week 40 when last tested. An IgM antibody to HTLV-III was not observed in CH132 (which showed only passive transfer of antibody) but was detected early in the course of HTLV-III antibody production in HTLV-III–infected CH114. CH133 manifested no clinical or immunologic evidence of AIDS.

The control animal, CH140, received plasma from three normal donors negative for antibodies to HTLV-III. No clinical or immunologic evidence of AIDS was noted, and no specific HTLV-III antibody response developed. As additional controls, 15 uninoculated chimpanzees housed at SFBR were tested, and all were found to be negative for antibody to HTLV-III.

Our results show that (i) the chimpanzee is susceptible to HTLV-III infection and can thus serve as an infectivity model for the study of AIDS; (ii) in addition to HTLV-III antibody seroconversion, the chimpanzee can develop a clinical syndrome of lymphadenopathy and immunologic impairment providing a disease model that simulates the human AIDS-related lymphadenopathy syndrome; and (iii) HTLV-III infection can be transmitted by lymphocyte-poor plasma, substantiating the potential AIDS risk of noncellular blood components such as pooled clotting-factor concentrates. The susceptibility of the chimpanzee to HTLV-III infection will provide an animal model of AIDS in which to assess antiviral agents and biologic response modifiers and, most importantly, in which to test the safety, immunogenicity, and efficacy of future vaccines.

HARVEY J. ALTER
Blood Bank Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20205
JORG W. EICHBERG
Virology and Immunology Department, Southwest Foundation for Biomedical Research, San Antonio, Texas 78284
HENRY MASUR
Critical Care Medicine Department, Clinical Center, National Institutes of Health
W. CARL SAXINGER
ROBERT GALLO
Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Maryland 20205
ABE M. MACHER
Laboratory of Pathology, National Cancer Institute
H. CLIFFORD LANE
ANTHONY S. FAUCI
Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205

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13. The stimulatory ratio was calculated as follows. A stimulatory index, representing the amount of label (in counts per minute) of cells plus mitogen divided by that of cells plus medium, was calculated in each test for each animal. The stimulatory ratio is that of the index for CH114 to the index for CH140 (a control animal), the index of each being determined simultaneously in the identical assay.
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Oncogene-Induced Transformation of C3H 10T1/2 Cells Is Enhanced by Tumor Promoters

Abstract. *The tumor promoters 12-O-tetradecanoyl-phorbol-13-acetate and teleocidin markedly enhanced the transformation of C3H 10T1/2 mouse fibroblasts when these cells were transfected with the cloned human bladder cancer c-ras^H oncogene. Transfection studies with the drug resistance marker* gpt *and time course studies indicate that this enhancement is not simply an effect on the process of DNA transfection. These findings, together with parallel studies with NIH 3T3 fibroblasts, also indicate that the competence of animal cells for DNA transfection is a function of the recipient cell line, the transfected marker, and the growth conditions. Our findings suggest that during multistage carcinogenesis tumor promoters may complement the function of activated cellular oncogenes.*

Development of a fully malignant tumor involves complex interactions between environmental and endogenous factors. In addition, carcinogenesis often proceeds through several discernible stages (initiation, promotion, progression) (*1*). DNA transfection studies with the NIH 3T3 cell line have revealed activated oncogenes in a number of human tumors and tumor cell lines (*2–5*). This approach does not in itself, however, indicate the types of interactions that might occur between environmental and endogenous factors in the de novo transformation of normal cells. We are intrigued by the possibility that, during the multistage carcinogenic process, tumor promoters might interact synergistically with cellular oncogenes, since promoters can induce mimicry of transformation, modulate differentiation, and enhance the transformation of cells previously exposed to chemical carcinogens, radiation, or certain DNA viruses (*1, 6–9*). It is of interest, therefore, to determine whether tumor promoters enhance the transformation of cell cultures transfected with an activated oncogene.

In examining possible synergistic interactions between tumor promoters and a cloned oncogene, we used C3H 10T1/2 cells as recipients since they have a more uniform fibroblastic morphology and a lower saturation density than NIH 3T3 cells. In addition, although they are aneuploid, they have an extremely low incidence of spontaneous transformation and are not tumorigenic in nude mice (*10*). They are also particularly well suited for studies of the action of phorbol ester tumor promoters, since they contain an abundance of high-affinity receptors for these and related compounds (*11*) and display striking changes in cell morphology and membrane-related properties in response to these agents (*11, 12*). Furthermore, these tumor promoters markedly enhance the outgrowth of transformed foci in C3H 10T1/2 cultures previously initiated by exposure to several types of chemical carcinogens (*6*) or radiation (*7*), thus mimicking the process of two-stage carcinogenesis on mouse skin.

We first assessed the general competence of C3H 10T1/2 cells for DNA-mediated transfection. For this purpose we chose the dominant drug resistance marker *gpt*, linked to the early region of SV40 to enhance its transcription (*13*). When C3H 10T1/2 cells were transfected with pSV2-*gpt* plasmid DNA (1 μg per plate) by the standard calcium phosphate precipitation technique and then selected

for Gpt^+ clones with mycophenolic acid, we obtained about 300 colonies per 5×10^5 recipient cells (first column in Table 1). Parallel studies with NIH 3T3 cells gave values that were about three times greater. These results and published *gpt* transfection frequencies obtained with other cell lines (*14*) indicated that the C3H 10T1/2 cells were reasonably competent for transfection. Therefore, we also assessed the ability of the mutated human bladder cancer c-ras^H oncogene (*5, 15, 16*) to induce foci of transformation on C3H 10T1/2 cells with the plasmid pT24 (*15*). This gene seemed particularly appropriate because its DNA sequence is well defined and it is extremely effective in transforming NIH 3T3 cells (*2–5*). On the other hand, when used alone, it has weak or negligible activity in transforming primary cell cultures (*17–19*), suggesting that its activity might be enhanced by tumor promoters. Parallel studies were done with NIH 3T3 cells as recipients. As previously reported (*5, 15, 16*), the oncogene yielded many transformed foci on NIH 3T3 cells. On the other hand, we obtained only a few transformed foci on C3H 10T1/2 cells (second column in Table 1).

It was of interest to determine whether the low frequency of oncogene-induced transformation obtained with C3H 10T1/2 cells could be enhanced by the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). We also ran parallel studies with the *gpt* marker plus TPA to determine the specificity of the effect. Exposure of C3H 10T1/2 or NIH 3T3 cells to TPA (100 ng/ml) during transfection with pSV2-*gpt* and selection in mycophenolic acid inhibited the number of Gpt^+ colonies obtained (third column in Table 1). On the other hand, TPA caused an approximately fivefold increase in the number of transformed foci obtained in C3H 10T1/2 cells transfected with the T24 oncogene (Table 1). Such enhancement was seen in five of five additional experiments, with the increase ranging from 5- to 15-fold. In all these experiments C3H 10T1/2 cells transfected with carrier DNA alone (that is, DNA from untransformed C3H 10T1/2 cells), with or without TPA treatment, were included as controls and no transformed foci were seen. In addition, the transformed foci appeared earlier and were often larger in the TPA-treated than in untreated cultures (Fig. 1). These results, as well as the time course studies (Fig. 2), indicate that the enhancement seen with TPA was not simply due to seeding of cells from primary foci of transformed cells. Growth of C3H 10T1/2 cells in the presence of TPA but without pT24 DNA transfection caused a slight (about 1.5-fold) increase in overall saturation density, but no transformed foci were seen. TPA inhibited the number of transformed foci seen with pT24 DNA transfection of NIH 3T3 cells (column 4 in Table 1), although TPA was not toxic to the growth of these cells. TPA is known to produce diverse and sometimes opposing effects on specific functions in different cell lines (*1*).

We next explored the effects of time and duration of exposure of C3H 10T1/2 cells to TPA with respect to the transformation induced by pT24 DNA transfection. If cells were grown in the presence of TPA (100 ng/ml)—beginning 4 days before transfection, during transfection, and for the subsequent 24 days (at the end of which time the cultures were stained and scored for transformed foci)—the frequency of foci was about 15 times greater than that obtained when the entire experiment was done in the absence of TPA. A similar enhancement was seen if TPA was removed from the culture 12 or 4 days after transfection. On the other hand, only a slight (two- to threefold) enhancement was seen if TPA was present only at the time of transfection, or if its addition was delayed until 4 days after transfection. No enhancement, and possibly even inhibition, was seen if TPA addition was delayed until 12 days after transfection. The results for groups 3 and 4 (Fig. 2) indicate that the enhancement of cell transformation by TPA is irreversible. It would appear, however, that TPA exerts its maximum action around the time of transfection and in the subsequent 4 days (Fig. 2).

It is difficult to interpret these results more precisely, since there may be lag periods after addition or removal of TPA. In addition, by days 4 to 6 the cultures had become confluent, which

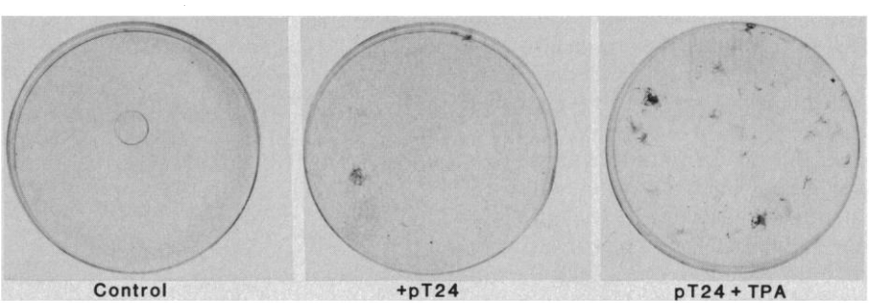

Fig. 1. Culture plates of C3H 10T1/2 cells stained with Giemsa 24 days after transfection with pT24 DNA. For experimental details, see the legend to Table 1.

Table 1. Transfection frequencies obtained with pT24 and pSV2-*gpt* DNA's in C3H 10T1/2 and NIH 3T3 cells in the presence and absence of TPA. The DNA transfection procedure used was essentially that described by Wigler *et al.* (*22*) and Parker and Stark (*23*). The indicated cells were seeded at a density of 5×10^5 per 9-cm-diameter plate in Dulbecco's modified Eagle medium with 10 percent calf serum (D10) and 1 day later were fed with fresh D10 medium 4 hours before transfection. Calcium phosphate–precipitated DNA (1 μg of plasmid DNA plus 20 μg of DNA from untransformed C3H 10T1/2 cells as carrier) was added to each dish and the cells were incubated for 4 hours at 37°C. The medium was then removed and the cells were treated with 15 percent glycerol in isotonic saline–Hepes for 2 minutes and then washed with phosphate-buffered saline. Cells transfected with pT24 DNA were fed D10 medium for 1 day, trypsinized, and replated in Dulbecco's modified Eagle medium with 5 percent calf serum (D5) at a density of 2.5×10^5 cells per 9-cm plate. The medium was changed the next day and the cells were fed every other day with D5 medium throughout the remainder of the experiment. Cells transfected with the pSV2-*gpt* DNA were kept in D10 medium for 12 days after transfection to allow phenotypic expression and then replated at a density of 5×10^5 cells per 9-cm plate in Gpt^+-selective D10 medium containing (micrograms per milliliter): hypoxanthine, 15; aminopterin, 0.2; thymidine, 5; xanthine, 25; and mycophenolic acid, 25. This medium was changed the next day and every other day throughout the remainder of the experiment. Colonies present in the *gpt*-transfected plates and foci of transformation (2 mm in diameter or greater) in the T24 plates were scored 3 to 4 weeks after transfection. Values represent colonies or foci per originally transfected plate, expressed as means for duplicate plates. Values in parentheses are transfection frequencies based on the total number of colonies or foci obtained from 5×10^5 recipient cells.

Recipient cells	Without TPA		With TPA*	
	gpt colonies	T24 foci	*gpt* colonies	T24 foci
C3H 10T1/2	313 (6×10^{-4})	8 (0.2×10^{-4})	82 (2×10^{-4})	41 (1×10^{-4})
NIH 3T3	933 (19×10^{-4})	706 (14×10^{-4})	303 (6×10^{-4})	182 (4×10^{-4})

*Cells were grown in the presence of TPA (100 ng/ml) beginning 4 days before transfection until 2 weeks after.

could limit their subsequent response to TPA if continued cell division is a factor. The solvent dimethyl sulfoxide (0.01 percent) and the compound phorbol (1, 10, and 100 ng/ml), which lacks tumor-promoting activity (*12*), had no effect on focus formation. At 10 rather than 100 ng/ml, TPA produced a two- to threefold stimulation of focus formation; no stimulation was seen at 1 ng/ml. In the same experiment, the indole alkaloid teleocidin—which is a potent tumor promoter on mouse skin, shares several other biological effects with TPA, and appears to bind to the same cell receptors (*12, 20*)—produced a fivefold stimulation of focus formation at 10 ng/ml and a twofold stimulation at 1 ng/ml. When calculated on a molar basis teleocidin was even more potent in this assay than TPA.

We picked and cloned four of the transformed clones obtained from C3H 10T1/2 cells transfected with pT24 DNA in the absence of TPA and six from experiments in which the transfected cells were grown in the presence of TPA. All these cultures retained their transformed morphology, grew to much higher densities than control C3H 10T1/2 cells, and, in contrast to normal cells, formed colonies in 0.3 percent agarose. When the DNA's of these clones were studied by Southern blot analysis with a probe to the T24 oncogene, they all revealed acquisition of this sequence (Fig. 3). Several discrete bands were seen on the original film in lanes 4 and 8, and these results were confirmed in an additional study with DNA digested by Sac I rather than Eco RI, yielding a 2.9-kb fragment diagnostic of the T24 DNA. Thus, none of these transformed cell lines represent "spontaneous" transformants. No reproducible differences in the Southern blot profiles were seen between transformants derived in the absence or presence of TPA in terms of the abundance of integrated copies of T24 sequences.

In DNA transfection studies it is often assumed that competence is a general property of the recipient cells and is unrelated to the specific marker being transfected. We believe, however, that competence is in part a function of the transfected marker and the physiological state of the recipient cells. Thus we found that C3H 10T1/2 and NIH 3T3 fibroblasts had similar competence (within a factor of 3) when transfected with the *gpt* marker, but differed by a factor of over 90 when examined for transformed foci induced by transfection with the T24 oncogene. It appears that variations between cell types in their competence for transfection by an oncogene, and the effects of TPA on enhancing T24 DNA–induced cell transformation, are not simply due to variations at the level of cellular uptake or integration of the transfected oncogene. The time course experiments (Fig. 2) and the Southern blot analyses (Fig. 3) also suggest that TPA does not act simply at these levels. Studies involving Northern blot analyses indicate that the enhancing effect of TPA on T24-induced cell transformation is not due to an effect on transcription of the T24 *ras* gene or expression of the endogenous *myc* oncogene. Further studies are required to determine whether TPA acts by enhancing the expression of other host genes in C3H 10T1/2 cells. It is of interest that TPA treatment can affect the expression of *myc* or *myb* oncogenes in human promyelocytic leukemia cell lines (*21*).

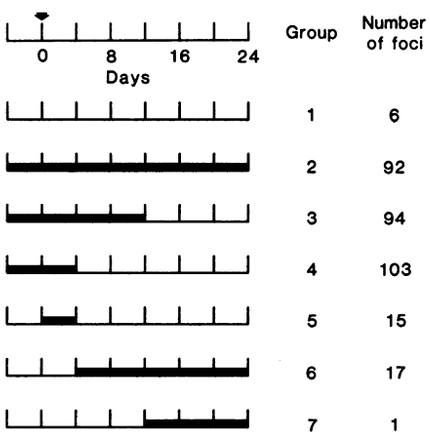

Fig. 2. Effect of time and duration of exposure of C3H 10T1/2 cells to TPA on transformation induced by transfection with pT24 DNA. The heavy lines indicate the times when TPA (100 ng/ml) was present during the transfection experiment. Fresh TPA was added at each refeeding with growth medium. The arrow indicates the time of transfection. Values are numbers of foci per plate on day 24.

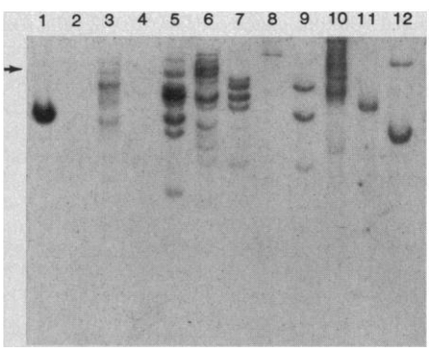

Fig. 3. Southern blot analysis of chromosomal DNA's extracted from C3H 10T1/2 cells transformed with pT24 DNA and probed with a ^{32}P nick-translated T24-specific fragment. The DNA's were cut with the restriction enzyme Eco RI before gel electrophoresis (*24, 25*). Lane 1, NIH 3T3 cell DNA containing a single integrated copy of the T24 gene (A5-1) (provided by M. Wigler); lane 2, normal C3H 10T1/2 cell DNA; and lanes 3 to 12, chromosomal DNA's from pT24-transformed C3H 10T1/2 cells transfected in the absence of TPA (lanes 3 to 6) or in its presence (lanes 7 to 12). The arrow indicates the position of the endogenous mouse c-ras^H gene (about 23 kb on the basis of λ DNA Hind III markers). This band was only faintly visualized with Eco RI digestion and the T24 probe. Discrete bands homologous to the T24 probe were seen in lanes 4 and 8 of the radioautograph, although these are not well displayed here.

Studies indicate that TPA and teleocidin also cause a five- to tenfold increase in transformed foci obtained when a near-diploid rat embryo fibroblast cell line is transfected with T24 DNA. Thus this phenomenon is not restricted to C3H 10T1/2 cells. Our results suggest that, during multistage carcinogenesis, the initiating carcinogen might function by activating a cellular proto-oncogene, whereas tumor promoters might enhance the outgrowth of the altered cells or the expression of other cellular genes that complement the function of the activated oncogene.

W.-L. W. HSIAO
S. GATTONI-CELLI
I. B. WEINSTEIN

Division of Environmental Sciences, Departments of Medicine and *Human Genetics,* and *Cancer Center/Institute of Cancer Research, Columbia University, New York 10032*

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Disulfide Bond Engineered into T4 Lysozyme: Stabilization of the Protein Toward Thermal Inactivation

Abstract. *By recombinant DNA techniques, a disulfide bond was introduced at a specific site in T4 lysozyme, a disulfide-free enzyme. This derivative retained full enzymatic activity and was more stable toward thermal inactivation than the wild-type protein. The derivative, T4 lysozyme (Ile*^{3} *→ Cys), was prepared by substituting a Cys codon for an Ile codon at position 3 in the cloned lysozyme gene by means of oligonucleotide-dependent, site-directed mutagenesis. The new gene was expressed in* Escherichia coli *under control of the* (trp-lac) *hybrid* tac *promoter, and the protein was purified. Mild oxidation generated a disulfide bond between the new Cys*^{3} *and Cys*^{97}*, one of the two unpaired cysteines of the native molecule. Oxidized T4 lysozyme (Ile*^{3} *→ Cys) exhibited specific activity identical to that of the wild-type enzyme when measured at 20°C in a cell-clearing assay. The cross-linked protein was more stable than the wild type during incubation at elevated temperatures as determined by recovered enzymatic activity at 20°C.*

In globular proteins, disulfide bonds provide conformational stability (*1, 2*), although the detailed mechanisms by which these bonds influence not only the stability of native structures but also pathways of folding and unfolding have yet to be defined. Until recently, investigators have been limited to one basic approach—the study of protein derivatives generated through the chemical cleavage of disulfide bonds. Partly because of this limitation, most folding and stability studies on disulfide-containing proteins have been designed so that disulfide bond formation is a component of the folding process (*3*). Yet it is also of interest to investigate the differences in stability and folding pathways between two proteins that differ throughout an experiment in the number of cross-links (*4, 5*). For example, it has been shown that a disulfide-free derivative of α interferon irreversibly loses elements of native conformation when incubated under conditions tolerated by the native disulfide-bonded molecule (*5*).

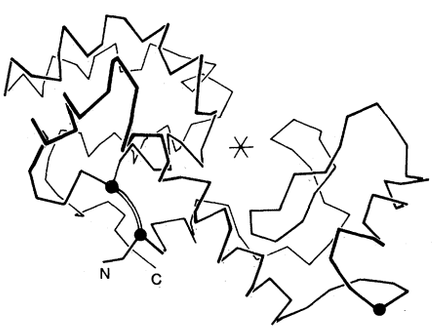

Fig. 1. Computer graphics simulation of T4 lysozyme (Ile^3 → Cys) α-carbon chain, showing the amino- and carboxyl-chain termini (N and C, respectively), the three cysteines (●), and the active site (star). Cys^3 and Cys^{97} are connected by a schematic disulfide.

Recent advances in techniques for specific mutagenesis (*6*) and efficient expression (*7*) of genes cloned into *Escherichia coli* make possible other experimental approaches. To study the modes by which disulfide bonds can stabilize proteins, we used these recombinant DNA methods to insert disulfide bonds into a normally disulfide-free protein. We chose as a model phage T4 lysozyme (*8*), a muramidase whose biological function is to break down the cell wall of an infected bacterium to facilitate the lytic release of replicated phage. The protein's x-ray crystal structure is known (*9*), and it has two unpaired cysteines and limited thermal stability (*8*). The thermodynamics of unfolding of T4 lysozyme have been studied through the thermal and solvent-effected reversible denaturation of the wild-type molecule and some temperature-sensitive mutants (*10*). T4 lysozyme also exhibits structural homology with egg-white lysozyme (*9*), a protein that contains four disulfide bonds. Recently we achieved efficient expression in *E. coli* of plasmid-encoded T4 lysozyme genes (*11*).

Theoretical calculations suggest that the degree of conformational stability conferred upon a protein by a cross-link increases with the number of amino acids of primary sequence that it spans (*1, 2*). We searched the T4 lysozyme crystal structure (*9*) for sets of amino acids widely separated in primary sequence but with side chain β carbons lying close (within 5.5 Å) in space. We further examined some of these by building simulations of disulfides with the use of computer graphics (*12*) (vector graphics PS300, Evans and Sutherland) and assessing them for the appropriate geometry (*1*). In no case was a simulated disulfide identical in structure to any protein disulfides characterized by x-ray diffraction (*1*). Several candidates, however, appeared to be particularly favorable by these criteria, and one of these was the disulfide produced between cysteines at positions 3 and 97. Examination of this simulated disulfide bond yielded the following values for its structural parameters (*1*): x_1, 117°; x_2, 25°; x_3, 134°; x'_2, 43°; x'_1, −162°; αC-αC distance, 5.71 Å; and βC-βC distance, 4.49 Å. Since residue 97 in wild-type T4 lysozyme is a cysteine, we chose to make a disulfide cross-link by introducing a cysteine at position 3 (Fig. 1).

Using the synthetic oligonucleotide

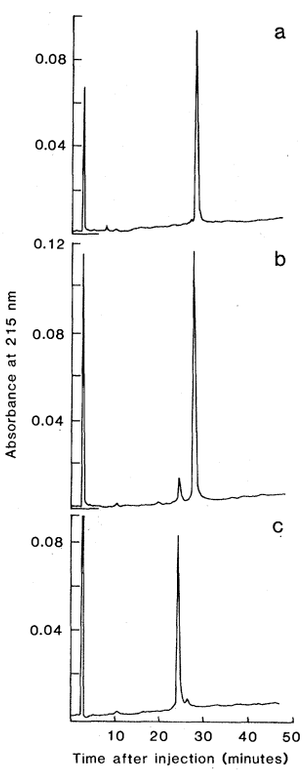

Fig. 2. Reversed-phase HPLC of lysozyme derivatives. Samples were loaded on a Vydak 218-TP546 C_{18} column (Rainin) and eluted at 1 ml/min with a linear gradient of 37 to 52 percent acetonitrile in 0.1 percent aqueous trifluoroacetic acid at 0.33 percent acetonitrile per minute. (a) Wild-type T4 lysozyme, (b) reduced T4 lysozyme (Ile^3 → Cys), and (c) oxidized T4 lysozyme (Ile^3 → Cys).