nism for amyloid formation may be by means of intralysosomal catheptic (*22*) digestion of light polypeptide chains of immunoglobulins. This mechanism is supported by the frequent close spatial relationship between amyloid deposits and cells of the macrophage system (*23*) and by the electron microscopic observations of fibrils within plasmalemmal invaginations and membrane-bound vesicles of macrophages (*24*). Since none of the patients whose Bence Jones proteins could be degraded to form "amyloid" fibrils in vitro were known to have amyloidosis, other mechanisms or factors in addition to those noted here may be necessary for the production of amyloid fibrils in vivo. The type (and homogeneity) of variable fragment of immunoglobulin protein deposited in tissues as amyloid fibrils may be dependent on a selection process occurring at the level of either immunoglobulin-synthesizing cells or immunoglobulin-degrading phagocytic cells or both. Furthermore, the possibility must still be considered that some cases of amyloidosis may result from tissue deposition of antiparallel, β-pleated sheet fibrils derived from portions of immunoglobulins other than light chains or of proteins other than immunoglobulins.

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DNA Constancy in Heteroploidy and the Stem Line Theory of Tumors

Abstract. *Cellular DNA was measured by high-speed flow microfluorometry in mammalian diploid and heteroploid cell populations stained by the fluorescent-Feulgen procedure. Heteroploid cells with elevated modal chromosome number showed the expected increase in modal DNA content. However, the variability of DNA content was the same in diploid and heteroploid cell populations despite the large variability of chromosome number in the latter populations. This suggests that heteroploidy may include defects in the chromosomal condensation and kinetochore development systems.*

Heteroploidy, abnormal variability of chromosome number and structure (*1*), is a common feature of the cell populations of tumors and of established cell lines in culture (*2*). By contrast, normal animal cells exhibit very little karyotypic variability either in vivo or in vitro (*2*). Many heteroploid cell populations have high modal chromosome numbers as well as high variability of chromosome number; studies of such populations show a positive correlation between modes for chromosome number and cellular DNA content (*3*). It has been assumed that, within each such population, high variability of chromosome number corresponds to high variability of DNA content per cell. It has thus been assumed that such cell populations have abnormal genetic variability, a notion that has encouraged acceptance and extension of Makino's stem line theory (*4*), which has important implications for tumor therapy (*5*).

We present evidence that heteroploid cell populations consist of one or more subpopulations, each with its characteristic DNA content per cell and modal chromosome number. The DNA content per cell is constant within a subpopulation, but chromosome number is variable. Thus, variability of chromosome number for the entire population is the sum of two contributions: one due to the presence of one or more subpopulations, each with the same ratio between modal chromosome number and DNA content per cell; and the other due to variability of chromosome number, despite uniform DNA content, within each subpopulation.

Much of the published data on DNA distribution in cell populations has been obtained by measurement of relatively small numbers of Feulgen-stained cells. Because of poor statistical precision, histograms of these populations have failed to reveal details of shape and fine structure. As a result, it has been difficult to distinguish among components of a population heterogeneous with respect to mitotic cycle phase, stem line, and proportion of cells of each stem line that are in cell cycle traverse. A

flow microfluorometry method for rapid measurement of the DNA content of individual cells has been described (*7*). Fluorescent Feulgen-stained cells in monodisperse suspension (*8*) flow individually at high speed through the intense blue light of an argon ion laser beam, and the resultant fluorescent light flash from each cell is converted to an electrical pulse by a photomultiplier. The signals are then amplified, analyzed, and stored in the memory of a multichannel pulse-height analyzer. In this way, the analyzer memory accumulates a record of the DNA content per cell of 20,000 to 50,000 cells in 1 minute (*9*).

Figure 1, A–D, shows distributions of cells according to their DNA content (left) and chromosome number histograms (right) (*10*) for diploid and heteroploid cells (*11*) of human and Syrian hamster origin. For each cell type, DNA content per cell was determined for proliferating cells. This data yielded bimodal cell distributions dominated by the 2C (*12*) peaks. These large peaks represent G_1 cells, which predominate in proliferating populations with a long G_1 period. In each cell population, there is a 4C peak with double the mode of the 2C peak. This 4C peak represents the G_2 plus M cells in the population; S phase cells, with varying degrees of completion of DNA replication, are distributed between the 2C and 4C peaks (*7*).

From the dominant 2C peak we have estimated the variability of DNA content of cells in G_1, expressed as a coefficient of variation (CV) (*13*). In addition, the high statistical precision of the data enabled us to estimate the CV of the second peak, representing the G_2 plus M population. Calculations were done by a computer method that fits two Gaussians and a second-order polynomial to the data. Since variability is the most important parameter in this study, it must be remembered that the calculated CV's represent upper limits, and include instrumental and staining variability.

If we compare human diploid WI-38 cells to the human heteroploid line HeLa (Fig. 1, A and B), we see that the latter cell population has higher modes for both DNA content (G_1 cells) and chromosome number. The HeLa cell chromosome number is highly variable from cell to cell, while WI-38 cells have a tight chromosome number distribution, as would be expected for heteroploid and diploid cells. Despite this difference, CV values for DNA content (G_1 cells) are the same (8.8 percent for WI-38, 6.0 percent for HeLa). Similarly, if we compare diploid Syrian hamster embryo cells (SHE) to the low heteroploid P183 line (Fig. 1, C and D), we see that the striking difference in variability of chromosome number is not reflected in the DNA distributions for G_1 cells (CV values are 9.1 percent for SHE and 7.3 percent for P183).

Data for these and many other cell populations are presented in Table 1. Modes of the G_1 peaks are normalized to 1.00 for the diploid cells; on that

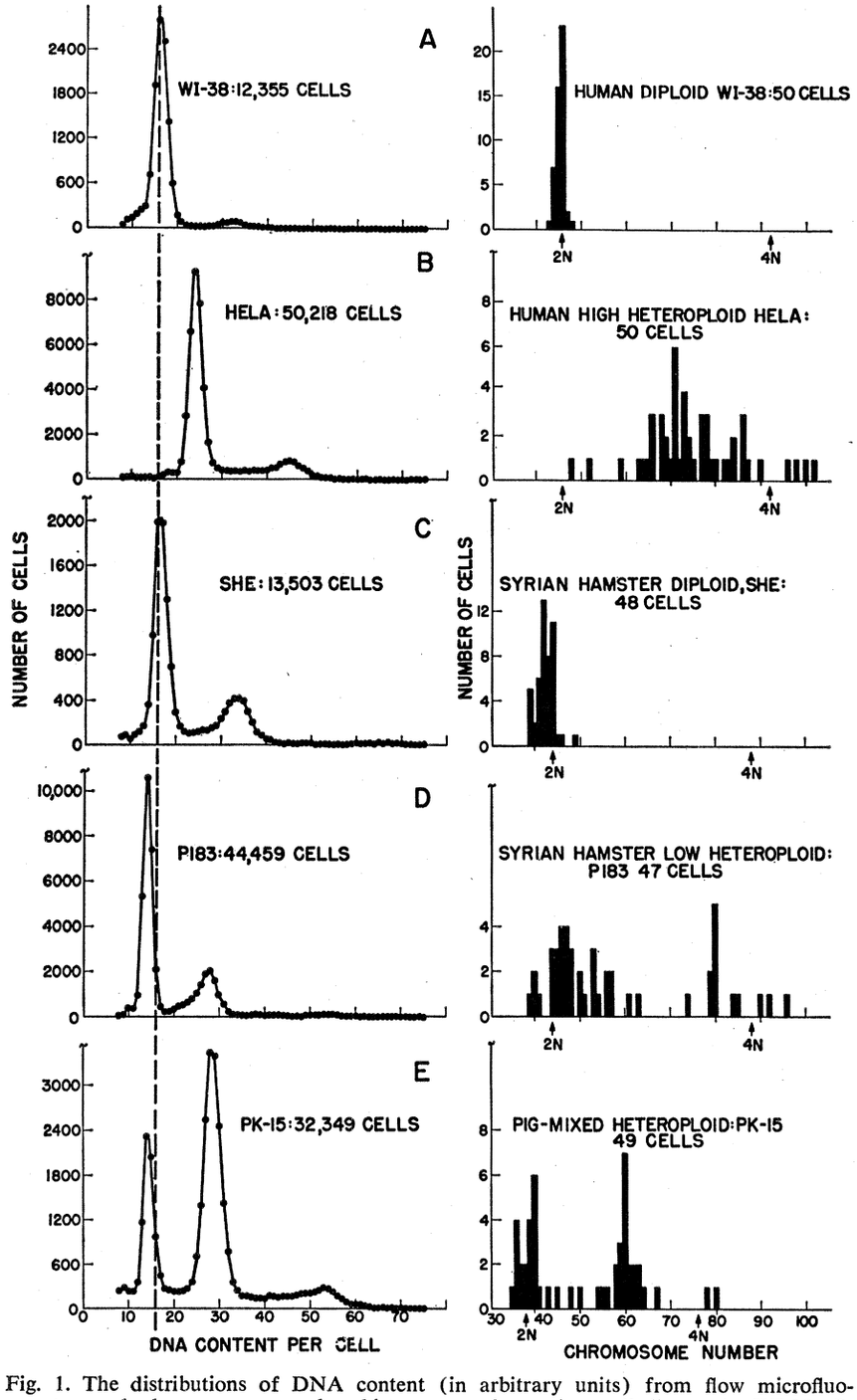

Fig. 1. The distributions of DNA content (in arbitrary units) from flow microfluorometry and chromosome number histograms of growing cell lines: A, WI-38; B, HeLa; C, Syrian hamster embryo; D, P183 [polyoma-transformed derivative of BHK-21(C13)]; and E, porcine PK-15. The dashed line at the left represents the average 2C DNA content of diploid mammalian cells. The abscissal scales for DNA content and chromosome number are not comparable.

basis, it is apparent that high heteroploid cell populations have modes for both DNA content and chromosome number near the 3C or triploid value (*14*). Although the variability of chromosome number in unimodal heteroploid cell lines is greater than that in corresponding diploid populations, the variability of DNA content is the same in both heteroploid and diploid populations. The 2C DNA content for all mammalian diploid lines studied is remarkably constant regardless of the species of origin; these results are consistent with the established clustering of cellular DNA values for mammals (*15*).

Figure 1E shows data for a heteroploid cell line (PK-15) that has retained a bimodal chromosome distribution for many years in vitro (*16*). While the CV value for the DNA content of the entire population is high, CV values for the two subpopulations are the same as those for diploid lines (Table 1). When PK-15 cells are subcultured, modes for DNA content of the two subpopulations always differ by a factor of 2. In contrast, the ratio of the areas beneath the peaks varies among subcultures. The findings indicate that for each subpopulation, the DNA content per cell is fixed, but the percentage of total cells varies.

Before drawing conclusions from our data, we must ask whether many of the metaphase cells of heteroploid populations consistently failed to divide. This possibility has been proposed by Stich and Steel (*17*) to explain the distribution of DNA content in a human breast adenocarcinoma. We consider this possibility unlikely, because very high plating efficiencies are often obtained with heteroploid cell lines, and because over 90 percent of mitotic HeLa cells are capable of division (*18*). Furthermore, our CV values for the G_2 plus M peak and for the G_1 peak are identical. In several cell lines, we studied DNA distributions after Colcemid treatment, which produces many arrested metaphase cells similar to those used for chromosome counts. As in untreated cells, CV values for the large G_2 plus M peak and for the G_1 peak were identical.

Our results indicate that heteroploid populations contain one or more subpopulations, each homogeneous with respect to DNA content per cell. We suggest that such a subpopulation corresponds to a stem line. A tumor with extravagantly variable metaphase chromosome numbers may consist of cells from a small number of stem lines (and may also include some aberrant metaphase cells that cannot divide). We do not know whether cells of a stem line have greater genetic variability than do normal diploid cells, or whether all stem lines are identical in this respect. We do know, however, that many heteroploid populations grow exponentially, respond readily to cell synchronization, and are damaged by selective agents, all in a quantitative manner that suggests genetic homogeneity grossly similar to diploid cells (*6*). Our discovery of uniform DNA content may account for this paradox.

We do not know what causes the variability of chromosome number despite uniform DNA content in the populations we studied. However, our findings cannot be explained by those mechanisms—for example, nondisjunction—which produce daughter cells of unequal DNA content. Kucheria showed that the DNA replication pattern of human tumor cells in culture remains unchanged despite changes in chromosome number and morphology (*19*). It is tempting to speculate that the defects in heteroploidy are in the mechanisms of chromosome condensation and kinetochore formation. Evidence for extensive linear arrays of connected chromosomes in metaphase (*20*) has encouraged DuPraw to suggest that interphase cells may have continuity of several chromosomes or even of entire haploid sets (*21*). We further speculate that the number of chromosomes formed at metaphase is controlled by DNA sequences, along a continuous chromosome fiber, that determine loci for breakage and loci for kinetochore placement and development. Somers and Hsu (*22*) have described long telomeric A-T sequences, which these authors (understandably) regard as "genetic nonsense." However, such regions might be relevant to chromosome condensation and separation of several chromosomes from a much longer DNA fiber. Mutations that caused an error in the function

Table 1. G_1 phase cellular DNA content and variability and chromosome number characteristics of various mammalian cell populations. Abbreviations are as follows: Dip., diploid; Hi. Het., high heteroploid; and Pseudodip., pseudodiploid.

Cell	Karyotype class	G_1 peak		Chromosome number*		
		DNA per cell†	CV (%)	Mode	Range	CV (%)
		Rhesus monkey				
Rh‡	Dip.	1.00	8.4	42	39– 42	2.2
LLC-MK_2	Hi. Het.	1.80	8.1	62	43–116	17.8
		Human				
WI-38	Dip.	1.00	8.8	46	43– 48	2.0
HeLa	Hi. Het.	1.47	6.0	71	38–106	17.9
HAT-1	Hi. Het.	1.47	6.8	62	45–113	20.7
LLB	Hi. Het.	1.40	6.0	59	56–121	26.1
Tu Wi	Hi. Het.	1.47	6.8	50	41– 65	7.9
		Syrian hamster				
SHE	Dip.	1.00	9.1	44	39– 49	4.5
BHK-21(C13)	Pseudodip.	1.03	7.8	44	42– 45	1.3
P183	Low Het.	0.94	7.3	46	37–175	43.0
P183 tumor	Low Het.	0.84	11.2			
P183 tumor recultured	Low Het.	0.93	8.4			
		Mouse				
WME	Dip.	1.00	7.0	40	37– 43	3.4
L-929	Hi. Het.	1.74	7.0	70	40–115	18.0
3T3	Hi. Het.	1.67	8.9	66	48–149	37.1
SV3T3	Hi. Het.	1.58	10.3	66	51– 77	8.22
PY3T3	Hi. Het.	1.55	9.5	62	55–200	36.1
SVPY3T3	Hi. Het.	1.57	9.4	62	55– 70	5.18
L5178Y ascites tumor	Pseudodip. (?)	0.90	8.3	40	37– 41	2.79
		Chinese hamster				
CHO	Near Dip.	0.81	6.6	21	20– 22	2.16
		Pig				
PK-15	Mixed-	0.89§	7.4	38	33– 41	4.6
	Het.	1.78	6.4	60	54– 67	4.7

* Chromosome number histograms were prepared from counts of 50 metaphase cells. † DNA content of G_1 cells normalized to unity for diploid cells of the particular species or to average diploid values for CHO and PK-15. ‡ Chromosome counts were supplied by Dr. J. C. Petricciani, Division of Biologics Standards, NIH. § PK-15 cells are bimodal with respect to both metaphase chromosome number and DNA distribution (Fig. 1E).

of this system, or of the kinetochore placement and development system, could cause variable chromosome number and morphology without attendant genetic variability.

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1. The term mixoploidy is now used in place of heteroploidy to describe karyological states of interest to this paper. A recent terminology statement, sponsored by the Tissue Culture Association, is published in *Nat. Cancer Inst. Monogr.* **29**, 587 (1968).
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8. Monodisperse cells were prepared from monolayer cultures by dispersal with trypsin, ethylenediaminetetraacetic acid (EDTA), and deoxyribonuclease, neutralization with soybean trypsin inhibitor and bovine serum albumin, and fixation in formalin-saline G (Puck). Hydrolysis and Feulgen staining with auramine-O were done as previously described (*7*).
9. We further validated the flow microfluorometric method by analyzing DNA distributions of CHO cells during prolonged Colcemid block. Peaks emerged with modes in the relationship 2C, 4C, 8C, and 16C (*12*) as anticipated from E. Stubblefield's data [in *Cytogenetics of Cells in Culture*, R. J. C. Harris, Ed. (Academic Press, New York, 1964), pp. 223–248].
10. Chromosome counts were made on 50 metaphase cells, which were treated with Colcemid and hypotonic sucrose, fixed in methanol—acetic acid, spread by the air-dry method, and stained by the method of Klinger and Hammond [*Stain Technol.* **46**, 43 (1971)].
11. Sources of cell lines used in this study were as follows: WI-38, Tu Wi, and LLC-MK$_2$, from American Type Culture Collection; Rh (FRhL-3), from Division of Biologics Standards, NIH; HAT-1, LLB, and HeLa, from Charles James, University of New Mexico School of Medicine, Albuquerque; BHK-21-(C13), 3T3, SV3T3, PY3T3, and SVPY3T3, from Dr. Alexander Kisch, University of New Mexico School of Medicine, Albuquerque; PK-15, from the National Animal Disease Laboratory, Ames, Iowa; L5178Y, from Dr. Lionel Manson, Wistar Institute, Philadelphia; P183 [polyoma-transformed derivative of BHK-21(C13)], from Dr. Vittorio Defendi, Wistar Institute, Philadelphia; L-929, from Dr. Angus Graham, Wistar Institute, Philadelphia; WME (whole mouse embryo) and SHE (Syrian hamster embryo), from our laboratory. Cells were grown in F-10 medium or Eagle's BME medium (supplemented with 10 percent calf serum) in Blake bottles or plastic T-75 flasks (Falcon).
12. Abbreviations used are as follows: C, the amount of DNA in a set of haploid mammalian chromosomes; G_1, the period of interphase that precedes DNA synthesis; G_2, the period of interphase that follows DNA synthesis; S, the period of interphase when DNA is synthesized; M, mitotic phase.
13. Coefficient of variation (CV) in percent equals 100 times the standard deviation divided by the mean.
14. Analysis of 10^7 cells by a modified Schmidt-Thannhauser procedure indicated that proliferating diploid cells contained 8 pg of DNA per cell; "high heteroploid" lines contained 10 to 15 pg of DNA per cell.
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Junctions between Cancer Cells in Culture: Ultrastructure and Permeability

Abstract. *Cell junctions between Novikoff hepatoma cells (N1S1-67) growing as small clumps or chains in suspension culture have been studied with ultrastructural, electrophysiological, and dye-injection techniques. Cells within clumps are commonly electrically coupled and can exchange dyes with a molecular weight of 332 to 500. Gap junctions and intermediate junctions are present, whereas true tight junctions and desmosomes are absent or very rare. This system should provide a useful model for studying the properties of "communicating" junctions.*

Certain intercellular junctions involving close membrane apposition or fusion are possible sites for the direct exchange of small inorganic ions and larger molecules (*1, 2*). It has been suggested that such junctions might normally pass regulatory molecules from cell to cell (*1, 2*) and that alterations in the junctions might result in faulty coordination of cellular activity (*3–5*). Such considerations have prompted study of cancer cell junctions, but no clear picture has yet emerged. Electrophysiological studies indicate that some cancer cells lack low resistance junctions—that is, they are not "electrically coupled" (*5–7*). Other cancer cells remain electrically coupled (*2, 4, 7, 8*) and certain of these cells are capable of directly exchanging molecules larger than small ions (*2*). Electron microscopic studies indicate that still other cancer cells have junctions that are less frequent or extensive although they may retain a normal fine structure (*9, 10*). None of these studies fully defines the degree of abnormality or normality of cancer cell junctions partly because each system has been studied with only one or at best two of the available techniques. Thus the presence or absence of coupling has not been directly correlated with ultrastructural evidence and in only a few cases has the coupling been correlated with permeability to larger molecules.

We report here the results of our initial experiments on a cancer cell system that is particularly amenable to study by electrophysiological, dye-injection, and electron microscopical techniques. Using all three methods, we have shown (*11*) that these cells readily exchange small ions and certain dyes and possess one of the junctions, namely, the "gap" junction, thought to mediate such exchange in "normal" systems (*12*).

Our studies have been carried out on Novikoff hepatoma cells (N1S1-67) grown in suspension culture in accordance with reported procedures (*13*). In a few initial experiments, we confirmed the cancerous nature of these cells by injecting them intraperitoneally into randomly bred rats (*14*). In suspension culture, the N1S1-67 cells grow in clumps of 2 to 100 or more cells. Clumps are characterized by marked intercellular adhesion remaining intact during centrifugation, transfer by micropipette, and impalements with microelectrodes. These clumps were conveniently prepared for electron microscopy after being centrifuged into loose pellets. The pellets were fixed in glutaraldehyde-paraformaldehyde and osmium tetroxide either accompanied by lanthanum hydroxide (*15*) or followed by uranyl acetate in the block (*16*).

By electron microscopy we have identified two types of contact specializations. The most conspicuous type resembles the *zonula adhaerens* (or