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8. A. Meylan, unpublished data.
9. Females: $\bar{x}$ = 95.2%, SD = 7.5, range 78.3 to 99.2, n = 7; males: $\bar{x}$ = 96.4%, SD = 7.7, range 72.3 to 99.9, n = 12, Mann-Whitney U test, P = 0.2067.
10. Panama: $\bar{x}$ = 96.3%, SD = 8.0, range 72.3 to 99.9, n = 11; Dominican Republic: $\bar{x}$ = 95.8%, SD = 2.2, range 93.4 to 97.9, n = 4; Lesser Antilles: $\bar{x}$ = 96.2%, SD = 5.8, range 78.3 to 99.6, n = 12; Kruskal-Wallis test, P = 0.1089. Sample includes nongravid turtles greater than 25-cm carapace length with complete digestive tract contents.
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Hormone Conjugated with Antibody to CD3 Mediates Cytotoxic T Cell Lysis of Human Melanoma Cells

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Cytotoxic T lymphocytes can be activated by antibodies to their antigen-specific receptor complex (TCR-CD3) to destroy target cells, regardless of the specificity of the cytotoxic T cells. A novel hormone-antibody conjugate, consisting of an analog of melanocyte-stimulating hormone chemically coupled to a monoclonal antibody to CD3, the invariant component of the T cell receptor complex, was used to target human melanoma cells for destruction by human cytotoxic T lymphocytes that bear no specificity for the tumor cells. As targeting components of such anti-CD3 conjugates, hormones or growth factors are expected to prove more effective than antibodies to tumor-associated antigens in focusing the destructive activity of cytotoxic T cells on tumor target cells.

IT IS WIDELY RECOGNIZED THAT THE specificity of antibodies for cell-surface ligands and of hormones for receptors on cells offers possibilities for delivering therapeutic agents to selected target cells. Thus, chemotherapeutic drugs and toxins have been attached to antibodies that are specific for tumor-associated antigens [reviewed in (*1*)], and hormones have been used as vectors for directing chemotherapeutic agents to tumor cells (*2*). In alternative approaches, antibody conjugates, referred to as heterobifunctional antibody duplexes, have been used to target tumor cells for destruction by killer T cells (*3*). In the duplexes, one antibody (the "targeting" antibody) is specific for a tumor antigen and the other (the "effector" antibody) binds specifically to the antigen receptor complexes on T cells, activating their effector functions regardless of the antigens they normally recognize. Activated cytotoxic T lymphocytes (CTLs) lyse the cells to which they are adherent, and the antibody duplexes can thus induce CTLs to kill tumor cells for which they bear no specificity. However, tumor cell variants that lack tumor-associated antigens are often found (*4*). In contrast, the growth of many tumors is dependent on their cell-surface receptors for hormones or autocrine growth factors (*5*). Hence, hormones or autocrine growth factors might offer advantages over antibodies to tumor-associated antigens as targeting reagents.

We describe a novel conjugate in which a hormone, substituting for the targeting antibody, is covalently joined to an antibody to the CD3 component of the T cell receptor (TCR)-CD3 complex. The hormone, termed MSHa, is an analog of α-melanocyte–stimulating hormone (MSH); it is specific for the MSH receptor on melanoma cells (*6*). We show that, in the presence of this conjugate, CTLs destroy melanoma cells. The conjugate may be regarded as a useful model for exploring the more general use of growth factor–antibody conjugates to mediate destruction of tumor cells and other factor-dependent cells by cytotoxic cells.

The MSHa–anti-CD3 conjugate (*7*) mediated specific lysis of the human melanoma line B16F10 (*8*) by the human $CD8^+$ cloned CTL cell line 6B7 (*9*) (Fig. 1). That these CTLs were not specific for the melanoma cells they killed was evident from their failure to lyse these cells in the absence of the conjugate. Individual components of the conjugates—that is, the free MSHa alone (*6*) and anti-CD3 alone (*10*)—did not mediate CTL lysis of the melanoma cells. Indeed, each of them, and also antibodies to MSH (*11*), blocked the effect of the conjugate (Fig. 1B) in a dose-dependent manner (Fig. 2, A, B, and C). These results indicate that conjugate-mediated lysis of the melanoma cells depends on the binding of the conjugate's anti-CD3 moiety to the CTL and the binding of its MSHa moiety to the MSH receptor on the B16F10 cells; unconjugated anti-CD3 blocked the former interaction and unconjugated MSHa and anti-MSH blocked the latter interaction.

As in conventional antigen-dependent lysis of target cells by CTLs, the conjugate-mediated lysis of melanoma cells also increased as the ratio of CTLs to melanoma cells increased. Thus, at ratios of 0.5, 1, 5, 10, and 20 CTLs to one melanoma cell, the amount of specific lysis was 9, 14, 30, 34, and 42 percent, respectively, in the presence of conjugate ($\sim 2 \times 10^{-8} M$). When the ra-

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tio was fixed (at 10:1) and the conjugate concentration was systematically increased from $7 \times 10^{-13}M$ to $7 \times 10^{-8}M$, the extent of lysis increased to a maximum and then decreased. Thus, at conjugate concentrations of 0.07×10^{-9}, 0.2×10^{-9}, 0.7×10^{-9}, 2×10^{-9}, 7×10^{-9}, 20×10^{-9}, and $70 \times 10^{-9}M$, specific lysis of B6F10 melanoma cells was 0.5, 5, 7, 35, 60, 55, and 42 percent, respectively. A maximum is understandable because at concentrations beyond the optimum ($\sim 7 \times 10^{-9}M$) those MSHa–anti-CD3 conjugates that are bound to one cell (CTL or target cell) have to compete with excess free conjugate for binding sites on the complementary cell, resulting in less CTL–target cell cross-linking.

In the absence of any antigen recognition, unconjugated antibodies to CD3 can stimulate CTLs to lyse those cells that have surface receptors for the Fc domain of the antibodies (*12*), probably because an array of anti-CD3 molecules on the prospective target cell brings about aggregation or cross-linking of TCR-CD3 complexes and activates the adherent CTL (*13*). Such a mechanism seemed at first to account for our observations with other melanoma cell lines—for example, M1313, whose lysis by CTL clone 6B7 was mediated not only by the MSHa–anti-CD3 conjugate (Fig. 3A), but also (though less extensively) by anti-CD3 alone. (For B16F10 cells, this effect appeared only at extremely high concentrations of anti-CD3, $2.5 \times 10^{-7}M$.) The inhibition pattern of the lysis that was mediated by anti-CD3 alone differed from that mediated by the conjugate; thus, the uncoupled MSHa and antibody to MSH inhibited conjugate-mediated lysis, but they did not affect the lysis mediated by anti-CD3 alone.

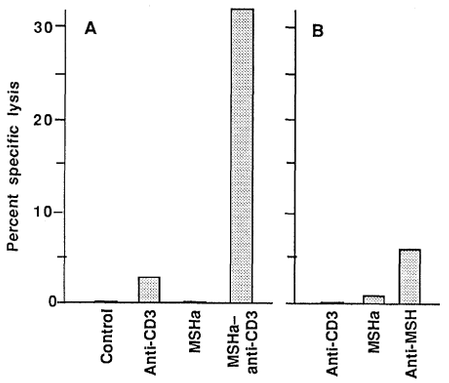

Fig. 1. (**A**) Cells of the human melanoma cell line B16F10 were targeted for lysis by cytotoxic T lymphocytes of the human cloned CTL cell line 6B7 ($CD8^+$, $CD4^-$) by a hormone-antibody conjugate. The 6B7 cells (2×10^5) (*9*) were incubated in U-bottom 96-well plates (Costar) with 2×10^4 B16F10 cells (*8*) in a total volume of 200 μl of culture medium containing various concentrations of either the MSH analog (*6*) or a monoclonal antibody to CD3 (*10*), or the MSHa–anti-CD3 conjugate (*7*). After 4 hours at 37°C, the plates were centrifuged at 1500 rpm for 5 minutes, and 75 μl of each supernatant was assayed for radioactivity. Percent-specific lysis was calculated as $[(a - b)/(c - b)] \times 100$, where a is the experimental ^{51}Cr release (counts per minute) from target cells in the presence of effector cells and antibody, peptide, or peptide-antibody conjugate; b is the ^{51}Cr release from labeled target cells in the absence of both CTLs and antibodies or peptides—that is, the spontaneous release (12 percent on average); and c is the maximal lysis determined by incubating 2×10^4 ^{51}Cr-labeled target cells in 200 μl of 1*N* HCl for the 4 hours (about 85 percent of their total ^{51}Cr content). The results are averages of duplicates, which generally agreed to within ±10 percent of the mean. The controls contained CTLs and B16F10 melanoma cells with no antibody or MSH analog. The final concentrations of anti-CD3, MSHa, and MSHa–anti-CD3 conjugates were $8 \times 10^{-9}M$, $7 \times 10^{-9}M$, and $\sim 10^{-8}M$, respectively (*6*). (**B**) Anti-CD3, MSHa, and anti-MSH inhibit the cytotoxic effect of the MSHa–anti-CD3 conjugate. The CTLs (6B7 cells) were incubated with ^{51}Cr-labeled B16F10 human melanoma cells at a 10:1 ratio in the presence of the MSHa–anti-CD3 conjugate (at a final concentration of $\sim 2 \times 10^{-8}M$) and unconjugated anti-CD3 ($3 \times 10^{-7}M$), MSHa ($4 \times 10^{-9}M$), or anti-MSH (a 1:200 dilution of the stock solution) (*11*). After 4 hours at 37°C, the ^{51}Cr release was measured as described in Fig. 1A.

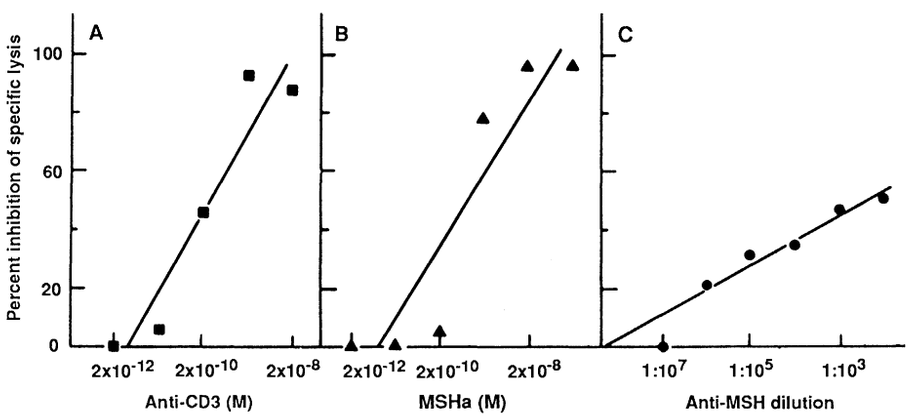

Fig. 2. Concentration dependence of the inhibitory effects of anti-CD3, MSHa, and anti-MSH on the MSHa–anti-CD3 conjugate–mediated lysis of B16F10 melanoma cells by CTLs. CTLs of clone 6B7 were incubated with B16F10 melanoma cells at a 10:1 ratio in the presence of the MSHa–anti-CD3 conjugate ($2 \times 10^{-8}M$) and the indicated concentrations of anti-CD3, MSHa, and anti-MSH. The stock solution of anti-MSH (*11*) was diluted 1:3 prior to the dilutions indicated. The percent-specific lysis for MSHa–anti-CD3 and CTLs in the absence of inhibitors (calculated as in Fig. 1A) in the 4-hour ^{51}Cr-release assay was 66 percent. The percent inhibition was calculated as $[(a - b)/a] \times 100$, where a is the MSHa–anti-CD3–mediated CTL lysis of B16F10 cells observed in the absence of inhibitors and b is the specific lysis in the presence of a given concentration of inhibitor.

Although the presence of Fc receptors on M1313 melanoma cells could explain their susceptibility to anti-CD3–mediated lysis by CTLs, we were unable to demonstrate these receptors on the M1313 cells by means (i) of flow cytometry using either antibodies to CD3 or to Fc receptor (antibody 3G8) (*14*) plus a fluorescein-labeled second antibody, (ii) by direct or indirect binding of ^{125}I-labeled anti-CD3, or (iii) by attempting to inhibit the reaction with a large excess of nonspecific antibody of the same immunoglobulin G subclass. In the absence of demonstrable Fc receptors on target cells, others have also observed that intact (Fc-bearing), ostensibly monomeric antibodies can activate T cell effector functions (*15*). Nevertheless, it remained possible that Fc receptors were present below the level of detection on M1313 cells and were responsible for their lysis by CTLs in the presence of unconjugated anti-CD3. Therefore we tested the activity of $F(ab')_2$ fragments of anti-CD3 (*16*). These fragments, which were free of intact antibody (inset, Fig. 3B) and which had been ultracentrifuged at >100,000*g* to remove aggregates, were still able to mediate (in the presence of CTLs) some lysis of M1313 cells, although less efficiently than intact anti-CD3 (Fig. 3B). These results suggest that even the minimal degree of cross-linking of TCR-CD3 receptors expected to result from the activity of monomeric bivalent anti-CD3 $F(ab')_2$ fragments may be able to induce low levels of cytolytic activity of CTLs. The differences in susceptibility of various melanoma cells to anti-CD3–mediated lysis by CTLs might reflect differences in the extent to which they adhere nonspecifically to T cells (*17*).

Although CTL lysis of tumor cells can be mediated by heteroantibody duplexes in which anti-CD3 is linked with antibodies to tumor-specific antigens (*3*), hormone-antibody conjugates offer potential advantages. For instance, some tumors secrete the antigens that might otherwise serve as cell-surface markers for targeting antibodies—for example, carcinoembryonic antigen (CEA). By competing with CEA on tumor

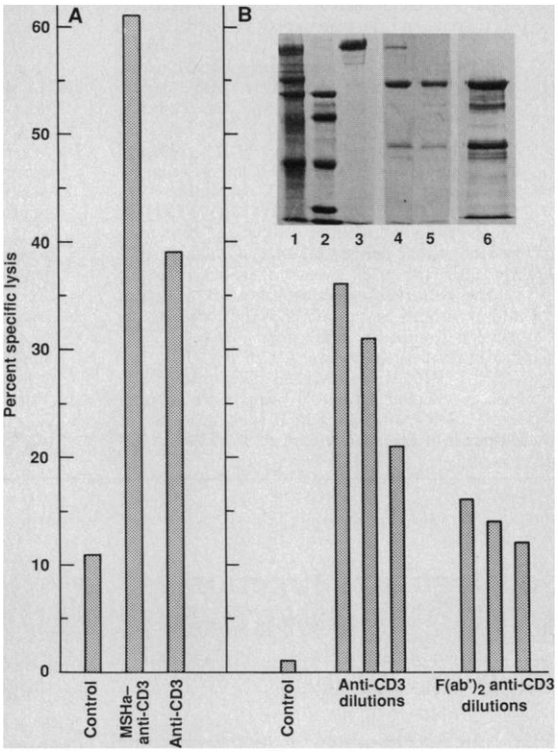

Fig. 3. (**A**) Unconjugated anti-CD3 mediates CTL lysis of human melanoma line M1313 but less than the MSHa–anti-CD3 complex does. A 4-hour ^{51}Cr-release assay was performed with human CTL clone 6B7 and human melanoma line M1313 (*8*) as described in Fig. 1A. The control, which contained only CTLs and M1313 cells, with no antibody, conjugate, or F(ab')$_2$ fragments present, was repeated for each experiment to reveal any natural killer–like activity by the CTLs; such activity is often expressed for up to about 10 days after stimulation of CTL cultures with phytohemagglutinin and fresh allogeneic peripheral blood lymphocytes (*9*). The results are the averages of duplicates, which agreed to within ±3 percent of the mean. (**B**) The ability of both intact anti-CD3 and F(ab')$_2$ fragments of anti-CD3 to mediate CTL lysis of human melanoma cells. The CTLs (6B7) and ^{51}Cr-labeled human melanoma cells (M1313) were incubated in the presence of intact or F(ab')$_2$ anti-CD3 for a standard 4-hour ^{51}Cr-release assay. The control (CTL plus M1313 cells in the absence of antibody) is lower than in (A), probably because the CTLs no longer expressed natural killer–like cytolytic activity. The results are the averages of duplicates, which generally agreed to within ±3 percent of the mean. The concentrations of anti-CD3 and F(ab')$_2$ anti-CD3 from left to right were $2.5 \times 10^{-7}M$, $8 \times 10^{-8}M$, and $8 \times 10^{-9}M$ for each. Inset: Evidence that the F(ab')$_2$ fragments of anti-CD3 were free of intact (Fc-bearing) anti-CD3 antibody. Polyacrylamide minigels (Bio-Rad, Mini-Protean II) (7 percent) were run at constant voltage (200 V) and stained with Coomassie brilliant blue (lanes 1 to 5 from left to right) or silver stain (*16*). (Lane 1) High molecular weight markers (Bio-Rad), 43,000 to 200,000; (lane 2) low molecular weight markers (Bio-Rad), 14,000 to 97,000; (lane 3) intact anti-CD3; (lane 4) F(ab')$_2$ digest before purification; (lane 5) F(ab')$_2$ after purification on a protein A column; (lane 6) F(ab')$_2$ after two passes over a protein A column (silver stain). No contaminating intact antibody is seen.

cells, high levels of circulating CEA would inhibit binding of the heteroantibody duplexes to the tumor. Moreover, the expression of many tumor-specific surface antigens is not essential to tumor cell survival, and tumor cell variants that lack these antigens are commonplace [for example, see (*4*)]. In contrast, receptors for hormones and growth factors may be expected to be related more consistently to the malignant state of the tumor cell.

In the hormone-antibody conjugate described here, both the hormone and antibody moieties are highly specific for their respective receptor and ligand; hence the conjugate can promote the adhesion and activation of CD3$^+$ T cells on target cells that the T cells would otherwise not recognize. How the anti-CD3 moiety of the conjugate induces the cytolytic activity of those T cells (CD8$^+$ T cells and some CD4$^+$ T cells) that inherently have this capability is not entirely clear, but it is likely that the simultaneous binding of the conjugate to CD3 on a CTL and to MSH receptors on a target cell results in aggregation of the CTL's TCR-CD3 complexes.

Although we have dealt with an analog of MSH as the targeting reagent, it is obvious that this peptide is representative of a large number of other peptide hormones and growth factors. Similarly, the anti-CD3 moiety can very likely be replaced by other effector antibodies capable of activating other cytotoxic cells—for example antibodies to Fc receptors that activate various cytotoxic cells (*18*).

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6. MSHa is a 13–amino acid peptide with the sequence Ac-Ser-Tyr-Ser-Nle-Glu-His-dPhe-Arg-Trp-Gly-Lys-Pro-Val-NH$_2$. It differs from α-MSH (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro Val-NH$_2$) at two positions. A norleucine (Nle) substitutes isosterically for L-methionine at position 4, and D-phenylalanine substitutes for phenylalanine at position 7. MSHa is more potent than α-MSH in stimulating adenylate cyclase activity [T. K. Sawyer *et al.*, *J. Med. Chem.* **25**, 1022 (1982)] and melanoma tyrosinase activity [M. M. Marwan *et al.*, *Mol. Cell. Endocrinol.* **41**, 171 (1985)]. The α-MSH analog was synthesized with a cysteine residue at its amino terminus to permit coupling to anti-CD3.
7. The MSHa–anti-CD3 conjugate was constructed as follows. A fivefold molar excess of maleimidobenzoyl sulfonate (Pierce) in tetrahydrofuran (5 mg/ml) or a sevenfold molar excess of *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pierce) in absolute ethanol (6.2 mg/ml) was added with vortexing to anti-CD3 (2.27 mg/ml) (*10*) in sodium phosphate buffer, *p*H 7.4 (0.14*M* NaCl, 1 m*M* KCl, and 3.7 m*M* sodium-potassium phosphate). After 65 minutes at room temperature, the reaction mixture was run over a G-75 Sephadex column in 0.1*M* sodium phosphate, *p*H 6.00. The void volume (detected by absorbancy at 280 nm) was concentrated with a Millipore CX-10 immersible filter to 0.5 ml. A molar excess of the cysteine-modified MSH analog (*6*) was added to the modified anti-CD3 after 45 to 60 minutes (15 minutes for the SPDP-modified antibody) at room temperature, with constant rotation on a mixing wheel. The reaction mixture was purified over a G-75 Sephadex column in sodium phosphate buffer, *p*H 7.4. The first peak was then loaded onto an affinity column containing antibody to MSH (*11*) coupled to an Affi-Gel-10 support. After the column was washed with sodium phosphate buffer, *p*H 7.4, the MSHa–anti-CD3 duplex was eluted with 0.23*M* glycine HCl, *p*H 2.6, dialyzed against sodium phosphate buffer, *p*H 7.4, and stored at 4°C. The concentration of MSHa–anti-CD3 conjugate was made similar to that of anti-CD3 by estimating the extinction coefficient of the conjugate (at 280 nm) to be 1.5 times that of anti-CD3.
8. Human melanoma lines B16F10 and M1313 were maintained in RPMI 1640 medium supplemented with 10 percent fetal calf serum, 50 μ*M* 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 m*M* L-glutamine, and 10 m*M* Hepes, hereafter referred to as K medium. The cells grew in adherent fashion and were passaged serially when confluent (every 4 to 7 days). Cells were removed from flasks with a cell scraper (Costar) or by incubating them with K medium containing 10 m*M* EDTA.
9. Human CD8$^+$ CTL clone 6B7 was previously described [T. G. Mayer *et al.*, *J. Immunol.* **134**, 258 (1985)]; it was maintained in RPMI 1640 supplemented with 5 percent fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and recombinant interleukin-2 (Biogen). The cells were stimulated every 2 to 4 weeks by adding phytohemagglutinin (5 μg/ml) and a 5:1 or 10:1 excess of fresh allogeneic peripheral blood lymphocytes that had been isolated on Ficoll-Hypaque and irradiated with 4500 rads. The day after stimulation, the cells were washed (to remove the phytohemagglutinin) and fresh medium was added every 2 to 4 days to maintain the cell concentration at about 2×10^5 cells per milliliter of medium.
10. The monoclonal antibody to CD3 (anti-CD3) has been described [J. T. Kurnick, A. I. Lazarovits, C. P. Leary, D. Camerini, R. B. Colvin, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 1318 (1985)]. It was stored at 3.3 mg/ml in 0.04 percent sodium azide at 4°C. Prior to use it was dialyzed into sodium phosphate buffer, *p*H 7.4 (0.14*M* NaCl, 1 m*M* KCl, and 3.7 m*M* sodium-potassium phosphate) and brought to a final concentration of 2.27 mg/ml.
11. Rabbit antiserum to MSH was generated by multiple injections of synthetic α-MSH coupled to thyroglobulin. The serum was assayed for binding activity to α-MSH in a radioimmunoassay. A stock solution of antibody to MSH (in phosphate-buffered saline, *p*H 7.4) was prepared as the total immunoglobulin

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16. The $F(ab')_2$ fragment of anti-CD3 12F6 was prepared by diluting the antibody to 1.35 mg/ml in 0.1*M* acetate buffer, *p*H 4.1 [P. Parham, in *Immunochemistry*, D. M. Weir, L. A. Herzenberg, C. Blackwell, L. A. Herzenberg, Eds. (Blackwell, Oxford, 1986), pp. 14.11 and 14.14]; 175 μl of pepsin (1 mg/ml of pepsin in 0.1*M* acetate buffer, *p*H 4.1) was added and the mixture was digested at 37°C for 19.5 hours. The reaction was stopped by adding 3*M* tris-HCl, *p*H 8.6, to bring the *p*H above 7. The reaction mixture was then passed over a protein A–Sepharose 4B column, and the material not retained [$F(ab')_2$ fraction] was dialyzed against phosphate-buffered saline, *p*H 7.4; before use in chromium-51 release assays, it was ultracentrifuged at >100,000*g* to remove aggregates. The same extinction coefficient (1.4 mg^{-1} ml) was used for the $F(ab')_2$ fragments and intact antibody. The purified $F(ab')_2$ contained no remaining intact immunoglobulin visible by silver staining [C. R. Merril, D. Goldman, S. A. Sedman, M. H. Ebert, *Science* **211**, 1437 (1981)] a 7.5 percent polyacrylamide gel that had been overloaded with the purified $F(ab')_2$.
17. The differences in adhesiveness of various melanoma sublines to certain tissues appears to be correlated with variations in the morphology, motility, and metastatic potential of the melanoma cells [P. A. Netland and B. R. Zetter, *J. Cell Biol.* **101**, 720 (1985)]. B16F10 and M1313 differ markedly in their morphology (B16F10 being more cuboidal and M1313 spindle-shaped). It is possible that the difference in their susceptibility to lysis by anti-CD3 (unconjugated) and CTLs might be related to differences in expression of adhesion molecules, enabling the CTLs to bind M1313 cells to a greater degree than B16F10 cells.
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19. We thank G. Matsueda for the synthesis of the cysteine-modified MSHa, R. Granstein for the human melanoma line B16F10, R. Bringhurst for the human melanoma line M1313, J. Kurnick for CTL clone 6B7, J. Unkeless for anti-FcR, Damon Biotech for anti-CD3 produced from hybridoma cells provided by R. Colvin, Biogen Research Corporation for rIL-2, and A. Hicks for help in preparing this manuscript. This work was supported by Cancer Center Core grant CA14051, by PHS grants CA28900, CA25472, and CA42504, and by Physician Scientist award AI00679 from the National Institutes of Health (to M.A.L.).

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The Cellular *src* Gene Product Regulates Junctional Cell-to-Cell Communication

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Overexpression of the cellular *src* gene in NIH 3T3 cells causes reduction of cell-to-cell transmission of molecules in the 400- to 700-dalton range. This down-regulation of gap junctional communication correlates with the activity of the gene product, the protein tyrosine kinase $pp60^{c\text{-}src}$. The down-regulation was enhanced by point mutation of Tyr^{527} (a site that is phosphorylated in $pp60^{c\text{-}src}$ and that inhibits kinase activity) or by substitution of the viral-*src* for the cellular-*src* carboxyl-terminal coding region. Mutation of Tyr^{416} (a site phosphorylated upon Tyr^{527} mutation) suppresses both the down-regulation of communication by Tyr^{527} mutation and that by gene overexpression. The regulation of communication by *src* may be important in the control of embryonic development and cellular growth.

THE CELLULAR *src* GENE (c-*src*) (*1*) encodes the membrane-bound protein tyrosine kinase, $pp60^{c\text{-}src}$ (*2*). The gene is present in all species and is highly conserved. Its functions are still unknown. We have explored the possibility of a function in junctional communication, prompted by the finding that this communication is reduced by polyomavirus middle T antigen (*3*). This antigen binds to $pp60^{c\text{-}src}$, enhancing the protein tyrosine kinase activity of $pp60^{c\text{-}src}$ (*4*). We now report that junctional communication is reduced when the level of $pp60^{c\text{-}src}$ is elevated by incorporating c-*src* expression plasmids into mammalian cells, and that this action is enhanced by modifications in the carboxyl-terminal region of $pp60^{c\text{-}src}$, which increase transforming activity.

Our expression vectors contained the chicken c-*src* coding sequences ligated to retroviral long terminal repeats which provided promoter-enhancer sequences and polyadenylation sites (Fig. 1). Mouse NIH 3T3 cells were transfected with these plasmids, and cell lines were cloned from foci (suffix *foc*) or coselected for expression of *Eco-gpt* (suffix *cos*) or *neo* (*5*). The $pp60^{c\text{-}src}$ expression levels were determined from immunoprecipitates of cells with monoclo-

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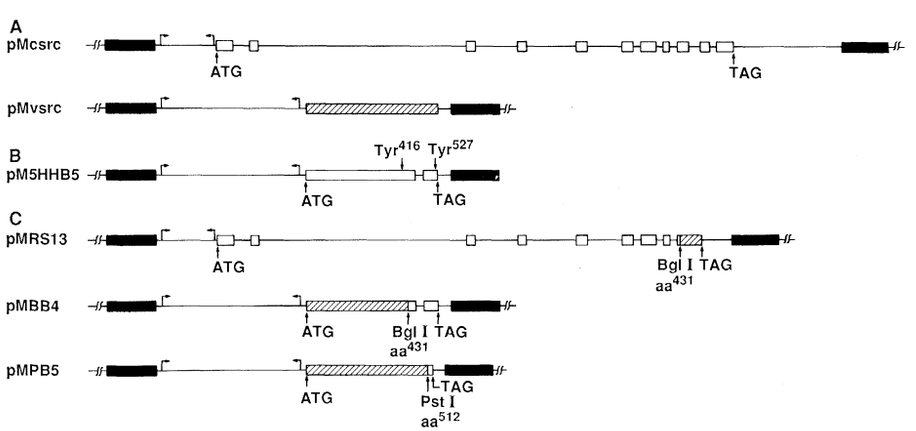

Fig. 1. The c-*src* and v-*src* expression plasmids. The plasmids contain the chicken c-*src*, v-*src*, mutant, and chimeric *src* genes ligated to transcriptional enhancers, promoters, and polyadenylation signals provided by flanking Moloney murine leukemia virus long terminal repeats (filled boxes) from the vector pEVX (*23*). The locations of Moloney splice donors and first *src* splice acceptors are denoted by bent arrows. Coding regions are indicated by open (c-*src*) or hatched boxes (v-*src*), and the locations of the initiating (ATG) and terminating (TAG) codons are marked. Only the functionally relevant eukaryotic regions of the plasmids are shown (*11, 20*). (**A**) pMcsrc, the plasmid used in the experiments on c-*src* overexpression, and the structurally similar v-*src* expression plasmid pMvsrc; (**B**) pM5HHB5, the plasmid used for site-directed mutagenesis. The Tyr → Phe codon exchanges were produced at amino acids 416, 527, or both, generating plasmids pcsrc416, pcsrc527, and pc416 527 by replacing the small fragments containing the codons to be exchanged with DNA fragments containing the appropriate mutations. These mutated plasmids have the same restriction map as pM5HHB5. (**C**) Plasmids pMRS13 and pMBB4 are mirror-image chimeric constructions with junctions at site Bgl I at amino acid (aa) codon 431. pMPB5 is similar to pMBB4, except that the junction is at the Pst I site at amino acid 512.