Double Minute Chromosomes and the Homogeneously Staining Regions in Chromosomes of a Human Neuroblastoma Cell Line

Abstract. *Four human neuroblastoma cell lines were studied by chromosome banding techniques. All of the lines contained a marker chromosome with a long nonbanding homogeneously staining region (HSR). The HSR-containing chromosome differed in each line. One line contained two classes of cells: one with an HSR marker chromosome and the other with double minute chromosomes. Each cell had one of these abnormalities; no cell had both. The presence of two additional chromosomal markers in all cells of this line indicates a common origin. These observations suggest that the double minute chromosomes are derived from the HSR.*

Cytogenetic studies of human neuroblastomas have repeatedly demonstrated two abnormalities: the presence of very long marker chromosomes and of double minute chromosomes (*1*). Double minute chromosomes are small, paired chromatin bodies whose number and size usually vary from cell to cell and whose origin and function are uncertain. The very long marker chromosomes have been found, in a recent study in which banding techniques were used, to contain a nonbanding, homogeneously staining region (HSR) whose origin and function are likewise unknown (*2*).

We have analyzed the chromosomes of four uncloned neuroblastoma cell lines derived from four independent human tumors. Chromosome preparations were studied after staining by modified trypsin-Giemsa (*3*) or quinacrine dihydrochloride (*4*) techniques, or for constitutive heterochromatin by C banding (*5*).

We confirmed the presence of an HSR in the short arm of chromosome 1 in the line designated IMR-32 (*2*). This line has been in continuous cell culture since 1967, and the long marker chromosome has been present since that time (*6*).

We have also identified HSR's in three recently established human neuroblastoma cell lines. One line, CHP-134, is pseudodiploid (modal chromosome number, 46) and contains an HSR in the short arm of chromosome 7 and another in the long arm of chromosome 6 (Fig. 1A) (*7*). Another line, NMB, is hypotetraploid (modal chromosome number, 83) and contains an HSR in the short arm of chromosome 13 (Fig. 1B). The chromosomes with HSR's which are characteristic of each line were found in virtually every cell of that line (*8*).

Examination of the remaining neuroblastoma line, CHP-126 (modal chromosome number, 46) in passage 8, revealed the presence of two classes of cells: one class had a marker chromosome 5 with an HSR in its long arm (Fig. 1, C and D) and the other contained cells with two normal No. 5 chromosomes and a varying number of double minutes (Fig. 1, E and F). Analysis of 110 cells from CHP-126 showed 66 cells with the HSR and 44 with the double minute chromosomes. Scanning of many more metaphases demonstrated that each cell had only one of these chromosome abnormalities; no cell had both. All of the cells examined also had two additional marker chromosomes (one with an extra band on the long arm of chromosome 2 and another with an extra band on the short arm of chromosome 7) indicating that both subpopulations had a common precursor.

The HSR in CHP-126 was located in

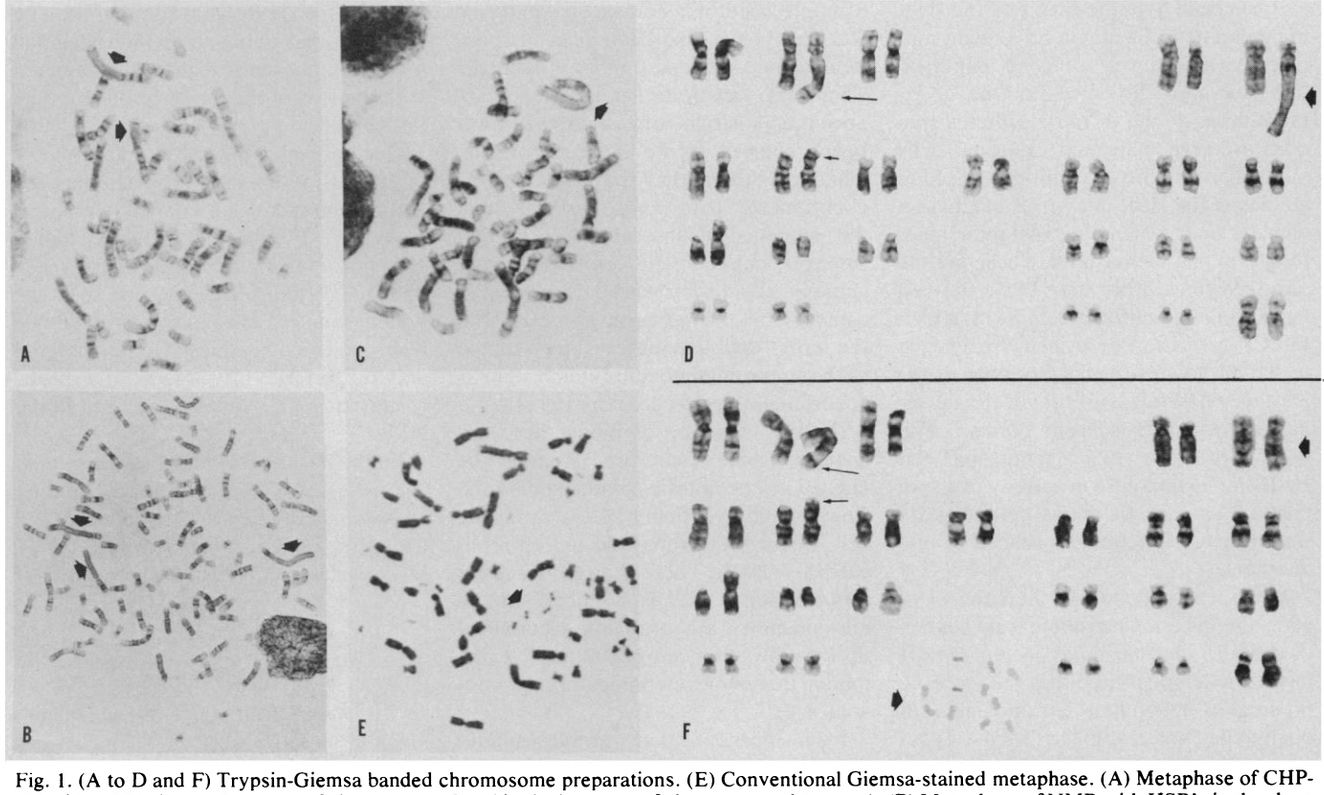

Fig. 1. (A to D and F) Trypsin-Giemsa banded chromosome preparations. (E) Conventional Giemsa-stained metaphase. (A) Metaphase of CHP-134 with HSR's in the short arm of chromosome 7 and in the long arm of chromosome 6 (arrows). (B) Metaphase of NMB with HSR's in the short arms of three No. 13 chromosomes, one of which also has a small translocation (arrows). (C) Metaphase of CHP-126 cell with an HSR on the long arm of one No. 5 chromosome (arrow). (D) Karyotype of a CHP-126 cell with an HSR on one No. 5 chromosome (large arrow) and two additional marker chromosomes (chromosomes 2 and 7) (small arrows). For a detailed analysis of the karyotypes in (D) and (F) see (*8*). (E) Metaphase of a CHP-126 cell with many double minute chromosomes of various sizes. (F) Karyotype of a CHP-126 cell with double minute chromosomes (bottom of figure) and two normal No. 5 chromosomes (large arrows). Marker chromosomes 2 and 7 are also present, as in (D) (small arrow).

a subterminal region of chromosome 5 (5q33) (Fig. 2) (*9*). In trypsin-Giemsa preparations, no bands were evident within the HSR and the HSR stained with intermediate intensity. The HSR did not contain constitutive heterochromatin (by C banding) and did not fluoresce brightly when stained with quinacrine dihydrochloride. These staining characteristics are identical to those reported for the HSR's in two other human neuroblastoma lines (*2*). The double minute chromosomes in CHP-126 also shared identical staining properties with the HSR and did not contain centromeres.

Double minute chromosomes and long marker chromosomes have been observed in direct preparations from neuroblastomas of untreated patients (*10*). No human cell lines other than those from neuroblastomas have been reported with an HSR. The cell lines used in this study are free of viruses and mycoplasma (*7*), and at least two of these lines (IMR-32 and CHP-126) were established from patients who had received no radiation or chemotherapy before the tissues were explanted. We, therefore, believe that the HSR and double minute chromosomes in these lines were present in the neoplasms and are not secondary to treatment received by the patients or the result of long-term growth in culture.

It has been hypothesized that the HSR in human neuroblastoma cells might represent a mechanism for gene amplification, perhaps involved in the overproduction of one or more proteins specific to these malignant neuronal cells (*2*). This possibility was initially raised in studies of the HSR in a series of Chinese hamster lines which are resistant to amethopterin and methasquin. These are the only cell lines other than those derived from human neuroblastomas in which HSR's have been described. Resistance to the antifolates results from the markedly elevated levels of dihydrofolate reductase which these cells contain. The finding of these two phenomena, the HSR and the increase in activity of a specific enzyme, in the same cell suggests that they are functionally related to one another.

Evidence in support of the concept of gene amplification has come from studies of meiotic chromosomes in *Acheta*. It has been demonstrated that the increase in size of individual chromomeres in pachytene chromosomes of *Acheta* is associated with the amplification of a specific portion of the genome coding for ribosomal RNA (*11*). As meiosis proceeds, these extra DNA copies are released into the nuclear sap and the chromomere becomes reduced in size.

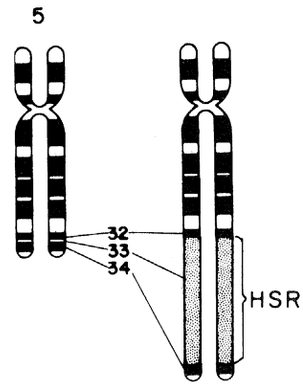

Fig. 2. Diagrammatic representation of a normal chromosome 5 and a chromosome 5 with the HSR, to show precise location of the HSR. The HSR is at band 5q33 [nomenclature as in (*9*)].

Chromomere formation and function are also postulated to be involved in the control of meiotic division in these cells (*11*). It is thus possible that amplification of chromomeres in pachytene chromosomes, "puffing" in polytene chromosomes, and the HSR in human neuroblastoma chromosomes are all phenomena of gene amplification and are critical to some specific cell function or functions (*12*).

Double minute chromosomes have been reported in a number of human tumors, mostly of neurogenic origin (for example, neuroblastomas and gliomas) (*1*). They have also been seen in spontaneous and induced tumors in animals. They may constitute heterogeneous aberrations, since in some lines they have been reported to be heterochromatic, while in others they have stained as euchromatin. It is likely, however, that the so-called double minute chromosomes which contain centromeres are actually small ring chromosomes. Because of this, it has been proposed that the term "double minutes" be restricted to those chromatin bodies which stain as euchromatin and lack centromeres (*13*).

Double minute chromosomes are acentric and, therefore, cannot be aligned on the mitotic spindle apparatus. They would eventually be lost from the cell unless they were able to self-replicate their DNA. There is no evidence for this capability. Thus, double minute chromosomes are probably maintained in a proliferating cell population by formation de novo from replicating chromosomes.

It is possible that double minute chromosomes are formed during the mitotic cycle from an unstable region of the chromosome with a concomitant reduction in chromosome size. If the HSR was the region of chromosome instability and was composed of amplified DNA, then the fragmentation of the HSR could result in the formation of double minute chromosomes and a morphologically normal chromosome by breakage and rejoining. Alternatively, an HSR might be formed when additional copies of a given gene or genes are built on a region of despiralized DNA in a particular chromosome. This is analogous to the mechanism proposed for gene amplification in *Acheta* (*11*). The extra DNA copies would then be released as double minute chromosomes, allowing the extended portion of the chromosome to compact and resulting in a morphologically normal chromosome. The independent segregation of HSR's and double minute chromosomes in CHP-126 and the finding of two normal No. 5 chromosomes in the cells which contain double minute chromosomes are consistent with either hypothesis.

It is not known whether double minute chromosomes have any function in the cell. Their association with tumors of neurogenic origin, especially neuroblastomas, and the propensity of neuroblastomas to regress spontaneously has led several investigators to propose that double minute chromosomes may inhibit the neoplastic process [Sandberg *et al.* (*1*)]. Alternatively, if the HSR represented the only chromosomal abnormality in the neoplastic cell, then the fragmentation of the HSR into double minute chromosomes followed by their eventual loss from the cell might be associated with the loss of the malignant phenotype in that cell.

Our findings confirm the initial report of HSR's in human neuroblastomas (*2*), and demonstrate the independent segregation of HSR's and double minute chromosomes in a neuroblastoma cell line (CHP-126). We suggest that the double minute chromosomes originate from preexisting chromosome material and that in CHP-126, at least, the specific chromosome segment involved is the HSR.

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14. The NMB line was kindly supplied by M. Goldstein of Washington University, St. Louis. We thank P. C. Nowell and W. J. Mellman for their critical reading of the manuscript. Supported by grants from the American Cancer Society (VC-189) and the National Institutes of Health (Pediatric Cancer Research Center grant to Children's Hospital of Philadelphia).

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Radioimmunoassay for Antibodies to Cytoplasmic Ribosomes in Human Serum

Abstract. *A radioimmunoassay for the detection of antibodies in human serum to tritium-labeled HeLa cell cytoplasmic ribosomes was developed with the use of Macaloid for the inhibition of endogenous ribonuclease activity. Antibodies were observed in the serum of patients with systemic lupus erythematosus in high incidence and titer. Patients with rheumatoid arthritis and chronic active hepatitis manifested a lower incidence and titer of antibodies to ribosomes, whereas serums from normal individuals and from patients with sarcoidosis, chronic glomerulonephritis, and malignant tumors showed no significant reactivity with cytoplasmic ribosomes. Maximum inhibition of the reaction was achieved with unlabeled HeLa cell ribosomes or rat liver ribosomes and partial inhibition by purified ribosomal RNA.*

Antibodies directed against cytoplasmic ribosomes have been observed in the serums of patients with systemic lupus erythematosus (SLE). A low incidence of antibodies has been detected (13 percent) by immunodiffusion (*1*) and a higher incidence of antibodies has been observed (25 to 50 percent) with bentonite flocculation (*2*) and fluorescent spot assays (*1*), whereas immunofluorescence studies with tissue sections demonstrated ribosomal antibodies in less than 1 percent of SLE serums (*3*). The specificity of the reaction for ribosomes appears to require both RNA and protein although the reactions of ribosomal antibodies demonstrable by immunofluorescence were not affected by treatment with ribonuclease. In addition, a soluble cytoplasmic ribonucleoprotein distinct from ribosomes has been described (*4*).

A radioimmunoassay provides a more sensitive and quantitative test for assaying ribosomal antibodies. Difficulties have been encountered in developing a radioimmunoassay because of the degradation of small quantities of labeled ribonucleoprotein by naturally occurring serum ribonuclease. We now report a radioimmunoassay procedure that has been adapted for the detection of antibodies in human serums reactive with labeled ribosomes derived from the cytoplasm of HeLa cells.

Tritiated ribosomes were obtained from HeLa cells grown for 24 hours in suspension medium containing ^{3}H-labeled uridine (1 μc/ml). Cytoplasm was obtained (*5*) and ribosomes were purified by discontinuous sucrose-gradient centrifugation (*6*). Ribosome preparations showed a specific activity of 12×10^3 to 20×10^3 count/min · μg with more than 97 percent of the radioactive material precipitable by trichloroacetic acid. Ribosomal RNA was prepared from unlabeled ribosomes by the sodium dodecyl sulfate–phenol procedure (*7*). Ribosomal portions retained activity at −70°C for more than 6 months and at 4°C for 10 to 14 days.

When human serum was incubated with labeled ribosomes at 37°C for 1 hour, more than 80 percent of the radioactivity became soluble in trichloroacetic acid. The ability of various inhibitors to inactivate serum ribonuclease activity as measured by trichloroacetic acid precipitability is shown in Table 1. Although rat liver cytoplasmic inhibitor prepared according to the procedure of Roth (*8*) was effective in preventing degradation of ribosomes, it was found to interfere with the Farr assay. Macaloid was an effective inhibitor that did not interfere with the radioimmunoassay procedure. Optimal conditions for the interaction of gamma globulin from human serum with labeled ribosomes were determined. Portions of serums from normal controls and patients were diluted with a buffer consisting of 0.15*M* NaCl and 0.2*M* sodium borate, *p*H 7.8, containing Macaloid (0.1 percent, weight to volume). The mixtures were incubated at 37°C in a gyratory water bath for 90 minutes, then cooled in ice and centrifuged at 8000*g* for 30 minutes. Samples (100 μl) of the supernatant were collected and mixed with 100 μl of borate-saline buffer containing ^{3}H-labeled ribosomes (6×10^3 to 8×10^3 count/min). The mixtures were incubated at 4°C overnight; 2 μl of 70 percent saturated ammonium sulfate were then added. After 1 hour at 4°C, the mixtures were centrifuged at 2000 rev/min for 1 hour, and the pellets were washed once with 2.5 ml of 35 percent saturated ammonium sulfate, dissolved in 1 ml of NCS solubilizer (Amersham/Searle, Arlington, Illinois) and counted in a Beckman scintillation counter. The percentage of precipitation of the ribosomes was calculated by dividing the pellet counts by the input counts. Under these conditions, the radioactivity not present in the pellet was detected in the supernatant and was precipitable by trichloroacetic acid.

Serums from normal individuals and from patients with active SLE and renal disease, active SLE but without renal disease, inactive SLE, rheumatoid arthritis, chronic active hepatitis, sarcoidosis, chronic glomerulonephritis, and malignant tumors were tested at 1:10 and 1:20 dilutions. The patients studied were receiving medical therapy at the Rockefeller University Hospital or at the University of Virginia Hospital. Patient selection was based on (i) fulfillment of the preliminary criteria of the American Rheumatism Association for classification as SLE (*9*) and (ii) presence of anti-

Table 1. Effect of ribonuclease inhibitors on interaction of serum ribonuclease and ^{3}H-labeled ribosomes. The result is expressed as the percent of radioactivity present in the fraction precipitated by trichloroacetic acid.

HeLa cell [^{3}H]ribosomes	Radioactivity precipitable (%)
Alone	> 97
+ serum	20
+ serum + heparin	60
+ serum + RLCI*	> 97
+ serum + Macaloid	95

*Rat liver cytoplasmic inhibitor.

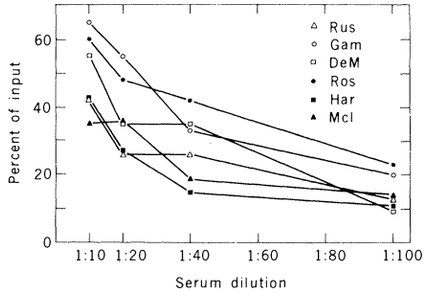

Fig. 1. Inhibition of precipitation of labeled ribosomes; 100 μl of a dilution of serum is added to 100 μl of ^{3}H-labeled HeLa cell cytoplasmic ribosomes.