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ARTICLES

Homologous Recombination in Variants of the B16 Murine Melanoma With Reference to Their Metastatic Potential

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Abstract Genomic instability has been accepted as providing a phenotypic variety of malignant cells within a developing tumour. Defects in genetic recombination can often lead to phenotypic differences; therefore, it is possible that metastatic variant cell lines exhibit their particular phenotype as a result of an altered ability to catalyse homologous recombination. We have investigated recombination efficiency in B16 melanoma metastatic variants, using a plasmid, pDR, as a recombination substrate. The plasmid contains two truncated, nontandem but overlapping segments of the neomycin resistance gene (*neo 1* and *neo 2*), separated by the functional *gpt* gene unit. Only a successful recombination of the two *neo* segments will generate a functionally intact neomycin gene. Extrachromosomal recombination here was a transient measure of the cells to recombine the *neo* fragments in an intra- or intermolecular manner. Extrachromosomal recombination frequencies were higher in the high metastasis variants (BL6, ML8) compared with the low metastatic F1 cells. On the other hand, the frequency of chromosomal recombination (after plasmid integration) was higher for the low metastasis (F1) cell line compared with the highly metastatic variants, BL6 and ML8. Since the recombination assay measures only successful recombination events, we have interpreted the observed higher incidence of chromosomal recombination in the low metastatic variant line as indicative of a more stable genome. Similarly, a higher inherent instability in the genome of the high metastasis variants would render these less efficient at producing and maintaining successful recombination events, and this was found to be true by Southern analysis. The results presented show that frequency of recombination may be adduced as evidence for implicating genomic instability in the generation of variant cell populations during metastatic spread. Such an interpretation is also compatible with the Nowell hypothesis for tumour progression.

Key words: DNA recombination, genomic instability, plasmid integration, metastasis, B16 melanoma

The metastatic spread of cancer is recognised as being a multistep process beginning with invasion of tumour cells from the primary tumour site via the blood or lymphatic system and ending in a progressive secondary growth at distant or metastatic sites. Tumours are heterogeneous in nature, and many of the malignant cells which are shed from the primary neoplasm do not give rise to metastatic lesions but either die, differentiate, or lie dormant [Alexander, 1984]. Therefore, the affiliation of tumour heterogeneity to tumour progression has been widely accepted as providing a phenotypic variety of malignant cells within the tumours, which are not all alike (even if the tumour is of monoclonal origin [Welch, 1989]), and from these, variant cell subpopulations are responsible for a progression in the malignant behaviour of the tumour. In this respect, Nowell's [1976] hypothesis for tumour progression postulated that the generation of variants from the primary tumour cell population with increased invasive and metastatic abilities was due to a genetic variability within the developing neoplasm. This genetic instability could arise by several mechanisms: for example, (1) inherent instability of DNA replication, manifested by sister chromatid exchange, gene amplifications, (2) host influences (i.e., selective pressure, nutritional requirements, therapy-induced mutation, etc.), and (3) an increased susceptibility to mutagenesis resulting from an inability to repair DNA damage. In fact, within variant cell populations of a B16

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murine melanoma, some of these indices do indeed correlate with the degree of metastatic ability [Usmani, 1993]. It has been shown that defects in genes involved in genetic recombination can often lead to phenotypic differences, one of which is a reduced ability to repair DNA damage [Ho and Mortimer, 1975]. Therefore, cell lines which are either UV or ionising radiation-sensitive could conceivably have been the result of a defect in recombination. Also, conversely, cell lines that have errors in aspects of DNA metabolism (e.g., XP cells [Lehmann and Norris, 1989], Fanconi's anaemia cells [Fujiwara et al., 1977], AT cells [Cox et al., 1986], and Bloom's syndrome cells) might be expected to exhibit altered recombination frequencies. Compatible with this view is the demonstration by Wahls and Moore [1990] of decreased extrachromosomal recombination efficiencies in mammalian DNA repair-deficient somatic cell lines. Furthermore, it would also seem likely that metastatic cell lines which show an altered ability to repair DNA damage may exhibit their particular phenotype as a result of an altered ability to catalyse homologous recombination. Recently, we have demonstrated such a difference in repair fidelity between metastatic variants of a B16 murine melanoma which correlates with their metastatic potential [Usmani et al., 1993]. Subramani and Rubnitz [1985], using a plasmid construct containing two segments of a neomycin gene, measured the frequencies of recombination events in cultured mouse cells and found that extrachromosomal recombination occurs at a frequency that is substantially higher than the frequency of chromosomal recombination. Chromosomal recombination, however, is measured after transfection and stable integration of vector DNA into the host genome and is a reflection upon the genetic stability of the mammalian genome itself.

In the present work we have used the extrachromosomal and chromosomal recombination assays to determine whether any intrinsic differences might exist in recombination efficiency between metastatic variants of the B16 murine melanoma, with a view to evaluating a possible correlation between differences in recombination frequency and the order of metastatic ability, in order to test whether genetic instability might provide a basic mechanism for the rapid generation of variants in highly metastatic tumour lines.

MATERIALS AND METHODS

Cells

A low metastasis variant, F1, and high metastasis variants, BL6 and ML8, of a mouse B16 murine melanoma were used. The ability of the variant cell lines to metastasize to the lungs has been measured at regular intervals [Lakshmi et al., 1988].

The F1 and BL6 cell lines were obtained from E.G. & G. Mason Research Institute (Worcester, MA). The variant ML8 line was isolated in our laboratory from metastatic lung deposits arising from a BL6 primary tumour grown subcutaneously in C57BL6/J mice.

Culture Conditions

Cells were grown in Eagles MEM (Gibco, Paisley, Scotland) containing 10% foetal calf serum (FCS) (NBL) glutamine (2 mM) (Gibco) and 1% nonessential amino acids (Gibco) at 37°C in a 5% CO_2 atmosphere. The cells were grown as monolayers, and cells from exponentially growing cultures were used throughout these experiments.

G418 medium contained Geneticin (Gibco) at a final concentration of 1 mg/ml. XHATM medium contained xanthine (Sigma, Poole, Dorset, UK) 250 μg/ml, hypoxanthine (Sigma) 15 μg/ml, adenine (Sigma) 25 μg/ml, thymidine (Sigma) 10 μg/ml, and mycophenolic acid (Sigma) 10 μg/ml.

Chromosomal Recombination Assay

To investigate chromosomal recombination in B16 cell variants, a plasmid, pDR, was used as a recombination substrate (see Fig. 1). The plasmid contains two truncated, nontandem but overlapping segments of the neomycin resistance gene (*neo*1 and *neo*2), separated by the *gpt* functional gene unit. Only a successful recombination of the two nonfunctional gene segments will generate an intact functional neomycin resistance gene. The plasmid was transfected into the B16 cells using the calcium phosphate/DNA coprecipitation method [Graham and Van der Eb, 1973]. Cells (5×10^5) were exposed to 10 μg DNA precipitate per 10 cm petri dish, and the precipitate was left overnight at 36°C and 3% CO_2 [Chen and Okayama, 1987]. The cells were then treated for 2 min with 27.5% DMSO (Dimethyl sulphoxide) (Sigma) in Dulbecco A and washed three times with Dulbecco A. The transfected cells were incubated for 24 h in the absence of selection to allow for recovery from the

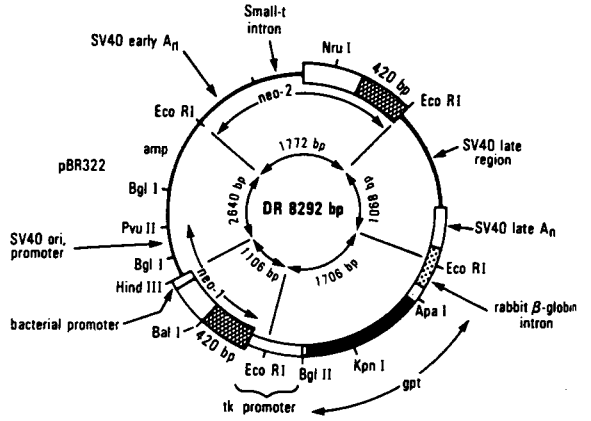

Fig. 1. Plasmid map of the DR construct [taken from Subramani and Rubnitz, 1985].

DMSO shock. Parallel control plates were seeded with cells minus the pDR plasmid. Initially, cells were screened for uptake and integration of plasmid DNA by selecting for the functional transcriptional product of the intact *gpt* gene in XHATM medium (2–3 weeks). Clones which had successfully incorporated the pDR plasmid into their chromosome were picked, using sterile toothpicks, and grown in 25 cm^2 tissue culture flasks (Falcon, Glasgow, Scotland). These cells were subsequently expanded into bulk cultures in 150 cm^2 flasks, and the whole of this process took approximately 2 weeks, under continuous XHATM exposure. Several of these clones from the first stage selection were tested for subsequent recombination events by plating 5×10^5 cells/plate in 100 × 10 cm petri dishes containing G418 medium. Colonies resistant to G418 will have undergone chromosomal recombination of the two nonfunctional *neo* segments into a functional *neo* gene. Recombinant cells were scored after 10 days, and the recombination frequency was expressed as a fraction of the total number of cells tested (Table II).

Extrachromosomal Recombination Assay

As above, the pDR plasmid was introduced into the B16 melanoma cells by $CaPO_4$/DNA coprecipitation. Following transfection, half of the cell population was seeded out into XHATM medium. This medium selects for cells which have taken up the pDR plasmid and stably integrated it into their genome to acquire a *gpt*-positive phenotype. The other half of the population was seeded into medium containing G418 (Geneticin). This selects for cells which have taken up the pDR plasmid and recombined the two truncated *neo* segments by homologous recombination (in an intra- or intermolecular manner) soon after transfection to generate an intact and functional *neo* gene. Parallel control plates were seeded without plasmid DNA to check the efficiency of the selection media. Extrachromosomal recombination frequency was expressed as a fraction of the total number of *gpt*-positive transfected clones, (Table I).

Southern Blot Hybridisation

Genomic DNAs from the metastatic variant cell lines were isolated using a model 340A nucleic acid extractor (Applied Biosystems, Warrington, UK). Cell DNA (20 μg) from each cell line was double digested with the HindIII and EcoRI restriction enzymes. DNA samples were electrophoresed through a 0.8% agarose gel at 80 V for 3 h. A 1 kb DNA ladder was included in the gel as a marker. The DNA was transferred onto Hybond-N nylon membrane filters (Amersham, Slough, Bucks, UK), hybridised to specific probe sequences in a Hybaid hybridisation oven at 65°C, and then washed repeatedly with 2× SSC (0.3 M NaCl, 30 mM Na Citrate) until the background radioactivity was appreciably low. The washed filters were exposed to Fuji RX-100 X-ray–sensitive film for 24 h at −70°C.

Neo specific probes were made by nick translation of the 1,339 bp HindIII-NruI DNA fragment from pSV2*neo*. DNA probes specific for the *gpt* gene were made by using the 930 bp BglII-ApaI segment from pSV2*gpt*.

RESULTS

Homologous Recombination

Extrachromosomal recombination frequencies were higher in the high metastasis variants (BL6, ML8) compared with the low metastatic F1 line (Table I).

The frequency with which *neo*-positive clones arose in the expanded population was taken as a measure of the chromosomal recombination frequency. Table II shows a higher frequency of successful recombination for the low metastasis (F1) cell line compared with the high metastasis variants, BL6 and ML8. The recombination frequency between the F1 clones varies over three orders of magnitude. The BL6 clones vary over one order of magnitude, and the ML8 clones hardly show any detectable recombination frequency.

In order to find out whether the XHATM-resistant clones selected for uptake and integra-

TABLE 1. Extrachromosomal Recombination Frequency Following Calcium Phosphate Transfection With the pDR Plasmid

Cell line	Frequency of XHATM-resistant colonies	Frequency of G418-resistant colonies	ERF[a]
F1	8.5×10^{-5}	2.5×10^{-6}	0.029[b]
	3.6×10^{-5}	1.3×10^{-6}	0.036[c]
	5.0×10^{-5}	1.25×10^{-6}	0.025[d]
BL6	2.75×10^{-5}	1.8×10^{-5}	0.654[b]
	2.4×10^{-5}	8.7×10^{-6}	0.362[c]
	2.5×10^{-5}	1.2×10^{-5}	0.48[d]
ML8	3.25×10^{-5}	4.0×10^{-6}	0.123[b]
	3.0×10^{-5}	2.1×10^{-6}	0.07[c]
	1.6×10^{-5}	9.8×10^{-6}	0.612[d]

[a]Extrachromosomal recombination frequency (ERF) = frequency of G418-resistant colonies/frequency of XHATM-resistant colonies.
[b]F1, BL6, and ML8 data from one experiment.
[c]F1, BL6, and ML8 data from a second experiment.
[d]F1, BL6, and ML8 data from a third experiment.

tion of the plasmid did in fact still have two separate *neo* fragments, Southern hybridisation analysis of *gpt*-positive clones with a full-length *neo* probe was carried out (Fig. 2). Two F1 clones (pDR1 and pDR2) show both *neo*1 and *neo*2 fragments; one F1 clone (pDR12) has only the smaller *neo* fragment (*neo*2), and one BL6 clone (pDR9) has retained the larger fragment (*neo*1). The remaining clones appear to have lost both fragments. None of the ML8 clones have either of the fragments *neo1* or *neo2*.

The *neo* probe was melted from the blots in Figure 2, and subsequent reprobing of the membrane with a *gpt* sequence again showed similar results (Fig. 3): three F1 clones (pDR1, pDR2, and pDR12) have bands corresponding to the *gpt* fragment; one BL6 clone has a *gpt* band (pDR9). All the ML8 clones appear to have retained a *gpt*-positive function.

Six recombinant G418 clones isolated from an F1pDR7 clone were scaled up and used for a further study to find whether they did indeed have a 2338 bp fragment coding for a complete *neo* gene. These clones are called R1, R2, etc., because they were picked in this order after switching the cells to G418 medium which would only allow cells with a functionally recombined *neo* gene to grow. Figure 4 shows that all the clones have generated a functional *neo* sequence. Rehybridisation to a *gpt* probe revealed that clones R2, R3, R4, and R5 have still retained *gpt* sequences, whilst clones R1 and R6 appear to have lost all *gpt*-related sequences (Fig. 5).

TABLE II. Observed Homologous Chromosomal Recombination Frequency in pDR Transformed Clones Derived From High (BL6, ML8) and Low (F1) Metastatic Cell Lines

Clone	Number of colonies	Recombination frequency[a]
F1 pDR1	557	1.1×10^{-5}
F1 pDR2	717	1.43×10^{-5}
F1 pDR3	33	6.6×10^{-7}
F1 pDR4	0	ND
F1 pDR5	0	ND
F1 pDR6	6	1.2×10^{-7}
F1 pDR7	270	5.4×10^{-6}
F1 pDR9	450	9.0×10^{-6}
F1 pDR12	1,386	2.77×10^{-5}
BL6 pDR1	41	8.2×10^{-7}
BL6 pDR2	2	4.0×10^{-8}
BL6 pDR3	0	ND
BL6 pDR4	0	ND
BL6 pDR5	0	ND
BL6 pDR6	0	ND
BL6 pDR7	0	ND
BL6 pDR8	1	2.0×10^{-8}
BL6 pDR9	30	6.0×10^{-7}
BL6 pDR10	0	ND
ML8 pDR1	0	ND
ML8 pDR2	0	ND
ML8 pDR3	1	2.0×10^{-8}
ML8 pDR4	0	ND
ML8 pDR5	2	4.0×10^{-8}

[a]Recombination frequency = number of G418-resistant colonies/total number of cells seeded.
ND, not detectable.

DISCUSSION

The process of metastasis has been considered to be due to an accumulation of somatic mutations and defective DNA repair and replication which, combined with the host's selective pressure, finally lead to the emergence of more aggressive clonal subpopulations within the neoplasm, with increased invasive and metastatic properties [Nowell, 1976]. In addition, gene duplication and rearrangement are thought to be of importance in genomic evolution [Maeda and Smithies, 1986] as well as in cancer development [Rodman et al., 1982]. In the past, attempts to associate changes in the genetic stability of the cell and its metastatic potential have revealed that highly metastatic cells have a

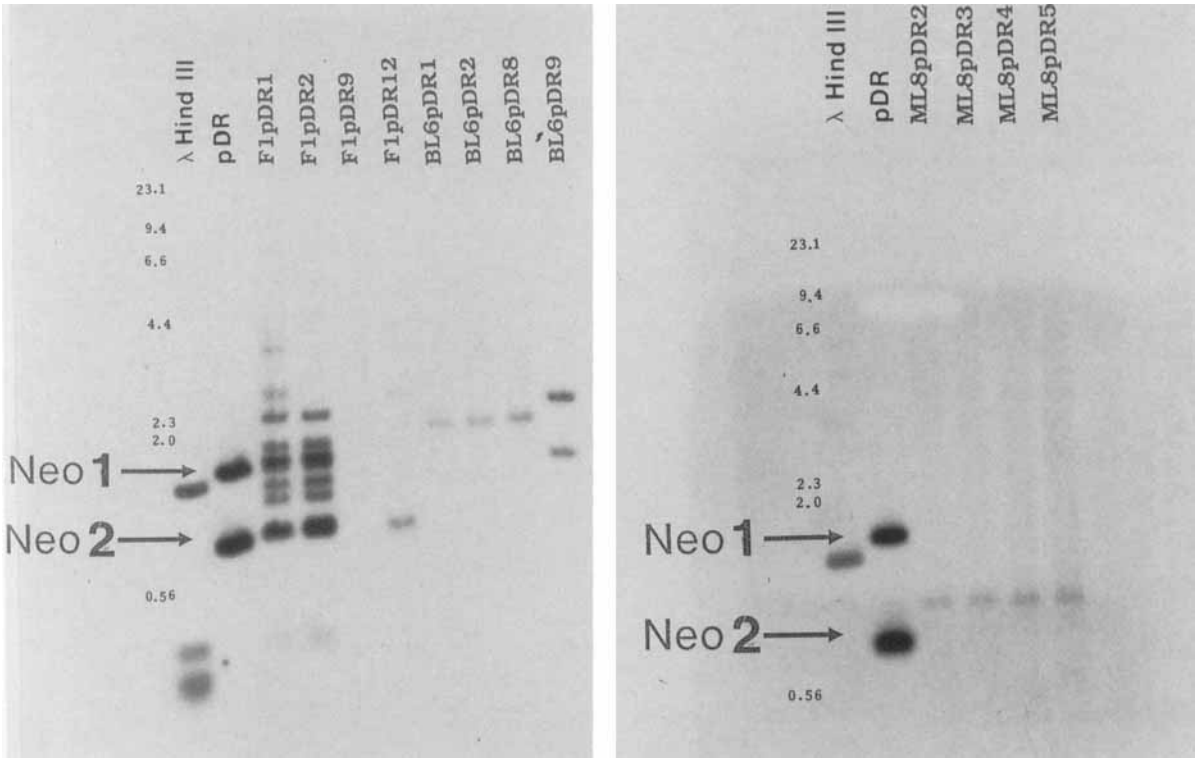

Fig. 2. Southern blot analysis of integrated pDR plasmid. Genomic DNAs from the above clones were digested with *HindIII* and *EcoRI* and then hybridised in the presence of a ^{32}P-labelled *neo* fragment obtained from a *HindIII/NruI* digest of the pSV2*neo* plasmid. *Lane 1:* λ markers. However, the bands which are highlighted in lane 1 are not marker bands but possibly contamination from the communal eppendorph containing the λHindIII marker, which have hybridised to the probe sequence. One microgram of *HindIII/EcoR1*-digested pDR plasmid DNA was also included to identify the two segments of the unrecombined *neo* gene *(lane 2).* The clones are numbered in the order in which they were picked (i.e., 1–12) and have the extension pDR to confirm that they were originally isolated under XHATM selection. In general, integration of the plasmid has resulted in the appearance or loss, or both, of other DNA fragments homologous to the *neo* probe.

higher spontaneous mutation rate than cells with a low metastatic potential [Cifone and Fidler, 1981]. We have further shown that highly metastatic variants of a B16 murine melanoma have a higher frequency of induced mutations than their low metastasis counterpart [Usmani et al., 1993]. Gene amplification is also an indicator of genomic instability and has been found to correspond to metastatic ability within tumour lines [Hill et al., 1984; Sager et al., 1985; Cillo et al., 1987]. Altered DNA repair properties have been associated with genomic instability in mammalian cell lines [Kohn, 1983; Kihlman and Andersson, 1985; Sherbet et al., 1986; Usmani et al., 1993], and phenotypic differences, such as the ability to repair DNA damage, can be the result of defects in genes involved in recombination [Ho and Mortimer, 1975]. It is possible, then, that variant cell lines which exhibit different metastatic phenotypes could have arisen as a result of defective recombination. Recently, Groden et al. [1990], whilst comparing Bloom's syndrome cells and normal cells, have shown a correlation between the degree of genetic instability and increased recombination frequency, and both are the result of altered cell proliferative control. The present studies were designed to determine whether the Nowell hypothesis for tumour progression [1976] would be compatible with any perceived differences in homologous recombination within the metastatic variants of the mouse B16 melanoma.

The plasmid based substrate, pDR [Subramani and Rubnitz, 1985], was utilised to screen the F1, BL6, and ML8 variant cell lines for potential differences in extrachromosomal and chromosomal recombination. Intra- or intermolecular reciprocal recombination between the *neo1* and *neo2* segments of plasmid(s) occurs extrachromosomally, prior to integration. Be-

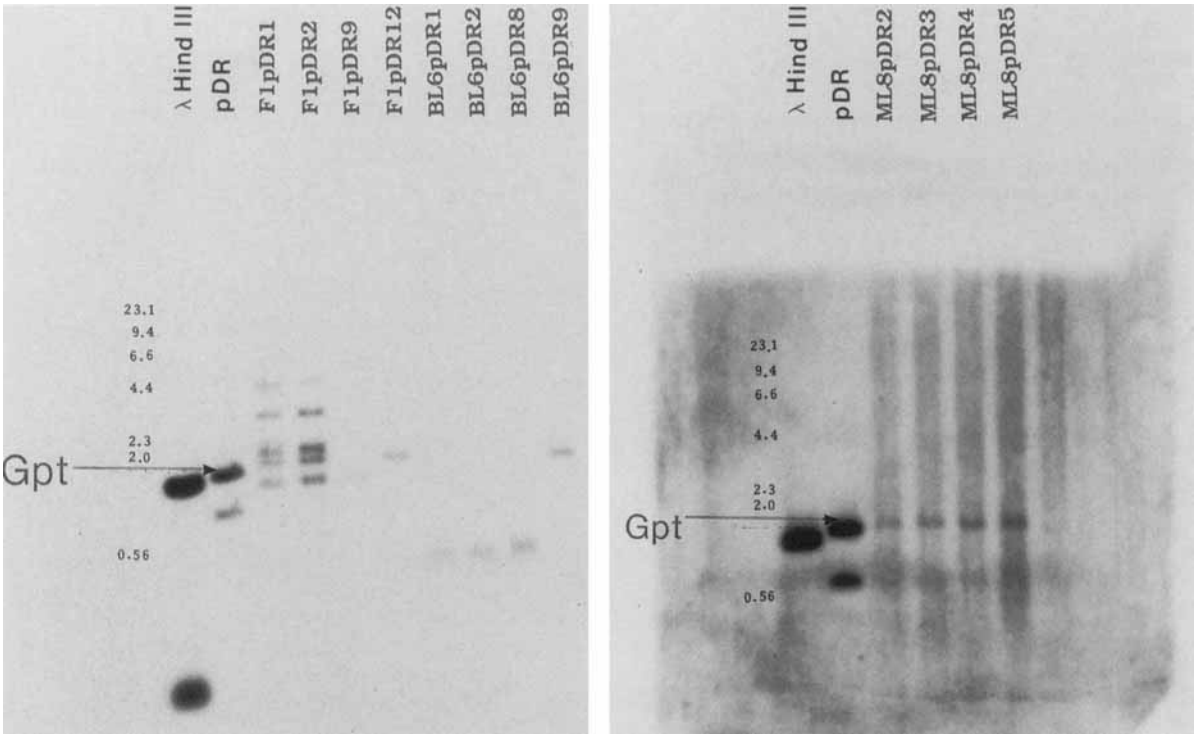

Fig. 3. The *neo* probe was melted from the blots in Fig. 2 and subsequently reprobed with *gpt* sequences (*BglII/ApaI* fragment of pSV2*gpt*). Again, in *lane 1,* visible bands are not marker bands but contaminating sequences which have hybridised to the probe. The position of the *gpt* coding fragment is shown in *lane 2* (*EcoR1/HindIII* digest of pDR).

sides reciprocal recombination, intermolecular gene conversion (nonreciprocal recombination) between either the *neo1* or *neo2* segment of one molecule and appropriate homologous segments of another molecule can also result in a G418-resistant product. In the B16 metastatic variants, extrachromosomal recombination occurred at a very high frequency when compared to chromosomal recombination (Tables I, II). Thus, the data reported here might show that the cellular events determining the degree of metastatic ability consequently reflect the degree of genetic instability and, in this particular case, the frequency with which extrachromosomal recombination is mediated. For example, Finn et al. [1989] have shown that immortally transformed cells mediate higher levels of extrachromosomal DNA rearrangement than normal diploid cells. However, the ability to maintain this successful recombination event comes into question when recombination after stable integration of the pDR plasmid into the host genome is examined.

Our observations for chromosomal recombination have clearly indicated a higher overall successful recombination frequency (which produces a functional *neo* gene) for the low metastasis F1pDR clones compared with the highly metastatic BL6pDR and ML8pDR clones (Table II). At first glance this suggests a greater genomic plasticity in the low metastasis variant and does not appear to correlate with the Nowell [1976] hypothesis for tumour progression. However, it may be that the highly metastatic cell lines do have a higher overall frequency of recombination but a lower frequency of successful recombination. The range of chromosomal recombination frequencies between the metastatic variants may be a reflection of either the site or the structure of the integrated DNA. Previous studies have demonstrated that some integration sites can be highly unstable, leading to rearrangements in the integrated plasmid as well as in the surrounding cell DNA [Murnane and Young, 1989]. Also, integration sequences commonly show instability after integration [Heartlein et al., 1988]. Therefore, in the highly metastatic cells, rearrangements near or within the integrated sites may occur more frequently due to a higher degree of genetic instability. The distance between integrated plasmid sites might also be reflected in the observed differences in

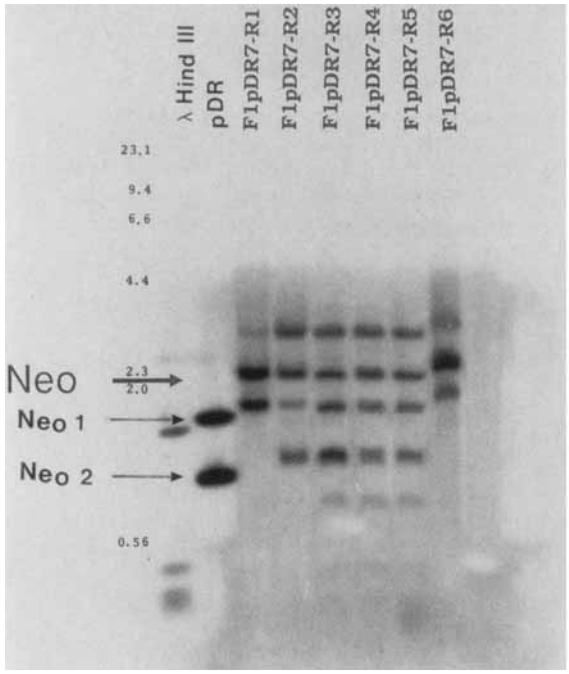

Fig. 4. Southern blot analysis of chromosomal recombination events. All of the G418-resistant recombinants have acquired a complete *neo* gene as identified by the appearance of a new 2,338 bp fragment which hybridises with the *neo* probe. *Lane 1:* λHindIII molecular wt. markers. *Lane 2:* pDR plasmid digested with *HindIII/EcoR1* (see Fig. 2). In general, the generation of an intact *neo* did not result in the loss of the *neo1* and *neo2* segments in R2 through to R5 and only the loss of the smaller (*neo2*) fragment in R1 and R6. Since R1 through to R6 are subclones, the patterns of hybridisation show a marked degree of similarity.

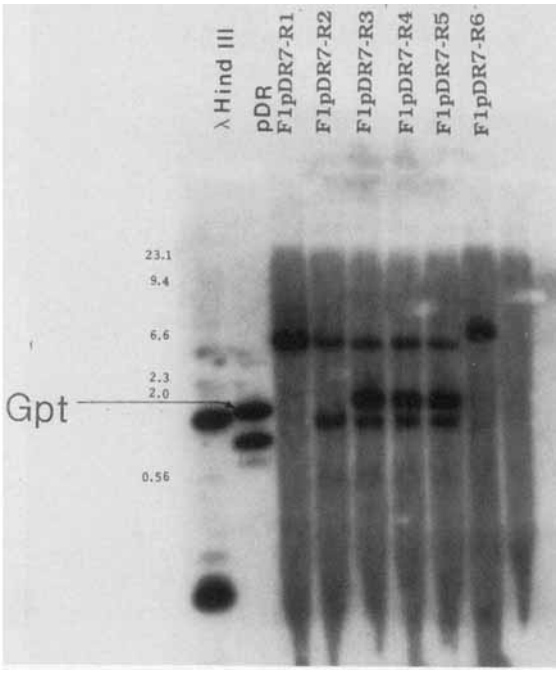

Fig. 5. The blot in Fig. 4 was probed with *gpt* sequences after melting off the *neo* probe. The sizes of the λHindIII molecular wt. markers are shown on the left side. The visible bands in *lane 1* are not λ bands but contaminating sequences. The fragment corresponding to the intact *gpt* coding sequence is shown in *lane 2*.

recombination frequency between the metastatic cell lines.

An outcome of these studies highly relevant to genomic instability is the observation that all the high metastasis ML8pDR clones have lost both *neo* fragments, and only one of the highly metastatic BL6pDR clones has managed to retain a *neo* band. However, of the low metastasis F1pDR clones, a proportionately greater number have retained one or both of the *neo* fragments. Subsequent analysis of several recombinant F1 clones did reveal that all had undergone a recombination to produce a full-length *neo* fragment (Fig. 4). A higher inherent instability in the genome of the high metastasis variants may result in the excision of these sequences.

In conclusion, the results presented here may be evidence that developing neoplastic tissue is composed of cells in which recombination and other mutations occur with increased frequency and that, depending on the degree of genetic instability, populations of cells arise with varying orders of metastatic ability. By this interpretation, the highly metastatic variants of the B16 mouse melanoma may have a higher inherent genetic instability and therefore be less efficient at stably maintaining successful recombination events, and this would be in accordance with the Nowell [1976] hypothesis for tumour progression. However, although such an interpretation would then indicate that increased homologous recombination is an event associated with an increased instability in highly metastatic cells, the data suggest the need for more incisive experiments to test whether chemical or other recombinogenic factors play a role in metastatic progression.

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