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Chromosomal alterations in cell lines derived from mouse rhabdomyosarcomas induced by crystalline nickel sulfide

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Summary. Prior studies have shown a preferential decondensation (or fragmentation) of the heterochromatic long arm of the X chromosome of Chinese hamster ovary cells when treated with carcinogenic crystalline NiS particles (crNiS). In this report, we show that the heterochromatic regions of mouse chromosomes are also more frequently involved in aberrations than euchromatic regions, although the heterochromatin in mouse cells is restricted to centromeric regions. We also present the karyotypic analyses of four cell lines derived from tumors induced by leg muscle injections of crystalline nickel sulfide which have been analyzed to determine whether heterochromatic chromosomal regions are preferentially altered in the transformed genotypes. Common to all cell lines was the presence of minichromosomes, which are acrocentric chromosomes smaller than chromosome 19, normally the smallest chromosome of the mouse karyotype. The minichromosomes were present in a majority of cells of each line although the morphology of this extra chromosome varied significantly among the cell lines. C-banding revealed the presence of centromeric DNA and thus these minichromosomes may be the result of chromosome breaks at or near the centromere. In three of the four lines a marker chromosome could be identified as a rearrangement between two chromosomes. In the fourth cell line a rearranged chromosome was present in only 15% of the cells and was not studied in detail. One of the three major marker chromosomes resulted from a centromeric fusion of chromosome 4 while another appeared to be an interchange involving the centromere of chromosome 2 and possibly the telomeric region of chromosome 17. The third marker chromosome involves a rearrangement between chromosome 4 near the telomeric region and what appears to be the centromeric region of chromosome 19. Thus, in these three major marker chromosomes centromeric heterochromatic DNA is clearly implicated in two of the rearrangements and less clearly in the third. The involvement of centromeric DNA in the formation of even two of four markers is consistent with the previously observed preference in the site of action of crNiS for heterochromatic DNA during the early stages of carcinogenesis.

Key words: Nickel tumorigenesis — Karyotic changes — Chromosomal aberrations

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Introduction

Extensive karyotypic alterations have been described for most types of human neoplasia, particularly for solid tumors. Although some chromosomal alterations have been causally related to a particular abnormality, such as the Philadelphia chromosome in chronic myelogenous leukemia (Nowell and Hungerford 1960; Rowley 1973) or the 8:14 translocation in Burkitt's lymphoma (Dalla-Favera et al. 1982), the basis for the majority of chromosomal changes in transformed cells is not clear. While some specificity might be expected for tissue type, attempts at classification along these lines have not provided unifying guidelines since tumors of a given type frequently do not have the same cytogenetic change (Nowell 1980). Studies of cytogenetic changes associated with human neoplasia are further complicated by the extensive changes in chromosomes that occur during tumor progression; therefore animal stud-

ies are needed in which early consistent chromosomal changes can be identified following specific carcinogen treatments.

Carcinogens of diverse chemical types are expected to produce different lesions in DNA; several studies have indicated that the nature of the carcinogen may be a factor in some of the chromosomal changes associated with tumorigenesis (Mitelman 1980). Tumors induced in rats by dimethylbenzanthracene (DMBA) had a specific karyotypic alteration involving chromosome A-2 (Levan et al. 1974). In leukemias induced by DMBA there was also a consistent effect on chromosome A-2, typically trisomy (Kurita et al. 1968). A less consistent involvement for the A-2 chromosome was found in rat sarcomas induced by 3-methylcholanthrene or benzo[*a*]pyrene (Levan and Levan 1975). It is thus possible that the mechanism of action of a carcinogen and, specifically, the type of initiating DNA lesion that it produces will be reflected in early chromosomal changes associated with tumorigenesis.

For the carcinogenic metals Ni(II) and Cr(VI) the lesions induced in DNA are less well defined than for polycyclic aromatic hydrocarbons, but DNA-protein complexes as well as single-strand breaks are induced by both metals (Christie et al. 1984; Ciccarelli et al. 1981). Carcinogenic nickel compounds have been shown to produce chromosomal aberrations including gaps, breaks, and exchanges (Sen and Costa 1985). Crystalline nickel sulfide (crNiS), a known human and animal carcinogen, causes preferential damage in heterochromatic regions of chromosomes of Chinese hamster ovary (CHO) cells (Sen and Costa 1985) or of mouse cells (Sen et al. 1987). In CHO cells, Ni(II) caused a preferential decondensation of the heterochromatic arm of the X chromosome, while in the mouse cell line C3H 10T1/2 Robertsonian fusions and other exchanges involving the centromere were the dominant aberrations observed. In the mouse all the chromosomes are acrocentric and the heterochromatic chromosomal regions are located exclusively in the centromeres. In the present study, we have investigated whether the specificity observed for the formation of aberrations by crNiS is maintained in karyotypic changes persistent after in vivo tumorigenesis. Intramuscular injections of crNiS produced predominantly rhabdomyosarcomas from which cell lines were initiated and used for chromosomal analysis. We present evidence that nickel carcinogenesis is associated with chromosomal aberrations involving heterochromatin and, further, that the preferential effect of crNiS on heterochromatin may be conserved during tumorigenesis.

Materials and methods

Induction of tumors by crNiS and derivation of cell lines. Two groups of female mice were injected intramuscularly with 5 mg crNiS as previously described (Pellis and Kahan 1976). All mice displayed initial inflammatory nodules at the site of injection which persisted for approximately 90 days and then subsided to a palpable 2 mm in diameter. By 200 days the tumor incidence was nearly 50% and at 9 months was 75%. Cell lines were derived from each tumor and assigned separate letters following the carcinogen designation, e.g., NS-A. These cell lines were kindly provided by Dr. Neal Pellis.

Chromosomal banding. Each cell line was karyotyped by its chromosomal Giemsa banding properties (Cowell 1984). Mitotic cells were collected following 1.5 h of Colcemid treatment (0.02 μg/ml). Cells were treated with a KCl hypotonic solution (0.56%) before fixation in methanol/acetic acid (3:1). Banding was performed on slides in the 2nd or 3rd week after preparation by the modified technique of Seabright (1971). Chromosomes were banded by trypsin treatment (0.25%) for 0.25–3 min at room temperature followed by an alcohol rinse and equilibration in water. Slides were stained with Giemsa for 5 min. Centromeric banding was performed by the barium hydroxide method of Sumner (1972). Freshly prepared slides were treated with 0.2 M HCl for 1 h at room temperature followed by a rinse with distilled water and a 5- to 15-min treatment with 5% aqueous barium hydroxide octahydrate at 50° C. Slides were then incubated in double the concentration of standard saline citrate (2XSSC) at 60° C for 1 h, rinsed in distilled water and stained for 5 min with Giemsa.

Results

Induction of chromosome aberrations by crNiS in the C3H 10T1/2 mouse cell line

Cultures were treated with crNiS for either 6 h or 24 h and examined for aberrations 24 h after removal of the nickel compound. The recovery period of 24 h in the absence of crNiS was essential for the attainment of mitoses because of the blockage of the cell cycle produced by Ni(II) (Costa et al. 1982). Table 1 shows the yields of aberrations in heterochromatin found in the centromeres and in the euchromatic arms. Since the heterochromatin in these cells comprises approximately 14% of the total chromosomal length, damage to these areas should not exceed that amount if an agent damages the genome randomly. For all three concentrations of crNiS used, the number of aberrations associated with the centromere was higher than the expected value of 14% of the total aberrations. The level of aberra-

Table 1. Chromosomal aberrations induced by crystalline NiS in C3H10T1/2 cells

Treatment time (h)[a]	NiS (μg/ml)	Number of cells		Heterochromatic aberrations[b]			Euchromatic aberrations[c]	Total aberrations
		Scored	With aberrations	Fusion	Exchange	Break		
Untreated		95	4	1	—	—	7	8
6	2.5	96	10	8	2	—	8	18
	5.0	128	30	26	8	—	17	41
	10.0	125	30	32	18	1	9	60
24	2.5	117	13	4	2	1	7	14
	5.0	96	17	6	2	3	9	20
	10.0	80	19	14	10	2	8	34

[a] Cells were treated as indicated in the table and then incubated in metal-free medium for 24 h. Mitotic cells were prepared as described in Materials and methods. The results presented are similar to three other experiments of similar design

[b] Fusions refer to centromeric exchanges and exchanges to dicentrics and exchanges refer to events when only one centromere was involved

[c] Euchromatic aberrations included exchanges and breaks

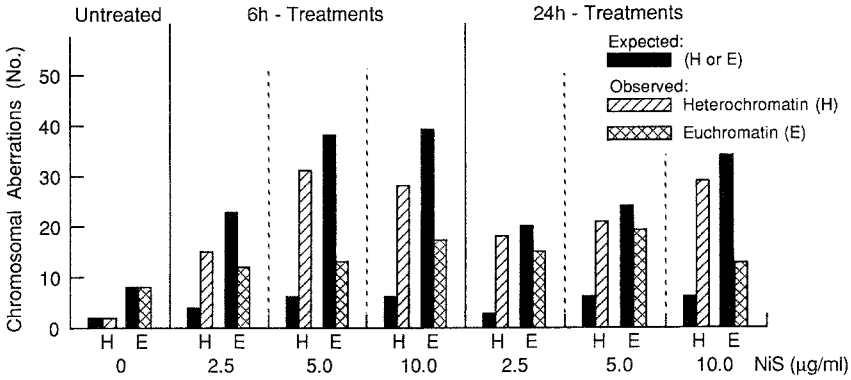

Fig. 1. Chromosome aberrations induced by crNiS in C3H 10T1/2 cells. Cell monolayers were treated with crNiS for the times indicated and at the indicated concentrations of the Ni(II) compound. Following treatment, cells were placed in fresh medium without Ni(II) for 24 h before addition of colcemid for collection of mitotic figures

tions in heterochromatin was always 50% of the total or greater. Although chromosomal breaks and exchanges did occur in the euchromatic arms, the alterations in these areas were reduced with increasing concentrations of crNiS compared to those in heterochromatin. For example, in the 6-h treatment of crNiS at 10 μg/ml, there were 85.0% of the aberrations found at the centromere compared to 15.0% along the arms. To emphasize this point, we have plotted (Fig. 1) the total aberration yield in heterochromatin and euchromatin adjacent to an expected yield of aberrations in each type of chromatin based on the amount of each type of chromatin. For every treatment of NiS tested, the observed aberration yield in heterochromatin exceeds the calculated expected value.

The strong preference for aberration formation in heterochromatic regions exhibited by crNiS prompted us to examine the type of chromosomal alterations associated with tumorigenesis by this compound. In this study tumors were induced in C3H mice by intramuscular injections of crNiS and cell lines were originated from the rhabdomyosarcomas produced (Christie, unpublished observations). The karyotypes examined were made at the third to the sixth passage to avoid chromosomal changes related to prolonged cell culture.

General characteristics of the NS cell lines

Three of the four cell lines studied were near-diploid with model numbers of 42 chromosomes (NS-A), 44 chromosomes (NS-E), and 39 chromosomes (NS-G). The fourth cell line was near-tetraploid with a modal number of 79 (NS-D). In Fig. 2 a karyotype of the NS-A cell line is shown. All four cell lines were characterized by the presence

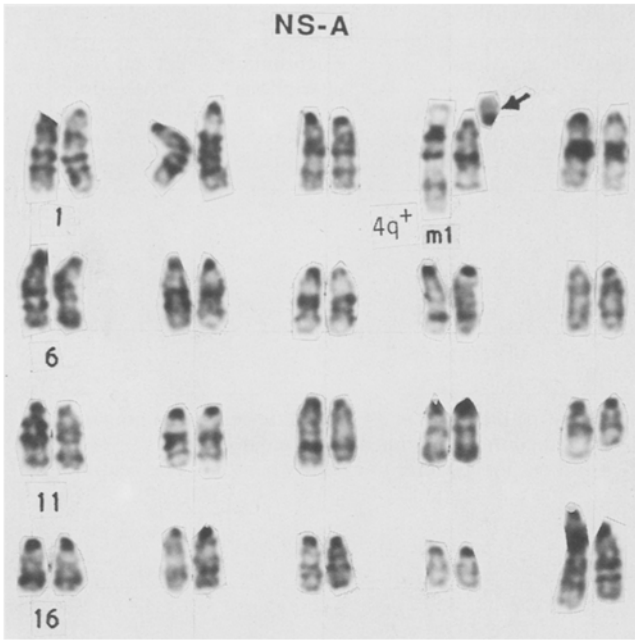

Fig. 2. A representative karyotype of the NS-A cell line

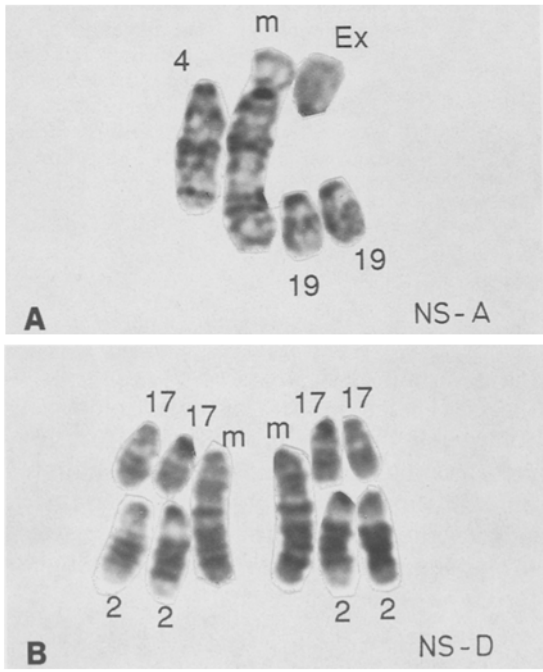

Fig. 3A, B. A composite analysis of the marker chromosomes of the NS-A and NS-D cell lines. The marker chromosomes (m) from the NS-A cell line **(A)** and from the NS-D cell line **(B)** have been taken from one cell of the respective cell lines. Also taken from the same cell are the chromosomes presumed to be the chromosomes from which the marker chromosomes were derived. The numbers of these chromosomes are indicated. Also for the NS-A cell line there is a minichromosome indicated as an extra (Ex) chromosome not representing a recognizable portion of a chromosome of the normal karyotype

of an extra small acrocentric chromosome not normally found in the mouse karyotype (see arrow in Fig. 2). Three of the cell lines had a single marker chromosome, consisting of a rearrangement of at least two chromosomes, that was present in at least 85% of the cells of the respective cell lines.

Composition of the marker chromosomes from the NS cell lines

The marker chromosomes from two NS cell lines are shown in Fig. 3. In two of the cell lines, NS-A and NS-E, the marker chromosomes were derived from chromosome 4 although the rearrangement in each case was quite different. In the NS-E marker, a Robersonian translocation of two chromosomes 4 is the only apparent event leading to this variant chromosome. In contrast, at least two events were responsible for the formation of the marker in the NS-A cell line. At the distal end of the NS-A marker chromosome there is extra chromatin material not typical of chromosome 4. We have tentatively identified this material as being derived from chromosome 19. Also, at the proximal end of chromosome 4 a centromeric fusion has occurred between chromosome 4 and a minichromosome. The minichromosome is an aberrant chromosome found in cell lines from all the rhabdomyosarcomas that we have examined, including ones induced by 3-methylcholanthrene (Christie, unpublished observations). Minichromosomes will be discussed further in the section below. When the NS-A cell line was initially karyotyped at passages 3–6, approximately 70% of the cells contained an acrocentric marker chromosome which did not have the minichromosome fused at the centromere. The remainder of the cells contained the submetacentric marker chromosome as shown in Fig. 3A. When karyotypes were analyzed from this cell line after approximately three months in culture, there were no cells with acrocentric marker chromosomes but only cells with the submetacentric marker. The order of events producing this marker can be surmised from these results. The fusion of the telomeric end of chromosome 4 with the centromere of a small chromosome or a broken chromosome must have preceded the centromeric fusion between chromosome 4 and the minichromosome. The increase in the frequency of the submetacentric marker chromosome suggests that a growth advantage was derived following fusion of the minichromosome.

The marker chromosome found in the NS-D cell line is shown in Fig. 3B in two copies, since both markers were found in a single cell. This was commonly observed in this cell line, presumably because the cell line was predominantly tetraploid. Only a small percentage (less than 10%) of the tetraploid cells had lost one of the marker chromosomes. The rearrangement producing this marker involves chromosome 2, an easily identifiable chromosome, and apparently occurred in a partial or very near the centromere and resulted loss of the chromosome 2 centromere. The chromosome that has been rearranged with this chromosome is tentatively identified as chromosome 17. This is based on the presence of the band that occurs immediately beneath the centromere of the marker chromosome. According to Cowell (1984), the presence of a band at this position is not always observed in chromosome 17, but its presence provides a strong positive indicator for chromosome 17. In the two marker chromosomes shown, only the one displayed on the left has a clear subcentromeric band characteristic of chromosome 17.

In summary, for all three marker chromosomes examined, rearrangements of centromeric regions were involved. These included the centromeric fusions involving chromosome 4 in NS-A and NS-E and a rearrangement involving chromosome 2 in NS-D. Evidence was also presented for the involvement of two telomeric regions: the telomere of chromosome 4 with a centromere of chromosome 19 (NS-A) and the telomere of chromosome 17 with a centromere of chromosome 2 (NS-D).

The presence of extra acrocentric chromosomes in NS cell lines

In each of the four cell lines one or more extra acrocentric chromosomes was observed, which we have referred to as minichromosomes. In all cases the minichromosomes were smaller than the chromosomes 19 from the same cell, as shown in Fig. 4. All of the chromosomes 19 and all of the minichromosomes from each of two cells for all four cell lines are displayed. In most cases the struc-

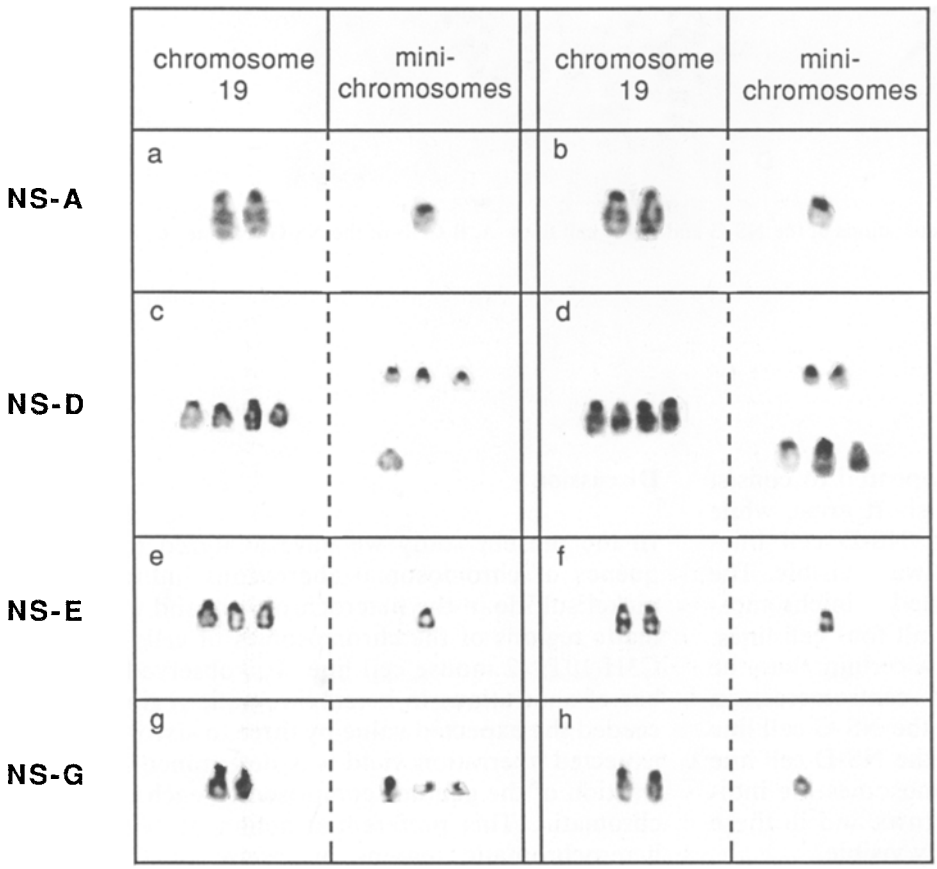

Fig. 4. The minichromosomes of the NS cell lines. For two cells from each cell line we have displayed all minichromosomes in each cell with both of the chromosomes 19 of the respective cell; NS-A *(a, b);* NS-D *(c, d);* NS-E *(e, f);* and NS-G *(g, h)*

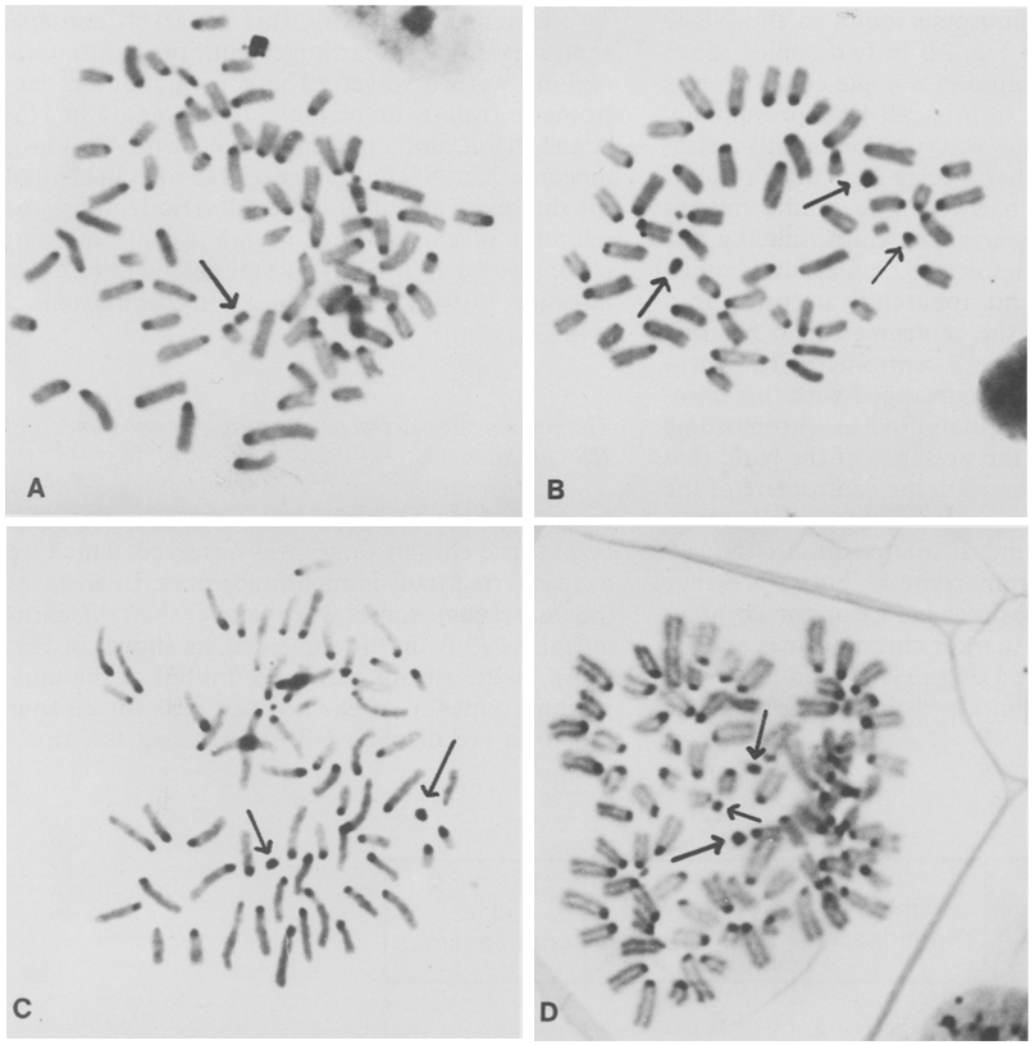

Fig. 5A–D. C-banded chromosomal preparations of the NS-G and NS-D cell lines. **A, B** Cells of the NS-G cell line; **C, D** cells of the NS-D cell line

ture of the minichromosome appeared to consist of a centromere and two very short arms, while occasionally in the NS-G and NS-D cell lines chromosomal arms were not always visible. The frequency of cells that contained minichromosomes ranged from 85–100% for all four cell lines. C-banding was performed to ascertain that the minichromosomes did contain centromeres. In Fig. 5 are shown two cells from the NS-G cell line (Fig. 5A, B) and two cells from the NS-D cell line (Fig. 5C, D). Several minichromosomes are indicated by the position of the arrows and in these instances centromeres are clearly visible.

Discussion

In the present study we have analyzed the frequency of chromosomal aberrations induced by nickel sulfide in the heterochromatic and euchromatic regions of the chromosomes of cells of the C3H 10T1/2 mouse cell line. The observed number of aberrations in heterochromatic regions exceeded the expected value by three to sixfold. The expected aberration yield was determined by the fraction of the genome comprised by each type of chromatin. This preferential action of Ni(II) in heterochromatic regions of mouse or Chinese

hamster chromosomes can be contrasted to results with Cr(VI) which showed random chromosome damage (Sen et al. 1987). The specificity of this effect is not typical of clastogens and therefore may provide an important clue to help understand the basis of the carcinogenicity of this metal ion.

Crystalline nickel sulfide has been clearly established as a human carcinogen by epidemiological studies, although at the present time there is little definitive knowledge concerning the mechanism of action of this compound. The unique nature of this carcinogen is illustrated by a weak or non-existent mutagenic response in bacterial or mammalian cells leading many investigators to rank this ion as a nonmutagen (Christie and Tummolo 1987; Hartwig and Beyersmann 1987; Heck and Costa 1982). These results, coupled with our previous finding that Ni(II) preferentially decondenses the heterochromatic X chromosome of Chinese hamster cells (Sen and Costa 1985), led to an examination of the tumors induced by Ni(II) to discover whether the acute damage occurring in heterochromatin persisted in the tumor cells.

We present the detailed analysis of three cell lines derived from tumors induced by nickel sulfide in which each cell line is characterized by a marker chromosome present in the majority of the cells. Each marker chromosome shows some involvement of centromeric heterochromatin in its formation. Since these three cell lines were selected at random, this finding supports the possible significance of heterochromatin in nickel carcinogenesis. In the fourth NS cell line analyzed a marker chromosome was present but at a much lower frequency. For comparison we also selected at random three cell lines derived in a similar manner by methylcholanthrene (MCA) (Christie et al., unpublished observations). There were no rearranged marker chromosomes in any of these cell lines. This result suggests that there is a greater probability for retention of rearranged marker chromosomes in the NS cell lines than in the MCA cell lines, particularly those involving heterochromatin. Definitive proof of this will require that greater numbers of tumors or clones of in vitro transformed cells be examined. The detailed analyses of marker chromosomes presented here have provided the impetus in our laboratory for further studies concerning this phenomenon.

Two types of heterochromatin have been described based upon their transcriptional activity. Constitutive heterochromatin has been considered to be permanently genetically inactive while facultative heterochromatin can exhibit some transcription. Recent studies, however, have demonstrated that constitutive heterochromatin can become transcriptionally active (Sperling et al. 1987). In addition to repetitive DNA, protein-coding sequences are also found in heterochromatin. It is possible that nickel carcinogenesis may be involved with activation of heterochromatin gene transcription. There are many lines of evidence pointing to nickel interaction with heterochromatin as an important step in its carcinogenesis and further work will focus on understanding this relationship.

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