplified fragments not detected by the *pvt-1* probe. According to the nucleic acid sequence data available (Bernard *et al.*, 1983; Stanton *et al.*, 1983, 1984) there are 3 SacII sites within the mouse *c-myc* locus. Thus, one may expect 4 bands hybridizing to the mouse *c-myc* cDNA probe after SacII cleavage if the SacII sites are completely unmethylated (SacII is a methylation-sensitive enzyme). Under the running conditions applied, the smallest fragment (approximately 0.1 kb) will run out of the gel. The 1.5-kb fragment shown in Figure 6 corresponds to an internal *c-myc* fragment. The positions of 5' and 3' SacII sites flanking the *c-myc* gene are unknown at present; it may be assumed, however, that 2 of the 3 high-molecular-weight fragments (150, 450 and 725 kb) correspond to the fragments generated by enzymatic cleavage at the SacII sites inside and outside of *c-myc*. The presence of an extra band could be a consequence of partial methylation of a SacII site or nucleic acid sequence heterogeneity. These data do not prove or exclude the existence of amplicons in SEWA-AS12-NA cells that contain *c-myc* but not *pvt-1* sequences.

DISCUSSION

Conversion of solid sarcomas and carcinomas into ascites tumors is due to the selection of cell variants with an increased capacity to grow in dissociated free cell form compared to the original majority (Klein and Klein, 1951; Klein, 1953; Klein, 1955). In addition to non-adherence, the gradual selection of solid into ascites tumors also involves an adaptation to the virtually anaerobic and nutritionally limiting conditions of the peritoneal fluid (Warburg and Hiepler, 1952; Christensen and Liang, 1966). Once selected, the ability to grow in the ascitic fluid remains stable, even after continuous passage as a solid tumor (Klein, 1953; Klein, 1955). Although ascitic growth is accompanied by tumorous infiltration of peritoneal tissues, it is not dependent on it. An ascites-adapted cell population can multiply in the peritoneal fluid before any infiltration can be detected (Klein and Revesz, 1953). In addition, the acquisition of increased capacity to grow in the peritoneal fluid may be accompanied by an impaired ability to become implanted and grow subsequently in the solid tissues of the peritoneal cavity (Ringertz *et al.*, 1957).

FIGURE 3 – (a) G-banded metaphase spread of SEWA-AS12-NA chromosomes. A metacentric Rb(5;5) marker chromosome is indicated by an arrow. (b) G-banded metaphase plate of SEWA-AS12-ADH chromosomes. An arrow indicates a metacentric Rb(5;5) marker chromosome. (c) Double minutes and premature chromosome condensation (arrows) in a metaphase spread of SEWA-AS12-NA cells. (d) Polymicronucleation in a plate of SEWA-AS12-NA cells.

We found that only the non-adherent cells could grow in the ascites form. The adherent subline (SEWA-AS12-ADH) grew only in the solid form even after i.p. inoculation. These findings suggest that selection of the non-adherent cells from the originally ascites-adapted SEWA line led to isolation of the ascites-adapted variants, even after the tumor had been reconverted to the solid form and carried by s.c. transfer for 12 passages. It is also clear, however, that a plastic-adherent variant arose during this growth. The adherent variant could not yet

be detected in the ascites tumor prior to s.c. passage (SEWA-AA7).

The biological difference between the adherent and non-

TABLE III – GROWTH OF *IN VITRO*-ESTABLISHED SEWA SUBLINES IN SYNGENEIC A.SW MICE

Designation	Site of inoculation	Number of cells inoculated			Tumor form
		10^6	10^5	10^4	
SEWA-AS12-ADH	sc	8/9 ¹	6/8	0/5	Solid
		4/9	9/9	0/6	Solid
	ip	1/9	0/9	0/6	Ascites
		5/9	9/9	0/6	(Total)
SEWA-AS12-NA	sc	7/10	3/8	0/5	Solid
	ip	8/10	3/8	0/5	Ascites
SEWA-AA7-NA	sc	1/9	0/5	0/3	Solid
	ip	7/7	1/5	0/3	Ascites

¹The figures designate the number of animals with progressively growing tumors over the total number inoculated.

FIGURE 4 – Total number of tumor cells in the peritoneal fluid of A.SW mice after inoculation of 5×10^5 cells of the cultured SEWA sublines. Each point represents one mouse. The total number of cells in peritoneal washings was counted in a hemocytometer and the total number of tumor cells was calculated after differential counting of tumor cells in Giemsa-stained smears of peritoneal fluid.

FIGURE 5 – Amplification of *c-myc* and *pvt-1* sequences in SEWA sublines. Ten micrograms of EcoRI-digested DNA from SEWA-AS12-ADH, SEWA-AA7-NA, SEWA-AS12-NA and A.SW spleen were electrophoresed in 0.8% agarose, transferred to a Hybond N membrane and hybridized with dCTP³²-labelled probes specific for mouse *c-myc* (a), *pvt-1* (b) and *int-1* (c) sequences.

adherent lines was paralleled by a major difference in *c-myc/pvt-1* amplification and also in cytogenetic parameters, like percentage of metaphases with more than 15 DMs, polychromatinization frequency and PCC frequency. These differences could be causal or coincidental. There is no evidence concerning the relationship between the DMs and *c-myc/pvt-1* sequences. SEWA-AS12-NA has less than the highest propor-

TABLE IV – AMPLIFICATION OF *c-myc* AND *pvt-1* IN SEWA SUBLINES COMPARED TO A.SW SPLEEN CELLS

Designation	Amplification of		
	<i>c-myc</i>	<i>pvt-1</i>	<i>int-1</i>
SEWA-AS12-ADH	8×	5×	1×
SEWA-AA7-NA	22×	25×	1×
SEWA-AS12-NA	63×	77×	1×
A.SW Spleen	1×	1×	1×

The degree of amplification of *c-myc* and *pvt-1* sequences was calculated on the basis of densitometric measurements using an LKB ULTROSAN XL Laser Densitometer.

FIGURE 6 – Pulsed field gel analysis of SacII-digested SEWA-AS12-NA DNA. The blot was hybridized with *c-myc* cDNA (a) and *pvt-1* (b) probes.

tion of cells containing double minutes (Table II) but it has the highest level of *c-myc/pvt-1* amplification (Table IV). This suggests that the double minutes may not contain *c-myc/pvt-1* sequences or that only some of them contain such sequences. It is possible that the micronuclei which are generated frequently in the non-adherent sublines fuse to the cells. The resulting premature chromosome condensation may favor the generation of DMs, as suggested by Sen (1989).

Amplification of *c-myc* or other members of the *myc* family showed a certain relationship to malignant behavior in other systems. The overwhelming majority of oncogene amplifications so far found in human solid tumors affect the *myc* family

(Alitalo and Schwab, 1986). Such amplifications appear to be related to more malignant phenotypes within certain tumor systems. Thus, more invasive and more metastatic small-cell lung carcinoma (SCLC) variants contained amplified *c*, *N* or *L-myc*, compared to their less invasive and metastatic counterparts (Little *et al.*, 1983; Nau *et al.*, 1985; Wong *et al.*, 1986). In neuroblastoma, amplification of *N-myc* was only found in stage-III and -IV tumors and was negatively correlated with patient survival (Brodeur *et al.*, 1984). The rare and highly malignant form of human plasma-cell tumor, PC leukemia, contained amplified *c-myc* genes, in contrast to solid plasmacytomas (Sumegi *et al.*, 1985). One may speculate that the amplification of *c-myc* in the SEWA ascites and non-adherent variants may have contributed to the ability of the cells to survive and grow in the freely dissociated form. The lack of contact communication and cross-feeding and/or the nutritional shortages that characterize ascitic fluid may impose serious restrictions on free cells, compared to their counterparts that live in a coherent community. Only cells with a high intrinsic ability for autonomous growth may proliferate in the face of these stringencies. Amplification of the proliferation-related *myc* gene may provide such an advantage, either by increasing the amount of *myc* protein which has been shown to stimulate replication in some systems (Classon *et al.*, 1987), or by titrating out negative regulatory factors that may counteract *myc* expression. As another alternative, a relationship may exist between *myc* amplification and changes at the cell surface, exemplified by the decreased expression of MHC class-I antigens in cells with amplified *N-myc* genes (Bernards *et al.*, 1986) or with overexpression of the *c-myc* gene (Versteeg *et al.*, 1989).

The possible significance of *pvt-1* amplification may be considered in geometric and in functional terms. *Pvt-1* is at least 72 kb distal to *c-myc* on chromosome 15 (region D2-D3) (Cory *et al.*, 1985; Adolph *et al.*, 1987, 1988). The *pvt-1* locus is identical to the proviral integration sites *Mis-1* and *Mlvi-1* (Vileneuve *et al.*, 1986; Koehne *et al.*, 1989) and one of the Moloney leukemia virus integration sites was estimated to be at least 300 kb distal to *c-myc* (Koehne *et al.*, 1989). We have

found that the amplified *c-myc* and *pvt-1* sequences are located on the same 725-kb *SacII* fragment of SEWA-AS12-NA DNA, suggesting that they are on the same amplification unit (amplicon). One of the 3 high-molecular-weight fragments (150, 450 and 725 kb) hybridizing to the *c-myc* cDNA probe cannot be explained on the basis of the sequence of the mouse *c-myc* gene. This extra band may be a consequence of partial methylation of a *SacII* site (*SacII* is a methylation-sensitive enzyme) or nucleic acid sequence heterogeneity; in addition, one cannot exclude the possibility of the existence of a smaller-sized amplicon containing *c-myc* sequences only.

Using a human PVT probe, Shtivelman and Bishop (1989) also detected co-amplification of *pvt* or part of *pvt* with *myc* in human colon carcinoma cells and in 2 SEWA sublines containing either double-minute chromosomes or C-bandless chromosomes. The co-amplification of *pvt-1* and *c-myc* may be coincidental to the close linkage of the 2 loci or it could have a functional significance. The role of the *pvt-1* locus is not known, but its possible importance has been suggested by 2 consistent findings. In the mouse plasmacytoma-associated variant (6;15) translocations, the constant region of the kappa gene is juxtaposed to *pvt-1*, rather than to *c-myc* itself (Cory *et al.*, 1985). In murine T-cell leukemias, the frequent retroviral insertion in the neighborhood of *c-myc* alternates with similarly clustered insertions near *pvt-1* (Graham *et al.*, 1985). It is conceivable that the co-amplification of *c-myc* and *pvt-1* has a similarly synergistic or alternating functional significance as in translocation and retroviral insertion.

ACKNOWLEDGEMENTS

This investigation was supported by a USPHS grant from the National Cancer Institute, by The Cancer Research Institute, by the Concern Foundation and by a grant from the Gustav V Jubileum Fund. J. Minarovits is on leave from the Microbiological Research Group of the National Institute of Hygiene, Budapest, Hungary, as a recipient of a fellowship from the Swedish Cancer Society.

REFERENCES

- ADOLPH, S., BARTRAM, C.R. and HAMEISTER, H., Mapping of the oncogenes *myc*, *sis*, and *int-1* to the distal part of mouse chromosome 15. *Cytogenet. Cell Genet.*, **44**, 65-68 (1987).
- ADOLPH, S., STRAUSS, P.G. and HAMEISTER, H., Localization of proviral integration sites (*Mlvi-1*, *Mlvi-2*, and *Pvt-1*) and the alpha-globin pseudogene, *Hba-3ps*, on murine chromosome 15. *Cytogenet. Cell Genet.*, **47**, 189-191 (1988).
- ALITALO, K. and SCHWAB, M., Oncogene amplification in tumor cells. *Advanc. Cancer Res.*, **47**, 235-281 (1986).
- BANERJEE, M., WIENER, F., SPIRA, J., BABONITS, M., NILSSON, M.-G., SUMEGI, J. and KLEIN, G., Mapping of the *c-myc* *pvt-1* and immunoglobulin kappa genes in relation to the mouse plasmacytoma-associated variant (6;15) translocation breakpoint. *EMBO J.*, **4**, 3183-3188 (1985).
- BERNARD, O., CORY, S., GERONDAKIS, S., WEBB, E. and ADAMS, J.M., Sequence of the murine and human cellular *myc* oncogenes and two modes of *myc* transcription resulting from chromosome translocation in B lymphoid tumors. *EMBO J.*, **2**, 2375-2383 (1983).
- BERNARDS, R., DESSAIN, S.K. and WEINBERG, R., *N-myc* amplification causes down-modulation of MHC class I antigen expression in neuroblastoma. *Cell*, **47**, 667-674 (1986).
- BOLDOG, F., ERLANDSSON, R., KLEIN, G. and SUMEGI, J., Long range restriction enzyme maps of *DNF15S2*, *D3S2* and *c-raf 1* loci on the short arm of human chromosome 3. *Cancer Genet. Cytogenet.*, **42**, 295-306 (1989).
- BRODEUR, G.M., SEEGER, R.C., SCHWAB, M., VARMUS, H.E. and BISHOP, J.M., Amplification of *N-myc* in untreated human neuroblastomas correlates with advanced disease stage. *Science*, **224**, 1121-1124 (1984).
- CHRISTENSEN, H.N. and LIANG, M., Transport of diamino acids into the Ehrlich cell. *J. biol. Chem.*, **241**, 5542-5551 (1966).
- CLASSON, M., HENRIKSSON, M., SUMEGI, J., KLEIN, G. and HAMMAR-
- SKJÖLD, M.-L., Elevated *c-myc* expression facilitates the replication of SV40 DNA in human lymphoma cells. *Nature (Lond.)*, **330**, 272-274 (1987).
- CORY, S., GRAHAM, M., WEBB, E., CORCORAN, L. and ADAMS, J.M., Variant (6;15) translocations in murine plasmacytomas involve a chromosome 15 locus at least 72 kb from the *c-myc* oncogene. *EMBO J.*, **4**, 675-681 (1985).
- FEINBERG, A.P. and VOGELSTEIN, B., A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **137**, 266-267 (1984).
- GRAHAM, M., ADAMS, J.M. and CORY, S., Murine T lymphomas with retroviral inserts in the chromosomal 15 locus for plasmacytoma variant translocations. *Nature (Lond.)*, **314**, 740-743 (1985).
- GUZELLA, J.F., KEYS, C., VARSANYI, B.A., KAO, F.T., JONES, C., PUCK, T.T. and HOUSMAN, D., Precise localization of human B-globulin gene complex on chromosome 11. *Proc. nat. Acad. Sci. (Wash.)*, **76**, 5239-5243 (1979).
- KLEIN, E., Gradual transformation of solid into ascites tumors. Evidence favoring the mutation-selection theory. *Exp. Cell Res.*, **8**, 188-212 (1955).
- KLEIN, G., Conversion of solid into ascites tumors. *Nature (Lond.)*, **171**, 398-401 (1953).
- KLEIN, G. and KLEIN E., The transformation of a solid transplantable mouse carcinoma into an "ascites tumor". *Cancer Res.*, **11**, 466-469 (1951).
- KLEIN, G. and REVESZ, L., Quantitative studies on the multiplication of neoplastic cells *in vivo*. I. Growth curves of the Ehrlich and MC1M ascites tumors. *J. nat. Cancer Inst.*, **14**, 229-277 (1953).
- KOEHN, C.F., LAZO, P.A., ALVES, K., LEE, J.S., TSICHLIS, P. and O'DONNELL, P.V., The *Mlvi-1* locus involved in the induction of rat T-cell

- lymphomas and the *pvt-1/Mis-1* locus are identical. *J. Virol.*, **63**, 2366–2369 (1989).
- LAW, L.W., Differences between cancers in terms of evolution of drug resistance. *Cancer Res.*, **16**, 698–716 (1956).
- LEVAN, G., MANDAH, N., BREGULA, U., KLEIN, G. and LEVAN, A., Double minute chromosomes are not centromeric regions of the host chromosomes. *Hereditas (Lond.)*, **83**, 83–90 (1976).
- LITTLE, C.D., NAU, M.M., CARNEY, D.N., GAZDAR, A.F. and MINNA, J.D., Amplification and expression of the *c-myc* oncogene in human lung cancer cell lines. *Nature (Lond.)*, **306**, 194–196 (1983).
- MARTINSSON, T., STÄHL, F., POLLWEIN, P., WENZEL, A., LEVAN, A., SCHWAB, M. and LEVAN, G., Tumorigenicity of SEWA murine cells correlates with degree of *c-myc* amplification. *Oncogene*, **3**, 437–441 (1988).
- NAU, M.M., BROOKS, B.J., BATTEY, J., SAUSVILLE, E., GAZDAR, A.F., KIRSCH, I.R., MCBRIDE, O.W., BERTNESS, V., HOLLIS, G.F. and MINNA, J.D., *L-myc*, a new *myc*-related gene amplified and expressed in human small cell lung cancer. *Nature (Lond.)*, **318**, 69–73 (1985).
- NOWELL, P.C., Mechanisms of tumor progression. *Cancer Res.*, **46**, 2203–2207 (1986).
- NUSSE, R., VAN OUYEN, A., COX, D., FUNG, Y.K. and VARMUS, H., Mode of proviral activation of a putative mammary oncogene (*int-1*) on mouse chromosome 15. *Nature (Lond.)*, **307**, 131–136 (1984).
- PRICE, J.E., AUKERMAN, S.L. and FIDLER, I.J., Evidence that the process of murine melanoma metastasis is sequential and selective and contains stochastic elements. *Cancer Res.*, **46**, 5172–5178 (1986).
- PURDOM, L., AMBROSE, E.J. and KLEIN, G., A correlation between electrical surface charge and some biological characteristics during the stepwise progression of a mouse sarcoma. *Nature (Lond.)*, **181**, 1586–1587 (1958).
- RIJSEWIJK, F.A.M., LOHUIZEN, M., VAN OUYEN, A. and NUSSE, R., Construction of a retroviral cDNA version of the *int-1* mammary oncogene and its expression *in vitro*. *Nucl. Acids Res.*, **14**, 693–702 (1986).
- RINGERTZ, N., KLEIN, E. and KLEIN, G., Histopathologic studies of peritoneal implantation and lung metastasis at different stages of the gradual transformation of the MC1M mouse sarcoma into ascites form. *J. nat. Cancer Inst.*, **18**, 173–199 (1957).
- ROTHFELS, K.H. and SIMINOVICH, L., An air drying technique for flattening chromosomes in mammalian cells grown *in vitro*. *Strain Technol.*, **33**, 73–74 (1958).
- SCHWAB, M., RAMSAY, G., ALITALO, K., VARMUS, H.E., BISHOP, J.M., MARTINSSON, T., LEVAN, G. and LEVAN, A., Amplification and enhanced expression of the *c-myc* oncogene in mouse SEWA tumor cells. *Nature (Lond.)*, **315**, 345–347 (1985).
- SEN, S., Personal communication, quoted by Wahl, G.M., in: The importance of circular DNA in mammalian gene amplification. *Cancer Res.*, **49**, 1333–1340 (1989).
- SHTIVELMAN, E. and BISHOP, J.M., The PVT gene frequently amplifies with *myc* in tumor cells. *Mol. cell. Biol.*, **9**, 1148–1154 (1989).
- SJÖGREN, H.O., HELLSTRÖM, I. and KLEIN, G., Transplantation of polyoma virus induced tumors in mice. *Cancer Res.*, **21**, 329–337 (1961).
- SOUTHERN, E., Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. mol. Biol.*, **98**, 503–517 (1975).
- STANTON, L.W., FAHRLANDER, P.D., TESSER, P.M. and MARCU, K.B., Nucleotide sequence comparison of normal and translocated murine *c-myc* genes. *Nature (Lond.)*, **310**, 423–425 (1984).
- STANTON, L.W., WATT, R. and MARCU, K.B., Translocation, breakage and truncated transcripts of *c-myc* oncogene in murine plasmacytomas. *Nature (Lond.)*, **303**, 401–406 (1983).
- SUMEGI, J., HEDBERG, T., BJORKHOLM, M., GODAL, T., MELLSTEDT, H., NILSSON, M.G., PERLMAN, C. and KLEIN, G., Amplification of the *c-myc* oncogene in human plasma-cell leukemia. *Int. J. Cancer*, **36**, 367–371 (1985).
- VERSTEEG, R., KRUSE-WOLTERS, K.M., PLOMP, A.C., VAN LEEUWEN, A., STAM, N.J., PLOEGH, H.L., RUITER, D.J. and SCHRIER, P.I., Suppression of class I human histocompatibility leukocyte antigen by *c-myc* is locus-specific. *J. exp. Med.*, **170**, 621–635 (1989).
- VILLENEUVE, L., RASSART, E., JOLICOEUR, P., GRAHAM, M. and ADAMS, J., Proviral integration site *Mis-1* in rat thymomas corresponds to the *pvt-1* translocation breakpoint in murine plasmacytomas. *Mol. cell. Biol.*, **6**, 1834–1837 (1986).
- WANG, H.C. and FEDOROFF, S., Banding in human chromosomes treated with trypsin. *Nature (Lond.)*, **235**, 52–53 (1972).
- WARBURG, O. and HIEPLER, E., Versuche mit Ascites-Tumorzellen. *Z. Naturforsch.*, **7b**, 193–194 (1952).
- WONG, A.J., RUPPERT, J.M., EGGLESTON, J., HAMILTON, S., BAYLIN, S.B. and VOGELSTEIN, B., Gene amplification of *c-myc* and *N-myc* in small cell carcinoma of the lung. *Science*, **233**, 461–464 (1986).