

DNA Content of Nuclei and Chromosome Number in Sublines of the Ehrlich Ascites Carcinoma*

JEROME J. FREED AND DAVID A. HUNGERFORD

(The Institute for Cancer Research and The Lankenau Hospital Research Institute, Philadelphia 11, Pa.)

The study of metaphase chromosomes of ascites tumors has shown that, for a given strain of tumor, there is found a characteristic modal chromosome number (referred to as s), which frequently lies near that of the diploid number for the host organism (3). Other tumors have modal numbers in the tetraploid range, this condition appearing to enhance the ability of the tumor cells to grow in host strains other than that in which the tumor originated. Recently, it has become possible to study the effect of such doubling of chromosome number in closely related sublines. From the predominantly hyperdiploid ($s = 45-46$) Lettré-Ehrlich ascites carcinoma, Kaziwara (4) was able to select sublines consisting largely of the hyper-tetraploid cells ($2s = 90-92$) normally present with low frequency in the parent strain. The subline EL88 appeared to be stable and suitable for use in investigations of the effect of doubled chromosome number on the physiology of the tumor cells. A recent study from this laboratory (10) is concerned with the differences in peptidase content of the s and $2s$ cells of these types. Biochemical and cytochemical (Feulgen) studies of DNA and chromosome number carried out in connection with this work showed nuclear phenomena in the EL88 subline not expected on the basis of its earlier history. There occurred a high rate of reversion to the s condition and a high frequency of cells with DNA contents beyond the octoploid range. The latter effect indicates that cells of high ploidy were present with frequencies not evident from analysis of the metaphase counts. This, as well as further work with the chromosome number and DNA content of these sublines, is reported below.

MATERIALS AND METHODS

The tumor sublines employed were those used by Patterson and Podber (10). The Heidelberg (Lettré-Ehrlich) strain of the

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Ehrlich hyperdiploid ascites carcinoma (1) was maintained by serial intraperitoneal injection of 0.2 ml. undiluted ascitic fluid into 1½-4-month-old female ICR Swiss mice. The EL88 sublines were maintained similarly, except that EL88-4 and EL88-5 were for a time transferred with an inoculum of 0.1 ml. ascitic fluid. The several sublines of EL88 were obtained by injection of 2×10^4 cells into mice of the above type. Such low cell dosage served to re-select tetraploid cells (4) and thus gave rise to a new subline (re-extraction). The history of the sublines thus derived is summarized in Chart 1. Animals were sacrificed, and ascitic fluid was sampled at various times as indicated below. Aliquots were used for chromosome counting and for the Feulgen reaction.

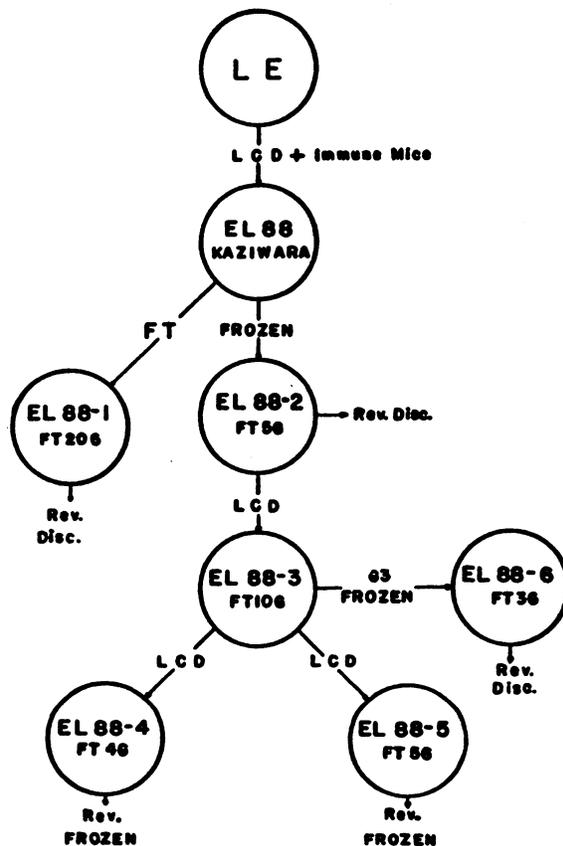


CHART 1.—Diagram showing the history of the ascites tumor strains used in this study. LCD = low cell dosage technic of re-extraction; FT = number of fluid transplant generations; Rev. = reversion, i.e., hyperdiploid metaphases at or near the 50 per cent level; Disc. = discarded.

Chromosome counts were made from acetic orcein preparations (9). In a few cases, the frequency of metaphases of the *s* and *2s* types was estimated from the relative sizes of the metaphase plates. Such estimates were in reasonable agreement with more exact counting methods.

Feulgen determinations were made on nuclei isolated in 1 per cent citric acid (2), with similarly isolated mouse kidney nuclei present on each slide as a standard. After hydrolysis in 1 N HCl for 11 minutes, preparations were stained as described by Swift (13) and mounted in oil of matching refractive index. With the use of a cytophotometer (11), absorption measurements were made at 546 m μ , through an area with a diameter slightly smaller than the lesser diameter of the nucleus. Since the nuclei were greatly flattened by being dried on the slide, the area was computed as an ellipse from the measurement of two diameters at right angles. The product of extinction and area (total ex-

RESULTS

In the Lettré-Ehrlich subline, the distributions of chromosome numbers and nuclear DNA values were constant in several samples examined. Chromosome counts of metaphases fell into two well defined classes—the first with a modal value of 45 chromosomes (the *s* class of metaphases), the latter having a doubled chromosome range of 90–92 (the *2s* class). The occurrence of aneuploidy and of metaphases with very high chromosome numbers corresponded with descriptions by other authors (1, 4). The frequencies of metaphases of the *s* and *2s* classes may be seen in Chart 2. The

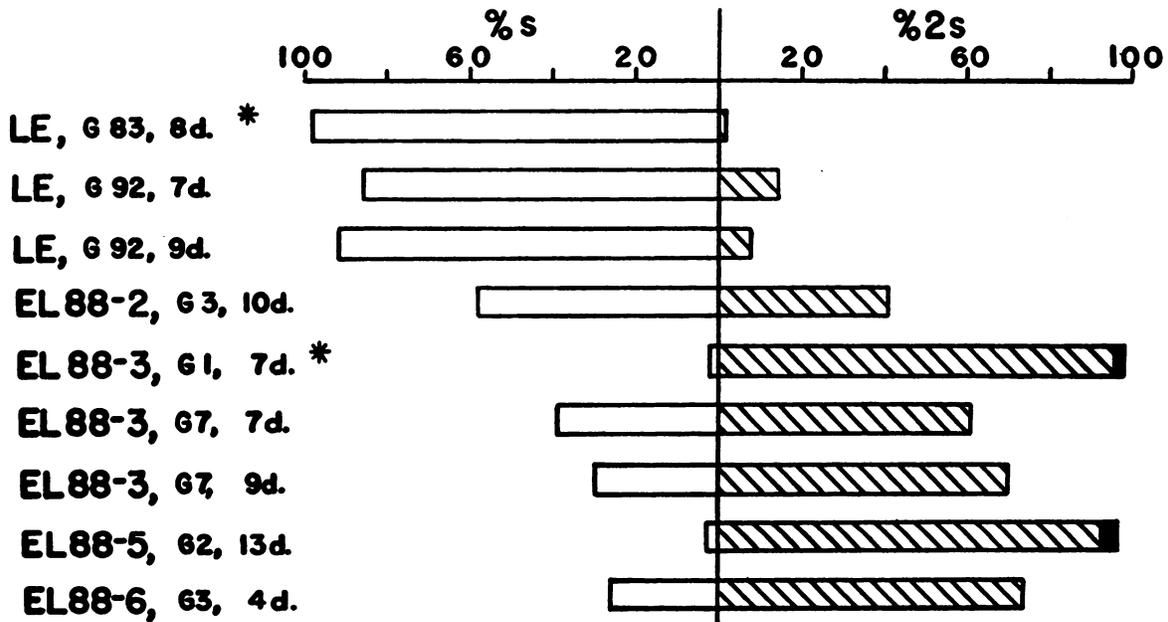


CHART 2.—Frequencies of chromosome counts falling into the hyperdiploid (*s*) and hypertetraploid (*2s*) classes, for samples of ascites tumors used in this study. G = generation

number; d = number of days from inoculation to sampling; black areas indicate *4s* and larger counts; * = based on estimates from metaphase plate size.

inction in sq. μ) was taken to be proportional to the amount of DNA per nucleus.¹ Variations in staining from slide to slide were never more than 10 per cent of the mean value for the standard kidney nuclei; such variations were compensated for by a factor of such size as to set equal the mean values for the kidney nuclei on each slide. As reported in Patterson and Podber (10), this technic of cytophotometry gave satisfactory agreement for the expected 1:2 relationship in DNA content between isolated spermatid and kidney nuclei of the mouse.

¹ If the mass extinction coefficient (E/gm/sq. μ) of the Feulgen-DNA complex is known, the total extinction as defined above may simply be divided by it to yield directly the weight of DNA in grams. Reliable determinations of the quantity E/gm/sq. μ are not at present available, but it has been repeatedly demonstrated that the Beer-Lambert law appears to apply to Feulgen-stained nuclei. Thus, the total extinction is a quantity proportional to the DNA content, and its use in this paper is analogous to the various systems of arbitrary units employed by other authors.

2s metaphases never amounted to more than 14 per cent of the metaphases examined. The distribution of DNA content in the population of interphase nuclei may be seen in Chart 3. The histograms show a primary mode at about 15 sq. μ in each case, with a secondary peak at about twice this value. Nuclei with intermediate values occur. Since the DNA content corresponding to a diploid mouse nucleus is 11 sq. μ (the $2C$ value), it may be seen that the mode at 15 sq. μ probably corresponds to the class of cells having the *s* number of chromosomes. The interphase nuclei of this modal value, then, may be said to have the S value of DNA. The S value of 15 sq. μ is greater than would be predicted on the assumption that the DNA content is directly proportional to the

number of chromosomes. On this basis, an S value of 12.4 sq. μ would have been expected. The difference, if real, may be due to the presence of two conspicuously large chromosomes in the Lettré-Ehrlich cells (1).

The series of EL88 sublines, on the other hand, showed variations of some magnitude during the course of this study. As has been described (10), EL88-1 began to show *s*-range chromosome num-

bers with increasing frequency during routine serial transplantation and was therefore discarded. From a frozen sample set aside by Kaziwara, EL88-2 was obtained; at the beginning of serial transplantation it was almost entirely hypertetraploid. In the third transplant generation, the frequency of *s* metaphases rose to 53 per cent, and the subline was considered to have reverted. Determinations of the distributions of DNA values in

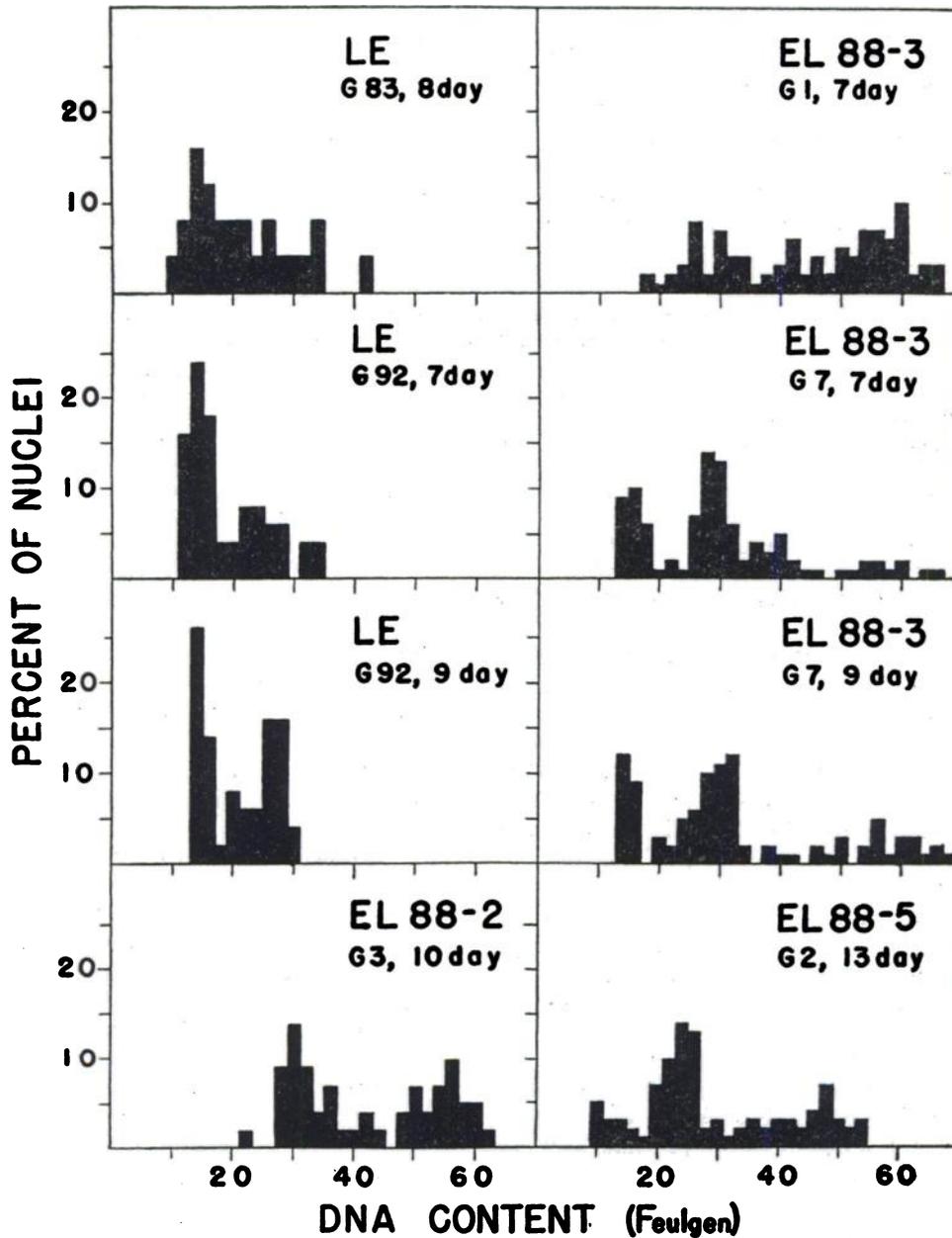


CHART 3.—Frequency distributions of DNA contents of individual nuclei from ascites tumors. DNA content expressed as total extinction per nucleus in sq. μ , after Feulgen reaction,

measured at 546 m μ . Samples consisted of 100 nuclei, except in the case of LE, G83, in which 56 nuclei were measured.

the population of interphase nuclei, however, gave the results shown in Chart 3. The distribution suggests two modes, in the 2S and 4S regions, respectively; nuclei having the S value appear to be practically absent.

Subline EL88-3 was then re-extracted by the method of low cell dosage. Metaphase counts made in the first generation (Chart 2) showed it to be almost completely 2s in chromosome constitution. The DNA histogram obtained (Chart 3) was, however, of the same type as that found in the preceding subline, with peaks at 2S and 4S. When further studies were made at the seventh generation, s-range metaphase counts were now present in appreciable numbers, and the DNA distribution had shifted markedly. Chart 3 shows histograms from tumor samples obtained 7 and 9 days after inoculation. Both show a peak corresponding to that of

quencies of polyploid cells. It seems unlikely from these data that the shift in DNA distribution encountered was due to variation in transplant procedure.

It may be observed, however, that the 8-day samples in all the age groups showed a greater frequency of s-range metaphases than did the 4-day ones. This difference may reflect the apparently greater rate of proliferation of the stem-cell type and account for the rapid reversion of the tumor to the hyperdiploid condition.

To eliminate the possibility that biased selection of nuclei, or similar technical errors, had been responsible for the appearance of the abnormal, predominantly polyploid type of histogram, comparison was made with biochemical determinations of the average DNA per cell, available for aliquots from some of the samples (10). The mean

TABLE 1
FREQUENCY OF DNA PLOIDY CLASSES, AND DISTRIBUTION OF CHROMOSOME COUNTS, IN HOSTS OF DIFFERING AGE, FOR SUBLINE EL88-6

Host age	Sample	PER CENT OF NUCLEI IN EACH DNA CLASS*			PER CENT OF METAPHASES IN EACH PLOIDY CLASS		PER CENT CELLS IN MITOSIS
		S	2S	4S	s	2s	
12 mo.	4-day	49	41	10	66	34	2.5
"	8-day	60	37	3	71	29	1.0
3½ mo.	4-day	47	49	4	56	44	1.6
"	8-day	46	48	7	86	14	1.3
6 wk.	4-day	54	39	8	72	28	1.6
"	8-day	43	42	15	87	13	1.0

* S class from 0-18 sq. μ , 2S from 19-40 sq. μ , 4S greater than 40 sq. μ .

the S mode in the Lettré-Ehrlich, a peak of larger area in the 2S region, and scattered values, with some suggestion of a peak in the 4S range.

EL88-5, obtained by a further re-extraction (Chart 1), was studied at the second generation. The chromosome counts were almost purely hypertetraploid, and the DNA histogram showed a corresponding reduction in the area of the S peak (Chart 3).

Inasmuch as the predominantly polyploid DNA distribution was encountered during a period when 3½-month-old mice were being used as hosts, and the subsequent work was done with 1½-month-old hosts, it was considered necessary to test for the effect of host age. Subline EL88-6, obtained from a frozen sample of EL88-2, was injected simultaneously into hosts of three age groups, with the results shown in Table 1. It may be seen that the tumor had largely reverted to the hyperdiploid condition and that the distribution of DNA values was comparable in all cases to that found for EL88-3 at the seventh generation. Neither variation in host age nor time of sampling can be correlated with the occurrence of high fre-

DNA value for the entire sample of measured nuclei may be expressed as a multiple of the value found for the standard mouse kidney nuclei or, conveniently, as a multiple of the haploid DNA value for the mouse (C). Similarly, the biochemical results, corrected for the presence of nontumor cells in the sample, were expressed as a multiple of the known DNA content of the diploid mouse nucleus, and the appropriate C value determined. Such comparison for the Lettré-Ehrlich tumor gave 3.2C and 2.9C for the Feulgen and biochemical methods, respectively. In the case of the first-generation sample of EL88-3, showing the aberrant type of DNA distribution, the results were 7.5C for the Feulgen method and 6.6C for the biochemical method. It was thus considered that the histograms are sufficiently accurate representations of the actual DNA distributions to permit the desired analysis.

DISCUSSION

In general, it seems that the DNA content of nuclei is proportional to the number of chromatid sets contained. For mouse ascites tumors specific-

ly, Richards *et al.* (12) have shown that the DNA contents of metaphases are proportional to the number of chromosomes per plate. Thus, it might be possible to make a quantitative comparison of metaphase chromosome numbers with the form of the interphase DNA frequency distributions. However, the process of premitotic synthesis, leading to the doubling of the DNA per nucleus, greatly complicates such analysis. The effect of synthesis on the shape of the DNA histogram may be calculated (14) for populations of euploid cells; however, the ascites tumor populations with which we have worked are mixtures of cells deriving from at least two aneuploid groups. As a result, such analysis appears impossible. We have been restricted, therefore, to qualitative comparisons in the interpretation of our data.

Lettré-Ehrlich samples.—The data obtained from these samples showed what appears to be the normal pattern of DNA distribution for ascites tumors. Richards *et al.* (12), working with the Krebs and Ehrlich tetraploid tumors, have obtained histograms of similar form, though in the 2S to 4S range. In the interpretation of these histograms, they ascribe the peak at the 4S value entirely to premitotic cells. In our own samples, since it is clear that 4S cells can and do divide successfully when carried in sublines such as the EL88, we must ascribe some of these values to hypertetraploid cells forming part of the mixed population.

Since the frequency of hypertetraploid metaphase counts in these samples ranged from 2 to 14 per cent, while one-third to one-half of the measured nuclei fell outside the S range in DNA value, it is apparent that neither possibility may be excluded. We consider that our 2S values derive both from premitotic hyperdiploids and postmitotic hypertetraploids.

It may be pointed out that our results and those of Richards *et al.* (12) are not consistent with the findings of Leuchtenberger *et al.* (5). The latter authors, working with Klein's tetraploid Ehrlich, reported a distribution symmetrical about the 4C value for the mouse. This was considered to be in agreement with the biochemical results, which showed the mean DNA per nucleus approximating twice the diploid value for the mouse. Such a distribution of DNA values could occur only in the absence of both premitotic synthesis and ploidy mixture in the population.

Behavior of the EL88 sublines.—The type of DNA histogram found in the EL88 samples did not appear to be a function of the relative frequencies of *s* and 2*s* metaphases. In one case in which the DNA contents were in the 2S and 4S

ranges the tumor was, with regard to chromosome counts, approximately 50 per cent hyperdiploid, while in the other it was almost 90 per cent hypertetraploid. Similarly, subsequent samples showed an entirely different type of DNA histogram, either when nearly completely 2*s* or when reverted to the 50 per cent *s*-condition. Perhaps the most likely process involved in the production of such large numbers of polyploid interphases is a change in the frequency with which stem cells divide to produce daughter cells not destined to divide further. If such daughter cells continued to increase in DNA content by endomitosis or endoreduplication (6), the result would be that the actively dividing stem cells would soon form only a small part of the population. Ascites tumors differ markedly in the frequency of occurrence of endopolyploidy (6), and it is possible to suppose that a shift in this character was permitted under the selective conditions used by Kaziwara in his original derivation of the EL88. Since the conditions used favored the growth of the hypertetraploid cells in the population, there might have been a related change in the frequency of endomitosis and/or endoreduplication. During subsequent serial fluid transplantation, selection would have favored those cells seldom giving rise to nondividing progeny. These would, therefore, have become the new stem-line cells. Thus, the production of nondividing cells may be regarded as an indication of the instability of the 2*s* tumor subline, these cells being selected against in the presence of competing *s*-range stem cells.

The change in the frequency of cells with polyploid DNA contents suggests a quantitative change in the make-up of the tumor population. We may say that in this work the change occurred during the period between the first and third transplant generations of the EL88-3 subline. This seems likely, since subline EL88-6, used for the age-effect experiment, showed DNA distributions of the S-2S type found in EL88-3 and EL88-5.

An alternative hypothesis to explain the occurrence of the elevated DNA values found in the aberrant EL88 samples may be based on the effect on the shape of the histogram of changes in the time of synthesis of the doubled DNA amount during the interphase. If the amount of DNA were doubled immediately after telophase, all interphase nuclei would show the amount of DNA usually associated with the next higher polyploid class. This has been found to be the case during cleavage in some embryos (7, 8). However, the number of intermediate values between classes should in this case be much reduced, since the majority of cells would have completed

synthesis at the time they were measured. As may be seen in Chart 3, this is not the case. Therefore, it seems more likely that the shift was due to endomitosis or to endoreduplication.

The use of metaphase chromosome counts alone to characterize the chromosome constitution of a population of ascites tumor cells may thus lead to error. While such counts are reliable in establishing the constitution of the stem line, physiological experiments on populations of ascites tumor cells may actually include large numbers of cells that differ in chromosome constitution or DNA behavior from the stem line. When tumors have been carried in routine transfer for long periods, selection may be expected to have stabilized the stem line to a considerable degree. However, in newly derived lines, the possibility of rapid evolutionary changes should not be overlooked.

SUMMARY

1. The distribution of nuclear DNA values in the Lettré-Ehrlich mouse ascites carcinoma, and in some of its hypertetraploid (EL88) sublines, was studied by Feulgen cytophotometry. Metaphase chromosome counts were obtained from the same samples.

2. In the Lettré-Ehrlich strain, the patterns of DNA distribution in the population and of chromosome number were constant and of the type expected from the work of others and from theoretical considerations.

3. The EL88 sublines showed pronounced changes in DNA distribution and chromosome counts during the study. In addition, the DNA determinations revealed the presence of numbers of nuclei of polyploid DNA content, not apparent from chromosome counts.

4. During subsequent culture of the EL88 sublines, the property of extensive polyploid production was lost from the population. After this shift, the pattern of DNA distribution was found consistent with that of the Lettré-Ehrlich strain.

5. These findings are discussed in regard to the experimental use of ascites tumor populations.

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