Reports

Tumor Cells Exhibit Deregulation of the Cell Cycle Histone Gene Promoter Factor HiNF-D

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Cell cycle–regulated gene expression is essential for normal cell growth and development and loss of stringent growth control is associated with the acquisition of the transformed phenotype. The selective synthesis of histone proteins during the S phase of the cell cycle is required to render cells competent for the ordered packaging of replicating DNA into chromatin. Regulation of H4 histone gene transcription requires the proliferation-specific promoter binding factor HiNF-D. In normal diploid cells, HiNF-D binding activity is regulated during the cell cycle; nuclear protein extracts prepared from normal cells in S phase contain distinct and measurable HiNF-D binding activity, while this activity is barely detectable in G1 phase cells. In contrast, in tumor-derived or transformed cell lines, HiNF-D binding activity is constitutively elevated throughout the cell cycle and declines only with the onset of differentiation. The change from cell cycle–mediated to constitutive interaction of HiNF-D with the promoter of a cell growth–controlled gene is consistent with, and may be functionally related to, the loss of stringent cell growth regulation associated with neoplastic transformation.

THE HUMAN H4 HISTONE GENE (FO108) proximal promoter contains two in vivo protein binding domains, sites I and II. These sites have been defined at single-nucleotide resolution, with the protein-DNA contacts confirmed by in vivo deoxyribonuclease (DNase) I protection analysis (*1*) and native genomic blotting (*2*). In vitro, the site I and II sequences form specific protein-DNA complexes with at least four distinct nuclear factors (HiNF-A, HiNF-C, HiNF-D, and HiNF-E), as shown by deletion analysis, DNase I footprinting, and dimethylsulfate fingerprinting (*3–5*). HiNF-A, HiNF-C, and HiNF-E bind independently to the distally located site I and are present in both actively proliferating and differentiating cells (*3, 5*) (Fig. 1A). Deletion of site I results in a four- to sixfold reduction in H4 histone gene transcription in vitro (*3*), suggesting that the interactions of these nuclear factors with site I have an auxiliary role in augmenting the H4 histone gene transcription rate.

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HiNF-D interacts with a highly conserved histone-specific element (Fig. 2) located in the distal part of site II (*3*). This protein-DNA interaction has been implicated as having an essential function in H4 histone gene transcription because deletion of the HiNF-D binding site abolishes expression in vivo (*6*). Furthermore, the HiNF-D–site II interaction is proliferation-specific. When HL60 promyelocytic leukemia cells are induced to differentiate into monocytes, the downregulation of H4 histone gene transcription at the completion of the proliferative phase is accompanied by a selective loss of interaction of HiNF-D with site II, as demonstrated both in vivo and in vitro (*7*) (Fig. 1A). A similar observation has been made in primary cultures of diploid rat calvarial osteoblasts at a transition point in their developmental sequence when proliferation and expression of cell growth–related genes are downregulated, and initiation of tissue-specific gene expression characteristic of the bone cell phenotype occurs (*8*) (Fig. 1A). Hence, the loss of the HiNF-D–site II interaction in the proximal promoter of the H4 histone gene may represent an important event in the process whereby proliferation ceases and the genes encoding phenotypic markers of differentiated cells are progressively expressed.

Because the occupancy of site II by HiNF-D appears to be essential for H4 histone gene expression, we reasoned that this protein-DNA interaction could be a rate-limiting step for the cell cycle–regulated expression of this gene. We have reported previously that in human HeLa S3 cells, the in vivo protein-DNA interactions at sites I and II of H3 and H4 histone genes persist throughout the cell cycle (*1, 9*) and that the binding activities of HiNF-A, HiNF-C, and HiNF-D are present during all phases of the cell cycle (*3*) (Fig. 1B). HeLa cells are continuously proliferating heteroploid cells derived from tumors and have lost the potential to differentiate. Therefore, we examined the HiNF-D–site II interaction during the cell cycle of diploid cell types in which normal cell growth control mechanisms are operative.

Initially, the site II binding activity of HiNF-D was monitored during the cell cycle of primary rat calvarial osteoblasts. Actively growing osteoblasts were synchronized by two cycles of 2 m*M* thymidine block, resulting in the accumulation of cells at the boundary between G1 and S phases of the cell cycle. After release from the second thymidine block, the cells progressed synchronously through the cell cycle, as reflected by DNA synthesis [monitored by pulse labeling with [^{3}H]thymidine and assayed by both in situ autoradiography and determination of trichloroacetic acid (TCA) precipitable radioactivity] and mitotic activity (Fig. 3). At intervals during the cell cycle, cytoplasmic RNA was analyzed for the presence of H4 histone mRNA and nuclear protein extracts were assayed for HiNF-D–site II binding activity by gel retardation assay.

Rat osteoblast cells actively engaged in DNA synthesis (S phase cells) showed ten times as much H4 histone mRNA as prerelease cells (those not released from the second thymidine block) or cells in the G1 phase, reflecting at a molecular level the high degree of synchrony obtained (Fig. 4). When nuclear extracts prepared from these cells were analyzed in gel retardation assays, a striking cell cycle–dependent alteration in HiNF-D binding activity became evident. While HiNF-D binding activity in S phase nuclear extracts is abundant, it is barely detectable in extracts prepared from prerelease cells and cells in the G1 phase of the cell cycle (Fig. 5A).

To determine whether the cell cycle–dependent alteration in HiNF-D binding activity is directly coupled to the process of DNA replication, we inhibited DNA synthesis in S phase rat osteoblast cells by treatment with hydroxyurea (1 m*M*) for 1 hour or 8 hours, after which nuclear protein extracts were prepared and analyzed for

HiNF-D binding activity. The HiNF-D binding activity persists after both short-term (1 hour) and long-term (8 hours) inhibition of DNA synthesis (Fig. 5A), suggesting that this activity is upregulated at the initiation of DNA replication and is not downregulated until the completion of this process.

In a second normal diploid cell type, WI-38 human fetal lung fibroblasts, we found a similar cell cycle–dependent fluctuation in HiNF-D binding activity. WI-38 cells were synchronized by either two cycles of 1 m*M* hydroxyurea block or two cycles of 2 m*M* thymidine block and monitored by in situ autoradiography of cells labeled with [^{3}H]thymidine for 1 hour. Both synchrony methods gave similar results: high levels of HiNF-D binding activity in nuclear extracts from S phase cells and barely detectable levels in G1 phase extracts (Fig. 5B). Also, in serum-stimulated cultures of quiescent diploid CF3 fibroblasts of human foreskin, high levels of HiNF-D binding activity were found exclusively in S phase cells (*10*).

Because HiNF-D binding activity appeared to vary in a cell cycle–dependent manner in normal diploid cell types, but was constitutively elevated in a tumor-derived heteroploid cell, we explored the possibility that this promoter factor is deregulated in tumor cells. Further synchrony experiments were performed with ROS 17/2.8 rat osteosarcoma cells and SV40-transformed WI-38 cells, the transformed counterparts of the normal diploid rat osteoblasts and human WI-38 cells. Nuclear extracts were prepared from these cell lines during the G1 and S phases of the cell cycle and analyzed for HiNF-D binding activity. Results were compared with those obtained from synchronized cultures of the two normal diploid cell types.

Synchronization of the rat osteosarcoma cells and SV40-transformed WI-38 cells required adaptations of the synchronization procedures due to the relatively short cell cycle times as compared to normal cell types (~16 hours versus 24 hours). Rat osteosarcoma cells were synchronized by three short cycles of 2 m*M* thymidine block. After release from the third block, cell cycle progression was monitored by [^{3}H]thymidine labeling for 1 hour and found to be highly synchronous. As was observed in the synchronized osteoblasts, osteosarcoma cells in S phase showed ten times more H4 histone mRNA than pre-release cells or cells in G1 (Fig. 4). In an alternative synchronization procedure, both rat osteosarcoma cells and SV40-transformed WI-38 cells were arrested in early mitosis by a 16-hour exposure at 0.1 μg/ml to nocodazole, a reversible inhibitor of microtubule polymerization (*11*). In both cell types, mitosis was completed within 2 hours after release from this mitotic block, and cells entered S phase after an additional 5 hours, as indicated by the pattern of [^{3}H]thymidine incorporation into DNA (Fig. 6A).

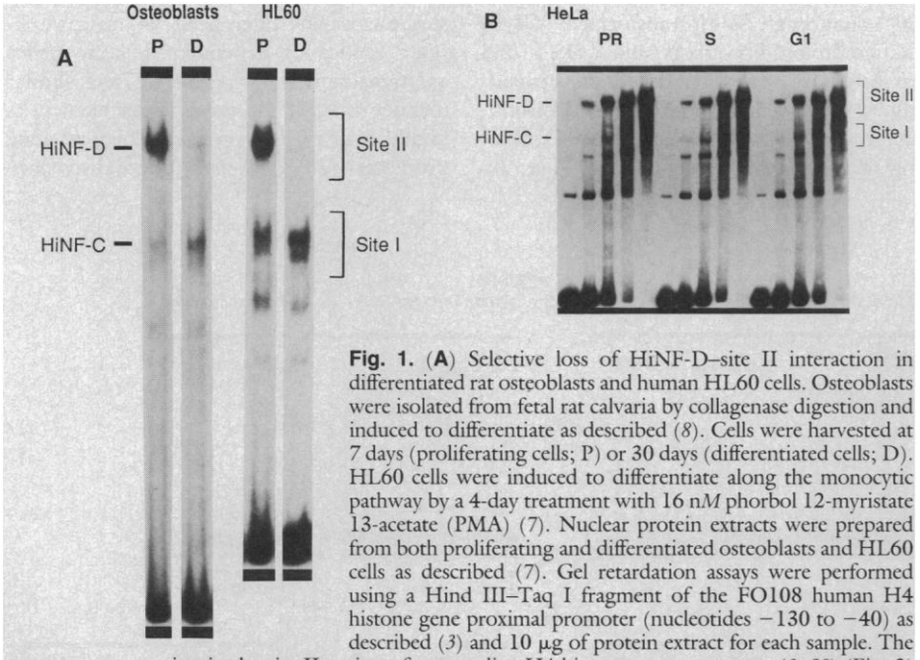

Fig. 1. (**A**) Selective loss of HiNF-D–site II interaction in differentiated rat osteoblasts and human HL60 cells. Osteoblasts were isolated from fetal rat calvaria by collagenase digestion and induced to differentiate as described (*8*). Cells were harvested at 7 days (proliferating cells; P) or 30 days (differentiated cells; D). HL60 cells were induced to differentiate along the monocytic pathway by a 4-day treatment with 16 n*M* phorbol 12-myristate 13-acetate (PMA) (*7*). Nuclear protein extracts were prepared from both proliferating and differentiated osteoblasts and HL60 cells as described (*7*). Gel retardation assays were performed using a Hind III–Taq I fragment of the FO108 human H4 histone gene proximal promoter (nucleotides −130 to −40) as described (*3*) and 10 μg of protein extract for each sample. The sequence conservation in the site II region of mammalian H4 histone gene promoters (*3, 29*) (Fig. 2) enabled us to use a fragment of the extensively characterized human gene assay for rat HiNF-D binding activity. Specificity of the interaction between the rat HiNF-D promoter factor and the human H4 site II probe was verified by competition studies using oligonucleotides specific to human site II (data not shown). (**B**) Constitutive HiNF-D–site II interaction during the cell cycle of human HeLa S3 cells. Cells were synchronized with respect to the cell cycle by double thymidine block (*30*). Nuclear protein extracts were prepared from cells prior to release from the second thymidine block (PR), during S phase (S; 4 hours after release), and during G1 phase (G1; 10 hours after release). Nuclear protein extracts were assayed for HiNF-D–site II interactions as described above except the lanes for each cell cycle phase contained, respectively, 2, 4, 6, 9, or 12 μg of protein.

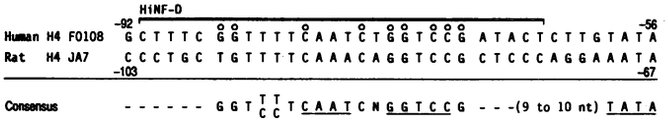

Fig. 2. Sequence homology between regions immediately upstream of the TATA boxes in the proximal promoters of the human H4 gene FO108 (*1*) and rat H4 gene (*31*), coinciding with the binding site of HiNF-D in the distal portion of site II. DNase I–protected domain of the HiNF-D binding site (*3*) is indicated by a bracket. Specific guanine-protein contacts determined in vivo are indicated by open circles (*1*).

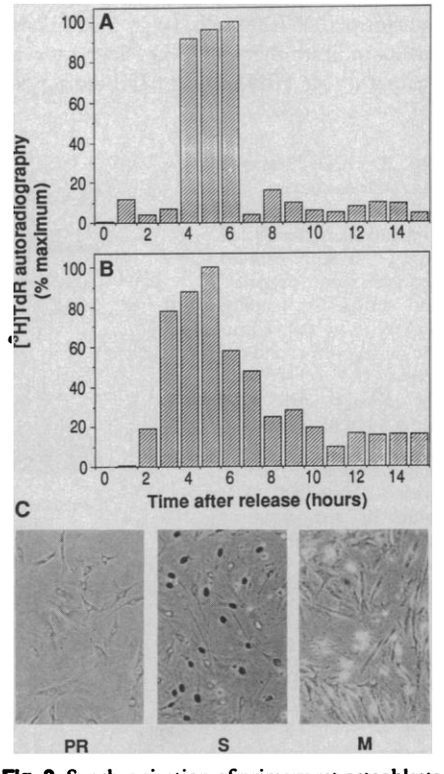

Fig. 3. Synchronization of primary rat osteoblasts by two cycles of thymidine block monitored by pulse labeling with [^{3}H]thymidine and analyzed by (**A**) determination of TCA-precipitable radioactivity and (**B**) in situ autoradiography. (**C**) Photomicrographs of in situ autoradiographed cells were taken at several time points during the cell cycle. Pre-release cells (PR) were not released from the second thymidine block. Number of cells undergoing DNA synthesis peaked at 5 hours after release (S), and mitotic activity was prominent 9 hours after release from the second block (M).

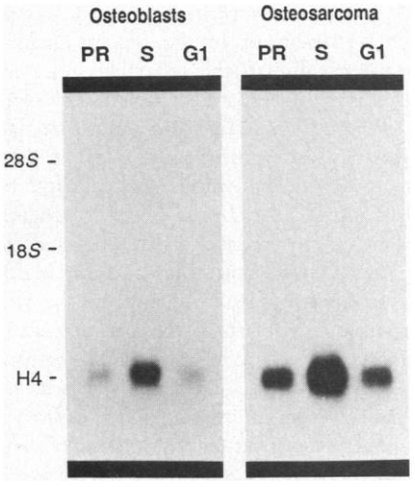

Fig. 4. Northern hybridization analysis of H4 histone mRNA following cell cycle synchronization of primary rat osteoblasts and ROS 17/2.8 rat osteosarcoma cells. Rat osteoblasts were synchronized by double thymidine block and ROS 17/2.8 cells were synchronized by three rounds of 2 m*M* thymidine block (10-hour block; 9-hour release). Total cytoplasmic RNA was isolated from cells prior to release from the final thymidine block (PR) and during S and G1 phases (*32*). For each sample, RNA (20 μg) was fractionated in a 1.2% agarose gel containing 6.6% formaldehyde, transferred (*33*) to Zeta Probe membrane (Bio-Rad, Richmond, California), and hybridized (*34*) using a ^{32}P-labeled 440-bp Hind III fragment of a rat H4 histone gene (pPS2) (*35*) as a probe.

High levels of HiNF-D binding activity were detected in all nuclear extracts derived from pre-release, S phase, and G1 phase rat osteosarcoma cells whether these cells had been synchronized by thymidine block or by mitotic arrest (Fig. 5C). Similarly, no difference between G1 and S phase was detectable in the high levels of HiNF-D binding activity in nuclear extracts prepared from SV40-transformed WI-38 cells (Fig. 5D). These results indicate that the cell cycle–regulated fluctuation in HiNF-D–site II binding activity that is observed in normal diploid cells is absent in both tumor cell lines. In another tumor-derived cell line, HL60 human promyelocytic leukemia cells, we also found constitutive elevation of HiNF-D binding activity during the cell cycle (Fig. 5E). In contrast to the other tumor and transformed cell lines we examined (HeLa human cervical carcinoma, SV40-transformed WI-38 human diploid fibroblasts, and ROS 17/2.8 rat osteosarcoma), HL60 cells have maintained the potential to terminally differentiate, a process during which HiNF-D binding activity is selectively lost (*7*) (Fig. 1). Thus, the absence of a cell cycle–dependent fluctuation in the HiNF-D–site II interaction in tumor cells appears to be a consequence of aberrant cellular growth control, rather than the loss (or preservation) of competency to differentiate.

The modular organization of mammalian histone gene promoters is reflected by multiple regulatory elements (*6, 12–16*) interacting with a series of sequence-specific promoter factors (*3, 17–22*) that together influence fidelity and levels of transcription. The binding activity of several histone gene promoter factors has been studied as a function of the cell cycle (*3, 4, 16, 21, 23–25*). Contrasting results were obtained with regard to the cell cycle dependency of the binding activity of the ubiquitous H2B-related octamer binding factor OTF-1. This activity was found to be cell cycle–regulated in Chinese hamster lung fibroblasts (*23*), but not in transformed chicken erythroid cells (*21*) or human HeLa S3 cells (*24*).

These results most likely do not reflect species-specific differences, because vertebrate replication-dependent histone genes are transcriptionally regulated in a similar manner (*26–28*) and the octamer element is highly conserved in vertebrate histone gene promoters (*29*). Moreover, these discrepan-

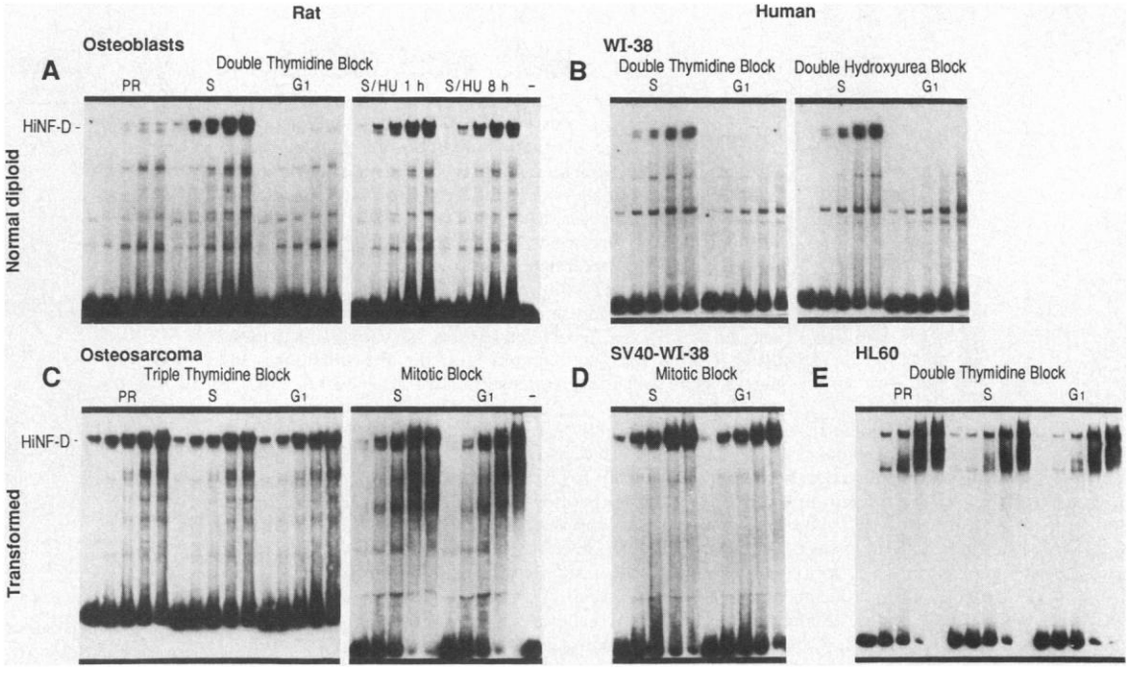

Fig. 5. HiNF-D–site II interactions during the cell cycles of normal diploid and transformed cell types. Nuclear protein extracts were prepared and HiNF-D binding activity was assayed in the rat osteoblast and osteosarcoma cells using the 195-bp Aha III–Hinf I fragment of a rat H4 histone gene proximal promoter (nucleotides +7 to −188) (*31*), which is analogous to the human FO108 site II region. In the human cell lines, HiNF-D binding activity was assayed as described in the legend to Fig. 1. For each cell cycle phase, the lanes contained, respectively, 2, 4, 6, 9, or 12 μg of protein. (**A**) Primary rat osteoblasts synchronized by double thymidine block (15-hour block; 9-hour release) and harvested prior to release from the second thymidine block (PR), 4 hours after release (S), 12 hours after release (G1), or after treatment during S phase with 1 m*M* hydroxyurea (HU) for 1 or 8 hours. (**B**) Normal diploid human WI-38 cells synchronized by either double thymidine block or double hydroxyurea block (for either type of block, 15-hour block; 9-hour release; S phase harvested 4 hours after release; G1 harvested 12 hours after release). (**C**) ROS 17/2.8 rat osteosarcoma cells synchronized by either triple thymidine block or by a 16-hour mitotic block with 0.1 μg/ml nocodazole as described for Figs. 4 and 6. For triple thymidine block; PR, cells harvested prior to release; S, cells harvested 5 hours after release; G1, cells harvested 12 hours after release. For mitotic block; G1, cells harvested 3 hours after release; S, cells harvested 12 hours after release. (**D**) SV40-transformed WI-38 cells synchronized by a 16-hour mitotic block with 0.1 μg of nocodazole per milliliter. G1, cells harvested 3 hours after release; S, cells harvested 12 hours after release. (**E**) HL60 human promyelocytic leukemia cells synchronized by two rounds of 0.1 m*M* thymidine block (16-hour block; 9-hour release). PR, cells harvested prior to release from the second thymidine block; S, cells harvested 4 hours after release; G1, cells harvested 12 hours after release.

cies are unlikely to be related to differences in cell synchronization protocols. For example, HiNF-D activity is cell cycle–regulated in normal diploid cells, whether synchrony is obtained by a variety of drug treatments (this report) or by serum stimulation (*10*). Here, we show a direct relationship between regulated activity of a nuclear factor–promoter element interaction (HiNF-D–site II) and maintenance of the diploid, growth-regulated phenotype. Hence, cell cycle regulation of other histone gene promoter binding activities (for example, OTF-1) may, like HiNF-D, strongly depend on the persistence of stringent cell growth regulation.

In conclusion, our results demonstrate that cell cycle–controlled transcription factors are potential targets for deregulation in the process of cellular transformation. The HiNF-D–site II interaction provides a molecular marker to study a component of the cellular mechanism by which growth control is deregulated during neoplastic transformation.

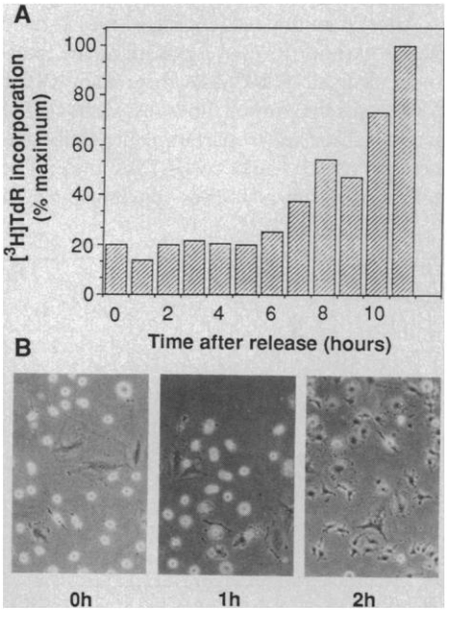

Fig. 6. (**A**) Synchronization of ROS 17/2.8 rat osteosarcoma cells by mitotic block with 0.1 μg of nocodazole per milliliter as monitored by labeling with [^{3}H]thymidine for 1 hour and determination of TCA-precipitable radioactivity. Cells were plated in F12 medium (Gibco) containing 5% NU-serum (Collaborative Research, Bedford, Massachusetts) on dishes coated with 0.1% gelatin and 0.1 μg of poly-L-lysine per milliliter. After 3 days, the cells were blocked in mitosis by addition of 0.1 μg of nocodazole per milliliter (*11*) and released 16 hours later by replacing the medium without nocodazole. Mitotic block synchrony of SV40-transformed WI-38 cells was performed in an identical way in DMEM medium supplemented with 10% NU-serum and 0.5% fetal calf serum. (**B**) Phase-contrast photo-micrographs of ROS 17/2.8 cells taken during the initial 2 hours after release from mitotic block.

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Tumor Resistance to Alkylating Agents Conferred by Mechanisms Operative Only in Vivo

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EMT-6 murine mammary tumors were made resistant to *cis*-diamminedichloroplatinum (II) (CDDP), carboplatin, cyclophosphamide (CTX), or thiotepa in vivo by treatment of tumor-bearing animals with the drug during a 6-month period. In spite of high levels of in vivo resistance, no significant resistance was observed when the cells from these tumors were exposed to the drugs in vitro. The pharmacokinetics of CDDP and CTX were altered in animals bearing the respective resistant tumors. The resistance of all tumor lines except for the EMT-6/thiotepa decreased during 3 to 6 months in vivo passage in the absence of drugs. These results indicate that very high levels of resistance to anticancer drugs can develop through mechanisms that are expressed only in vivo.

THE DEVELOPMENT OF RESISTANCE of malignant tumors to the chemotherapeutic alkylating agents used in the treatment of neoplastic disease is a major factor responsible for treatment failure (*1–3*). To elucidate the mechanisms of drug resistance, most investigators have utilized cell lines that have acquired drug resistance in vitro as a result of repeated or continuous exposure to increasing concentrations of the drug (*3*). Many fewer investigations have utilized tumor lines made resistant in vivo, which may more closely model the clinical situation (*4*).

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