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.

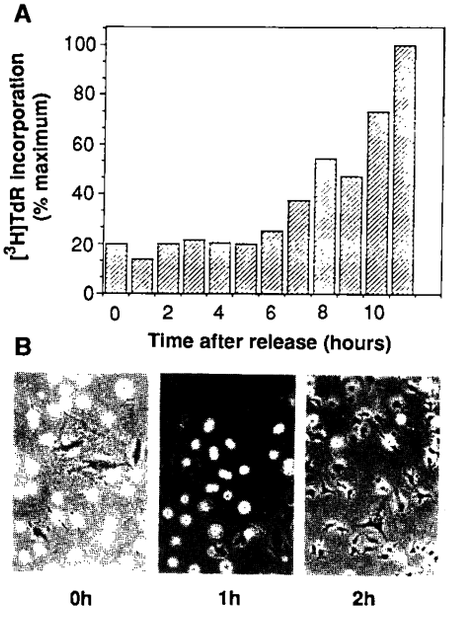

Fig. 6. (**A**) Synchronization of ROS 17/2.8 rat osteosarcoma cells by mitotic block with 0.1 μg of nocodazole per milliliter as monitored by labeling with [^{3}H]thymidine for 1 hour and determination of TCA-precipitable radioactivity. Cells were plated in F12 medium (Gibco) containing 5% NU-serum (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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36. We thank S. Pockwinse and M. Giorgio for photographic assistance. Supported by grants from NIH, NSF, and the March of Dimes Birth Defects Foundation. J.H. was also supported by the Scrinerius Stichting, Utrecht, the Netherlands.

10 November 1989; accepted 26 January 1990

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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The EMT-6 mouse mammary carcinoma grown for these studies as a solid tumor subcutaneously in the flanks of BALB/c mice (*5*) has been used widely in radiobiologic and chemotherapeutic studies (*1, 6*). We have established four alkylating agent–resistant EMT-6 tumor lines by repeated treatment of tumor-bearing animals with *cis*-diamminedichloroplatinum (II) (CDDP) (20 mg/kg) cyclophosphamide (CTX) (300 mg/kg), *N*, *N'*, *N''*-triethylenethiophosphoramide (thiotepa) (15 mg/kg), or carboplatin (100 mg/kg) injected intraperitoneally (ip) 24 hours before passage of each tumor line into fresh host animals. The parent tumor line was passaged in the same manner in the absence of drug treatment. After ten treatments and passages of the four tumor lines over a 6-month period, the sensitivity of each tumor line to the appropriate treatment agent was assessed. After treatment of the animals with two different doses of each drug, tumor cells were removed from the mice, and their ability to form colonies in vitro was examined (*7*). The plating efficiencies and cell yields of the EMT-6 parent and resistant tumor lines were similar, 8 to 12% and 18×10^6 to 22×10^6 per tumor, respectively. Resistance was measured as the surviving fraction (6 months) relative to the untreated parent (time 0). The EMT-6/CDDP tumor line was 54× and 21,000× more resistant, respectively, to 20 and 50 mg of CDDP per kilogram than the EMT-6 parent tumor line (Fig. 1). The EMT-6/CTX tumor line was 16- and 150-fold resistant to CTX at 300 and 500 mg/kg, respectively. The EMT-6/thiotepa tumor line was 5.8- and 38-fold resistant to thiotepa at 15 and 30 mg/kg, respectively. The EMT-6/carboplatin tumor line was 60- and 800-fold resistant to carboplatin at 100 and 500 mg/kg, respectively. Thus, the resistance was characterized by a much shallower slope of the curve of tumor cell survival versus drug dose compared with that of the parent tumor.

Cells from the EMT-6 parent tumor line and each of the four alkylating agent–resistant lines were established as monolayers in culture and several characteristics of these early passage, heterogeneous cell populations were determined (Table 1). The survival in culture of each of the four EMT-6 alkylating agent–resistant tumor lines after exposure for 1 hour to the appropriate alkylating agent (1 to 500 μ*M*) was compared to that of the parent EMT-6 tumor line (*8*). After three independent determinations, essentially no resistance to the treatment agent was observed in cells from the four resistant lines in vitro at drug concentrations that killed 90 or 99.9% of the cells in vivo. Neither the generation times nor protein content of the five lines in cell culture differed significantly from each other (*9*).

Table 1. Characteristics of cells from EMT-6–resistant tumor lines grown in monolayer culture. The resistance ratio equals the drug concentration required to kill 90 or 99.9% of the resistant cells divided by the drug concentration required to kill the same percentage of the parent cells. Exponentially growing cells were exposed to the appropriate alkylating agent for 1 hour, then plated for colony formation. 4-Hydroperoxycyclophosphamide was used as an activated form of cyclophosphamide in vitro. Generation times were calculated by the method of Rheinwald (*9*). Cellular protein was measured by a modified biuret assay (*9*). Measured by fluorescence emission at 420 nm of an OPT derivative (*10*). Measured by the difference between total sulfhydryl content and nonprotein sulfhydryl content with Ellman's method. Absorbance was measured at 412 nm (*10*). Values are means ± SEM.

Source	Resistance ratio		Generation time (hours)	Cellular protein (mg/10^7 cells)	Sulfhydryl (nmol/10^7 cells)	
	IC_{90}	$IC_{99.9}$			Nonprotein	Protein
EMT-6 parent			17 ± 2	1.84 ± 0.07	57 ± 7	230 ± 25
EMT-6/CDDP	1.0	1.2	22 ± 3	1.77 ± 0.08	34 ± 3	336 ± 39
EMT-6/CTX	1.1	1.2	21 ± 3	1.88 ± 0.05	73 ± 8	372 ± 47
EMT-6/thiotepa	1.2	1.1	18 ± 2	1.84 ± 0.06	35 ± 3	243 ± 27
EMT-6/carboplatin	1.1	1.2	19 ± 3	1.59 ± 0.05	31 ± 2	345 ± 45

Increased sulfhydryl content is often associated with alkylating agent resistance in cell lines. Three of the resistant lines, EMT-6/CDDP, EMT-6/thiotepa, and EMT-6/carboplatin had lower nonprotein sulfhydryl (primarily reduced glutathione) content, by 1.7-fold, than the EMT-6 parent tumor line, whereas the EMT-6/CTX line was not significantly different in nonprotein sulfhydryl content (*10*). The protein sulfhydryl content of three resistant lines, EMT-6/CDDP, EMT-6/CTX, and EMT-6/carboplatin was significantly increased compared to the EMT-6 parent line by 1.5- to 1.6-fold, whereas the protein sulfhydryl content of the EMT-6/thiotepa line was not significantly different. These changes, however, conferred no significant resistance when tumor cells were exposed to the alkylating agents in vitro.

After maintenance in vitro for 4 to 6 weeks without further exposure to the treatment drug, 2×10^6 cells from each of the four resistant tumor lines as well as the parent tumor line were reimplanted subcutaneously in the flanks of BALB/c mice. The tumor cell survival after alkylating agent

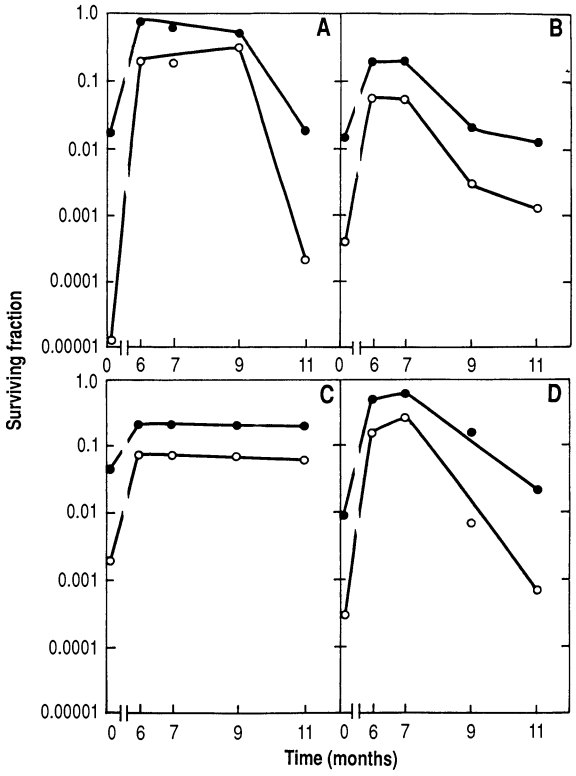

Fig. 1. Survival of EMT-6 tumor cells from tumors treated in vivo with (**A**) CDDP (●, 20 mg/kg; ○, 50 mg/kg), (**B**) CTX (●, 200 mg/kg; ○, 500 mg/kg), (**C**) thiotepa (●, 15 mg/kg; ○, 30 mg/kg) or (**D**) carboplatin (●, 100 mg/kg; ○, 500 mg/kg). From time 0 to 6 months tumor-bearing animals were treated ten times with CDDP (20 mg/kg), CTX (300 mg/kg), thiotepa (15 mg/kg), or carboplatin (100 mg/kg) 24 hours before tumor passage into fresh host animals (*7*). The parent tumor line was passaged in the same manner without drug treatment (shown as time zero). No additional drug treatments were administered before tumor passage between 6 and 11 months to determine the stability of the drug resistance of each of the tumors. When the tumors were approximately 100 mm^3 in volume (about 1 week after tumor cell implantation), the drugs were administered by intraperitoneal injection. Mice were killed 24 hours after treatment, in order to allow for expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised under sterile conditions and a single-cell suspension was prepared (*7*). The results are expressed as the surviving fraction ± SEM of cells from treated groups, compared to untreated controls.

treatment in animals bearing the tumors grown from these postculture cells was then compared to survival of cells in the preculture tumors (Fig. 2). Passage in vitro did not affect the in vivo drug resistance of the resistant tumor cells. The sensitivity of the parent tumor line to the alkylating agents was unchanged.

The morphology of the EMT-6 parent and four alkylating agent–resistant tumors was examined by light and electron microscopy (*11*). All of the tumors were poorly differentiated. The size distribution of cells was the same in all populations. The cells were large and multishaped, round to ovoid-spindle, and closely adherent with occasional tight junctions, consistent with an epithelial origin. Collagen fibers in extracellular matrix were seen in all resistant lines (especially in EMT-6/thiotepa) but not in the parental line. The EMT-6/CTX tumor line also had a significantly increased number of mitoses per ten high-power fields [parent tumor, 9 ± 1; CTX tumor, 17.5 ± 1 (SD)] versus the EMT-6 parental tumor line ($P < 0.005$), whereas the other three alkylating agent–resistant EMT-6 tumors showed no significant increase in mitoses compared with the parental tumor line. Consistent with this observation, the tumor volume doubling time of the EMT-6 parental, EMT-6/CDDP, EMT-6/thiotepa, and EMT-6/carboplatin tumor lines was 2.8 ± 0.3 days, whereas the tumor volume doubling time of the EMT-6/CTX tumor line was 2.2 ± 0.3 days (*12*).

The distribution of platinum over time after ip injection of CDDP (10 mg/kg) was examined in the tumors and several normal tissues of BALB/c mice bearing EMT-6/CDDP tumors or EMT-6 parent tumors and in the normal tissues of non–tumor bearing BALB/c mice (*13*). Fifteen minutes after CDDP administration, serum levels of platinum in all of the treatment groups were essentially identical. Over a 24-hour period, however, several differences in the platinum pharmacokinetic parameters in both the tumors and in normal tissues of animals bearing the EMT-6 parent tumor, the EMT-6/CDDP tumor, or no tumor became evident (Table 2) (*14*). The absorption of platinum into the EMT-6 parent tumor was 1.7-fold more rapid than in the EMT-6/CDDP. On the other hand, the $t_{1/2}$ distribution and $t_{1/2}$ elimination of platinum in the EMT-6 parent tumor were longer than those for platinum in the EMT-6/CDDP tumor. The peak level of platinum in EMT-6 parent tumor was 2.2-fold higher than that in the EMT-6/CDDP tumor. In addition, the area under the curve (AUC) of concentration versus time, which is a measure of total drug exposure, was twofold greater for the parent tumor than that of the EMT-6/CDDP tumor. Although the $t_{1/2}$ absorptions in the kidneys and livers of animals bearing either tumor were the same, the $t_{1/2}$ eliminations of platinum in both of these organs were longer in animals bearing the EMT-6 parent tumor compared with animals bearing the EMT-6/CDDP tumor. The absorption and distribution of platinum in the livers of normal BALB/c mice was the same as in the tumor-bearing animals but the elimination was much more rapid than in animals bearing either tumor. The absorption and distribution of platinum into the kidneys of the normal mice was slower and the elimination of platinum was more rapid than in animals bearing either the parent or CDDP-resistant tumor. Overall the renal pharmacokinetics of the animals bearing the CDDP-resistant tumor was more like that of normal mice (more rapid elimination and lower AUC for the kidneys) than for animals bearing the EMT-6 parental tumor. The tumor growth delay produced by CDDP (10 mg/kg) in the EMT-6 parent tumor was 5.6 ± 0.8 days, whereas there was no growth delay in the EMT-6/CDDP tumor by the same treatment.

We also examined pharmacokinetics for [^{14}C]CTX (150 mg/kg intraperitoneally) in EMT-6 parental tumors, EMT-6/CTX tumors, and normal BALB/c mice (Table 2). The ^{14}C content of tissues was assessed during 24 hours after drug administration (*15*). The serum levels of ^{14}C 15 min after drug administration were the same in all groups. The $t_{1/2}$ absorption of ^{14}C into both tumors was the same; however, the drug was distributed 5 times more rapidly into the EMT-6 parent tumor and was eliminated twice as fast from the EMT-6/CTX tumor resulting in an AUC for the EMT-6 parent tumor that was 3.4 times as great as for the EMT-6/CTX tumor. The drug was absorbed and distributed similarly in the livers of animals bearing either tumor but was eliminated 1.4 times as fast from the livers of animals bearing the EMT-6 parent tumor. The drug was absorbed and distributed twice as fast into the kidneys of animals bearing the EMT-6/CTX tumor and eliminated ten times as rapidly from the kidneys of these animals compared to those bearing the EMT-6 parent tumor. The [^{14}C]CTX was absorbed, distributed, and eliminated more rapidly from both the kidneys and livers of non–tumor bearing BALB/c mice than from the organs of mice bearing either the EMT-6 parental or the EMT-6/CTX tumor. As was the case for animals bearing the CDDP-resistant tumor, the renal pharmacokinetics of animals bearing the CTX-resistant tumor was more like that of the normal mice with more rapid elimination of ^{14}C and a lower AUC for the kidneys than for animals bearing the EMT-6 parental tumor. The tumor growth delay produced by CTX (150 mg/kg) in the EMT-6 parent tumor was 6.3 ± 1.1 days, whereas there was no growth delay in the EMT-6/CTX tumor by the same treatment.

The stability of the in vivo resistance developed in these tumors was tested by

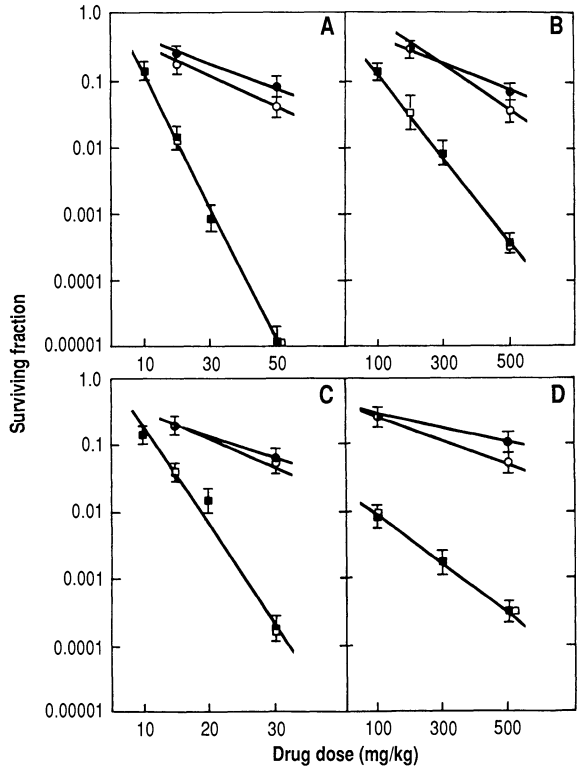

Fig. 2. Survival of EMT-6 tumor cells from tumors treated in vivo with a dosage range of each alkylating agent. (**A**) CDPP, (**B**) CTX, (**C**) thiotepa, and (**D**) carboplatin. ■, The survival of the parent EMT-6 tumor maintained by continuous biweekly passage in vivo; □, survival of the parent EMT-6 tumors established from cells maintained in culture for 4 to 6 weeks then reimplanted in vivo and allowed to grow to 100 mm^3 tumors; ●, survival of cells from the resistant tumors maintained by continuous biweekly passage in vivo with drug treatment 24 hours before each passage; ○, survival of the resistant tumors established from cells maintained in culture for 4 to 6 weeks in the absence of drug then reimplanted in vivo and allowed to grow to 100 mm^3 tumors. Tumor cell survival after in vivo treatment was assessed as in Fig. 1. The experiments were performed three independent times. Bars are SEM.

Table 2. Pharmacokinetic parameters for CDDP or [^{14}C]CTX administered ip to animals bearing parent or resistant EMT-6 tumors. These parameters were derived from curves based on 11 data points measured during 24 hours. The data were analyzed with a computer program based on a two-component model (*14*). The data are expressed as micrograms of platinum equivalents or micrograms of ^{14}C-labeled equivalents per gram of tissue (wet weight).

Group	Tissue	$t_{1/2}$ absorption (min)	$t_{1/2}$ distribution (min)	$t_{1/2}$ elimination (hour)	Peak levels (μg/g tissue)	AUC [(μg hour)/g tissue]
		Platinum from CDDP				
EMT-6 parent	Tumor	2.2	5.9	40	4.2	110
	Liver	0.6	2.7	24	4.3	108
	Kidneys	1.0	3.0	33	5.5	200
EMT-6/CDDP	Tumor	3.7	5.0	30	1.9	55
	Liver	0.6	2.7	17	5.6	98
Nontumor	Liver	0.6	2.7	1.2	6.3	14
	Kidneys	5.3	9.4	4.2	10.3	24
		[^{14}C] from [^{14}C]CTX				
EMT-6 parent	Tumor	0.33	1.50	2.5	156	581
	Liver	0.30	0.07	1.8	126	354
	Kidneys	0.16	0.04	0.90	206	536
EMT-6/CTX	Tumor	0.33	7.50	1.25	124	172
	Liver	0.30	0.07	2.6	105	429
	Kidneys	0.08	0.02	0.09	395	321
Nontumor	Liver	0.22	0.05	1.4	185	137
	Kidneys	0.05	0.15	0.08	539	178

continued biweekly passage of the tumors into fresh hosts without further drug treatment and assessment of drug sensitivity by tumor cell survival assay (Fig. 1) (*17*). Resistance to CDDP in the EMT-6/CDDP tumor was stable for 3 months (six passages) but was lost by 5 months (eight passages) after treatment. The resistance of the EMT-6/CTX tumor to CTX and the EMT-6/carboplatin tumor to carboplatin was more labile and was largely lost by 3 months without further treatment. On the other hand, even after 5 months without further treatment, the EMT-6/thiotepa tumor line remained stably resistant to thiotepa.

The emergence of in vivo drug resistance in these tumor lines supports the theories developed by Goldie and Coldman (*2*), which mathematically described the genetic instability of neoplasms and favored survival of phenotype or genetic variants that are resistant to a particular drug. We believe that the mechanism of drug resistance that developed in each of these tumor lines, however, involved an interaction between the tumors and the host normal tissues rather than resulting from changes in the cellular sensitivity of the tumors to the alkylating agents. The very high levels of resistance evident in the four resistant tumor lines in vivo were not reflected by cellular resistance to the drugs in vitro. Significant changes were observed in the distribution of drug after CDDP administration and [^{14}C]CTX administration in both the tumors and normal tissues of mice bearing the resistant tumors as compared to mice bearing the parental EMT-6 tumor. These changes resulted in lower AUCs in the CDDP- and CTX-resistant tumors and would seem to provide a partial explanation for the induced in vivo tumor resistance to these agents. In addition, the presence of either the parent or resistant tumors altered the pharmacokinetics of CDDP and CTX in the livers and kidneys of the host animals as compared with non–tumor bearing animals. In three of four cases, resistance was lost 3 to 5 months after drug treatment was stopped. Therefore, this type of in vivo resistance appears to be due to an inducible change in the tumors, which is capable of abscopally altering drug disposition in fresh host animals.

It is well recognized that some tumors can secrete hormonal substances, many of which are peptide hormones, capable of far-reaching effects on the normal tissues of host animals (*16*). It is possible that such a mechanism is operative in animals bearing these EMT-6 tumors and is responsible for the altered drug distributions observed. While the study of such in vivo mechanisms will necessarily be more difficult than the study of in vitro effects, an understanding of these factors may be critical to the development of strategies to overcome or prevent the expression of this type of tumor resistance in the clinic.

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8. In vitro monolayer cultures from each of the five tumors were maintained in exponential growth in Waymouth's medium (Gibco), supplemented with 15% newborn calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco) at 37°C in a 5% CO_2/95% air atmosphere. In vitro plating efficiencies of control cultures for each tumor were 55 to 80%. 4-Hydroperoxycyclophosphamide was used in vitro as an activated form of cyclophosphamide. Exponentially growing cells were exposed to varying concentrations of each drug for 1 hour at 37°C. Non–drug-treated controls were handled identically. Drugs were prepared in sterile phosphate-buffered 0.9% saline immediately before use and added to the flasks in media without serum. After treatment, the medium was removed and the cultures were washed twice with phosphate-buffered 0.9% saline and the cells were suspended by treatment with trypsin (0.01% for 2 to 3 min). Known numbers of cells were plated into dishes for colony growth formation. After 8 to 10 days, colonies were stained with crystal violet in methanol containing 3.7% formaldehyde and were counted manually. Each experiment was repeated three times and the cycle from tumor to culture and back to tumor was repeated three times.
9. Cellular protein was measured by a modified biuret assay (Sigma). Generation times were determined by the method of Rheinwald [J. G. Rheinwald, *Methods Cell Biol.* **21**, 229 (1980)].
10. Cells (4×10^6) from the EMT-6 parent and each of the resistant lines in exponential growth were lysed in 2 ml of 5% perchloric acid. The protein was removed by centrifugation at 1000*g* for 3 min at 5°C. The supernatant was neutralized with 5*M* potassium phosphate, then passed through a 0.2-μm filter before derivatization. Glutathione was assayed at four dilutions of the supernatants. For the fluorescence assay, 0.1 ml of the sample was added to 3 ml of 0.1*M* potassium phosphate buffer containing 5 m*M* EDTA (*p*H 8), then 0.15 ml of an *ortho*-phthaldialdehyde (OPT; Aldrich) solution (1 mg/ml in methanol) was added. The derivatization was allowed to continue for 15 min at room temperature in the dark. Fluorescence was measured with an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The calibration curve was linear from 0.05 to 50 nmol of (reduced) glutathione per milliliter. The data shown are the mean of five determinations. P. C. Jocelyn and A. Kimminga, *Anal. Biochem.* **37**, 417 (1970); V. H.

Cohen and J. Lyle, *ibid.* **14**, 434 (1986); M. E. Varnes and J. E. Biaglow, *Cancer Res.* **39**, 2960 (1979). Nonprotein and total sulfhydryl content was determined by a modification of the Ellman method [G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959); J. Sedlak and R. H. Lindsay, *Anal. Biochem.* **25**, 192 (1968); T. W. Wong and G. F. Whitmore, *Radiat. Res.* **71**, 132 (1977); M. E. Varnes and J. E. Biaglow, above]. Cells (10^7) from the EMT-6 parent tumor line and each of the resistant lines were lysed in 6 ml of 0.02*M* EDTA. For nonprotein sulfhydryl determination, 5 ml of the cell lysate was treated with an equal volume of 10% trichloroacetic acid, then centrifuged at 500*g* for 5 min at 5°C. Four milliliters of the supernatant was adjusted to *p*H 8.9 with 0.2*M* tris buffer. Ellman's reagent [0.01*M* 5,5′-dithiobis(2-nitrobenzoic acid)] in methanol (Aldrich) (12.5 μl of sample per milliliter) was added, and color was allowed to develop for 30 min at room temperature. Total sulfhydryl content was determined by adding cell lysate (0.5 ml) to 0.02*M* tris buffer (1.5 ml, *p*H 8.2), followed by 0.1 ml of 0.01*M* 5,5′-dithiobis(2-nitrobenzoic acid) in methanol. After bringing the sample volume to 3 ml with methanol, the color was allowed to develop for 30 min at room temperature. All of the samples were filtered (0.45 μ*M*), and absorbance was read at 412 nm. Protein sulfhydryl content was determined from the difference between the total sulfhydryl content and nonprotein sulfhydryl content. Each measurement was repeated at least three times.

11. Animals were killed by cervical dislocation, and tumors were quickly excised for fixation in phosphate-buffered formalin (light microscopy) or Kamovsky's fixative (electron microscopy). After tissue processing by routine methods, sections were examined in a random fashion.
12. Tumor volume measurements began on day 7 after implantation of 2×10^6 tumor cells subcutaneously in the legs of BALB/c mice 8 to 10 weeks of age. We made three measurements (length, width, and depth) on each tumor using calipers and calculated the tumor volume assuming the tumor to be a hemiellipsoid. Data from the tumor volume measurements were analyzed with a computer program written for an Apple microcomputer. The program first derives the best-fit curve for each individual set of tumor volume data, then calculates the median, mean, and SEM for each experimental group. Measurements were made on nine individual tumors of each type.
13. BALB/c mice bearing EMT-6 parental tumors or EMT-6/CDDP tumors and non–tumor bearing animals were injected ip with CDDP at 10 mg/kg. Animals were killed at 11 time points after drug administration. Known wet weights of tumor, liver, kidney, skin, skeletal muscle, and blood were dissolved in a tissue solubilizer (Protosol; DuPont Biotechnology Systems), then analyzed by flameless atomic absorption spectroscopy. Platinum from a 15-μl sample injection volume was atomized from the walls of pyrolytically coated graphite tubes. A Perkin-Elmer Model 2380 atomic absorption spectrophotometer was used in conjunction with a Perkin-Elmer Model 400 graphite furnace to measure the absolute mass of platinum in the cell samples. Each measurement was made in triplicate in three independent experiments. [O. H. Drummer, A. Proudfoot, L. Howes, W. J. Louis, *Clin. Chim. Acta.* **136**, 65 (1984); T. S. Herman *et al.*, *Cancer Res.* **48**, 5101 (1988); T. S. Herman *et al.*, *ibid.*, p. 2335.]
14. The data were fitted to a triexponential equation of the form $C = A_0 e^{-kat} + B_0 e^{-\alpha t} + C_0 e^{-\beta t}$, which allowed derivation of the half times ($t_{1/2}$ for absorption, distribution, and elimination of the drug as well as calculation of the area under the curve of concentration versus time [R. L. Furner, R. K. Brown, G. Duncan, *Cancer Treat Rep.* **61**, 1637 (1977); R. K. Brown, G. Duncan, D. L. Hill, *ibid.* **64**, 643 (1980)].
15. BALB/c mice bearing EMT-6 parental tumors or EMT-6/CTX and non–tumor bearing animals were injected ip with 6 μCi of [*ring*-4-^{14}C]CTX (150 mg/kg; 52.5 mCi/mmol). The animals were killed at 11 time points after drug administration. Known wet weights of tumor, liver, kidney, skin, skeletal muscle, and blood were dissolved in a tissue solubilizer (Protosol; DuPont Biotechnology Systems), then counted by liquid scintillation in Aquasol (DuPont Biotechnology Systems).
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17. CDDP and carboplatin pure powders were a gift from M. Abrams and D. Picker, Johnson-Matthey, Malvern, PA; 4-hydroperoxycyclophosphamide, used in vitro as an activated form of cyclophosphamide (Sigma), was a gift from O. M. Colvin; thiotepa was a gift from Lederle Laboratories, Pearl River, NY. Supported by National Cancer Institute grant P01-CA38497 and a grant from Bristol-Myers Co., Wallingford, CT.

20 November 1989; accepted 25 January 1990

Engineering Human Prolactin to Bind to the Human Growth Hormone Receptor

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A strategy of iterative site-directed mutagenesis and binding analysis was used to incorporate the receptor-binding determinants from human growth hormone (hGH) into the nonbinding homolog, human prolactin (hPRL). The complementary DNA for hPRL was cloned, expressed in *Escherichia coli*, and mutated to introduce sequentially those substitutions from hGH that were predicted by alanine-scanning mutagenesis and other studies to be most critical for binding to the hGH receptor from human liver. After seven rounds of site-specific mutagenesis, a variant of hPRL was obtained containing eight mutations with an association constant for the hGH receptor that was increased more than 10,000-fold. This hPRL variant binds one-sixth as strongly as wild-type hGH, but shares only 26 percent overall sequence identity with hGH. These studies show the feasibility of recruiting receptor-binding properties from distantly related and functionally divergent hormones and show that a detailed functional database can be used to guide the design of a protein-protein interface in the absence of direct structural information.

THE RECRUITMENT OF FUNCTIONAL properties within a family of homologous proteins by site-directed mutagenesis is a powerful approach for identifying determinants that modulate protein function. This is a useful design strategy because it makes it possible to combine into one homolog the most desirable functional characteristics (such as specificity, catalysis, stability, and immunogenicity) that are contained in the family. Previous recruitment studies (*1–4*) have relied on high-resolution structural data to target specific contact residues or entire units of secondary structure in order to exchange binding (*1, 2*) or enzymatic functions (*3, 4*) between homologs. We now show that the identification of receptor-binding determinants in hGH by high-resolution mutagenic analysis can be used in place of a structural model of the hormone-receptor complex to engineer hPRL, a distantly related and nonbinding homolog, so that it will bind to the hGH receptor.

Prolactin is a member of a family of homologous hormones that includes growth hormones, placental lactogens, and proliferins (*5*). Collectively, this group of hormones regulates a vast array of physiological effects important in growth, differentiation, and electrolyte balance, for example (*6*). These pharmacological effects are initiated by the binding of the hormones to specific cellular receptors. For example, hGH binds to both the lactogenic and somatogenic receptors and stimulates both lactation and bone growth. In contrast, hPRL binds only to the lactogenic receptor and stimulates lactation but not bone growth. The molecular basis for these differences in receptor-binding specificity is not understood.

Scanning mutagenesis methods (*7, 8*) have identified residues in hGH that strongly modulate binding to the extracellular fragment of the hGH receptor (also called the hGH-binding protein). The hGH-binding protein is more convenient and accurate for the assay of binding affinity because it can be expressed and purified to homogeneity in large amounts from *Escherichia coli* (*9*) and it retains binding specificity virtually identical to that of the full-length somatogenic receptor (*9, 10*). The amino acid side chains that modulate binding to the hGH-binding protein are located in three discontinuous segments of hGH. Together, these residues form a patch when mapped onto a crude

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