(1991)] with DGII in the INSIGHT II package [NMRchitect, version 2.3; Biosym Technologies, San Diego (1993)] based on the experimental restraints described in (*16*). In addition, the sugar residues were forced into their lowest energy chair conformations with 68 dihedral angle restraints. Embedded structures were optimized with a simulated annealing protocol [M. Nilges, M. G. Clore, A. M. Gronenborn, *FEBS Lett.* **229**, 317 (1988)] and subsequently energy minimized with experimental restraints according to (*3*). The average root-mean-square deviation from the mean structure was 0.78 Å for all backbone heavy atoms of residues 7 to 103 and the heavy atoms of the trisaccharide core with an SD of 0.07 Å. The 16 structures had on average 16 violations of distance restraints above 0.1 Å with no violation greater than 0.3 Å and on average 21 violated dihedral angle restraints with no violation greater than 4°. Atomic coordinates have been deposited in the Brookhaven protein data bank (access code 1GYA).

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23. Digestion of $hsCD2_{105}$ with endo H and PNGase F was as follows: $HsCD2_{105}$ was digested with PNGase F and purified as described (*14*). Endo H digestions were done in 50 mM citrate (pH 5.5) at a protein concentration of 400 μg/ml. Endo H was added at a concentration of 20 mU and the sample was incubated overnight at 37°C. Digested $hsCD2_{105}$ was then purified with a TSK 2000SW gel filtration column equilibrated in 20 mM sodium acetate, 200 mM $NaSO_4$ (pH 5).
24. For site-directed mutagenesis, the full-length CD2 sequence in M13mp18 vector was used as the parental plasmid for generating the CD2 mutant (*10*). Mutagenesis was done according to the thionucleotide method (Amersham), with sense oligonucleotides and antisense template. The mutants were confirmed by sequencing the mutated single-stranded M13-CD2. Mutant CD2 inserts were subcloned to CDM8 expression vector at the Xba I site (*10*). The entire mutant CD2 adhesion domain in CDM8 was resequenced to ensure the correct mutation in each construct. For transfection, SRBC rosetting, and immunofluorescence assays, COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Bio*whittaker) containing 10% fetal calf serum (FCS), 2 mM glutamine, and 1% penicillin and streptomycin. Cells were seeded in six-well dishes at 5×10^4 per well and subsequently transfected the next day with CDM8-CD2 as described [N. R. Landau and D. R. Littman, *J. Virol.* **66**, 5110 (1992)]. For immunofluorescence, transfected cells were released after 48 hours by incubation in 0.02% EDTA–phosphate-buffered saline (PBS) for 5 to 10 min. The released cells were analyzed by indirect immunofluorescence, with anti-CD2 mAbs 3T48B5 and 35.1 (1:100 dilution of ascites), anti-$T11_2$ (10 μg/ml), and M32B (*10*) rabbit anti-human CD2 heteroantiserum (1:300 dilution). COS-7 cells transfected with the CDM8 vector alone were used as a negative control. The SRBC rosetting assay was done as described (*10*), with 1 ml of 1% SRBC in 5% FCS-RPMI added to each well 48 hours after transfection. After a 1-hour incubation at 37°C followed by four PBS washes, 200 COS-7 cells were examined for rosette formation. A rosette-positive cell was defined as one COS 7 cell binding to ≥ five SRBCs. Vector alone–transfected COS-7 cells served as the negative control.
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Commitment of CNS Progenitors Along the Dorsoventral Axis of *Drosophila* Neuroectoderm

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In the *Drosophila* embryo, the central nervous system (CNS) develops from a population of neural stem cells (neuroblasts) and midline progenitor cells. Here, the fate and extent of determination of CNS progenitors along the dorsoventral axis was assayed. Dorsal neuroectodermal cells transplanted into the ventral neuroectoderm or into the midline produced CNS lineages consistent with their new position. However, ventral neuroectodermal cells and midline cells transplanted to dorsal sites of the neuroectoderm migrated ventrally and produced CNS lineages consistent with their origin. Thus, inductive signals at the ventral midline and adjacent neuroectoderm may confer ventral identities to CNS progenitors as well as the ability to assume and maintain characteristic positions in the developing CNS. Furthermore, ectopic transplantations of wild-type midline cells into *single minded* (*sim*) mutant embryos suggest that the ventral midline is required for correct positioning of the cells.

The development of the CNS in insects begins shortly after gastrulation, when about 30 neuroblasts (NBs) per hemisegment delaminate from the neuroectoderm according to a stereotypical spatial and temporal pattern (*1*–*3*). Each of these NBs gives rise to a characteristic cell lineage (*4*–*6*). Although the genetic network that regulates NB formation is well understood [reviewed in (*7*)], the mechanisms leading to the specification of the individual NBs are unknown. Candidate genes for NB specification include segmentation genes (*8*, *9*), homeotic genes (*10*), and dorsoventral patterning genes (*11*, *12*). Here, we investigated to what extent the fate of progenitors is determined before their delamination from the ectoderm.

By labeling individual early gastrula cells with the lipophilic fluorescent tracer DiI (*6*) or by isotopic transplantation of single cells labeled with horseradish peroxidase (HRP) (*13*) at the same stage, we have clarified the embryonic lineages of the mesectodermal midline progenitors (*6*) and of several NBs. The type of lineage formed by the various NBs is clearly identifiable by the morphology, number, and position of the neuronal or glial progeny (or of both) and is dependent on their dorsoventral site of origin from the neuroectoderm (Fig. 1, A through E) (Table 1) (*5*, *14*).

To examine the degree to which neuroectodermal progenitor cells (nPCs) and midline cells of the early gastrula [stage 7; stages are described according to (*15*)] are specified to produce specific neural lineages, we performed heterotopic transplantations (Fig. 2 and Table 1). The experiments were designed to test determination as a function of dorsoventral but not of anteroposterior position in the ectoderm. In one experiment (I in Fig. 2), single nPCs were transplanted from dorsal sites [30 to 50% ventrodorsal perimeter (% VD)] of HRP-labeled donors to ventral sites (0 to 20% VD) of unlabeled hosts that were allowed to develop until late embryonic stages (stage 17). From these transplantations, 129 CNS clones were obtained; 81 of these lineages corresponded to ventral NBs (*16*), and 38 clones corresponded to midline lineages (*17*) (Fig. 1, A, B, and E). In 10 cases, identification of the lineage was not possible. The switch to ventral fates of transplanted dorsal nPCs is also corroborated by the expression of a molecular marker. The enhancer-trap line X55 specifically expresses the *lacZ* reporter gene in ventral midline cells (*18*). Dorsal nPCs (30 to 50% VD), which normally do not express the marker, were taken from HRP-labeled X55 donors and transplanted into ventral midline positions of wild-type hosts. Midline clones derived from these transplants stained positive for *lacZ* expression (Fig. 1G) [for *single minded* (*sim*)–*lacZ* expression, see (*19*)]. Thus, dorsal nPCs of the early gastrula are not firmly specified, inductive signals exist at ventral sites to confer ventral neural identities, and dorsal nPCs are receptive to

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the ventral inductive signals.

In a second experimental series, transplantations were performed in the reverse direction (II and III in Fig. 2 and Table 1). Cells were selectively removed either from the ventral midline, which can be unambiguously identified in the early gastrula by position and shape (6), or from the adjacent ventral neuroectoderm (5 to 20% VD). In both cases, the cells were individually implanted at dorsal sites of the neuroectoderm (30 to 50% VD). In contrast to the heterotopically transplanted dorsal nPCs, the ventral cells behaved according to their origin in that they gave rise to clones typical of ventral progenitors. In total, we obtained 98 CNS clones from these transplantations. The ectopically transplanted midline progenitors gave rise to midline clones (n =

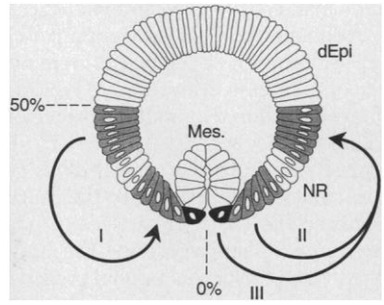

Fig. 2. Diagram of heterotopic transplantations along the ventrodorsal axis of the early gastrula; shown is a cross section of the abdominal region. The neurogenic region (NR) covers the ventral half, the dorsal epidermal anlage (dEpi) the dorsal half of the ectoderm (*25*). Cells were taken from HRP-labeled donors and transplanted into unlabeled hosts (*13*) (experiments I, II, and III) (Table 1). Black, midline cells; shaded, ventral (5 to 20% VD) and dorsal area (30 to 50% VD) of the NR from or to which cells were transplanted. Mes., mesoderm.

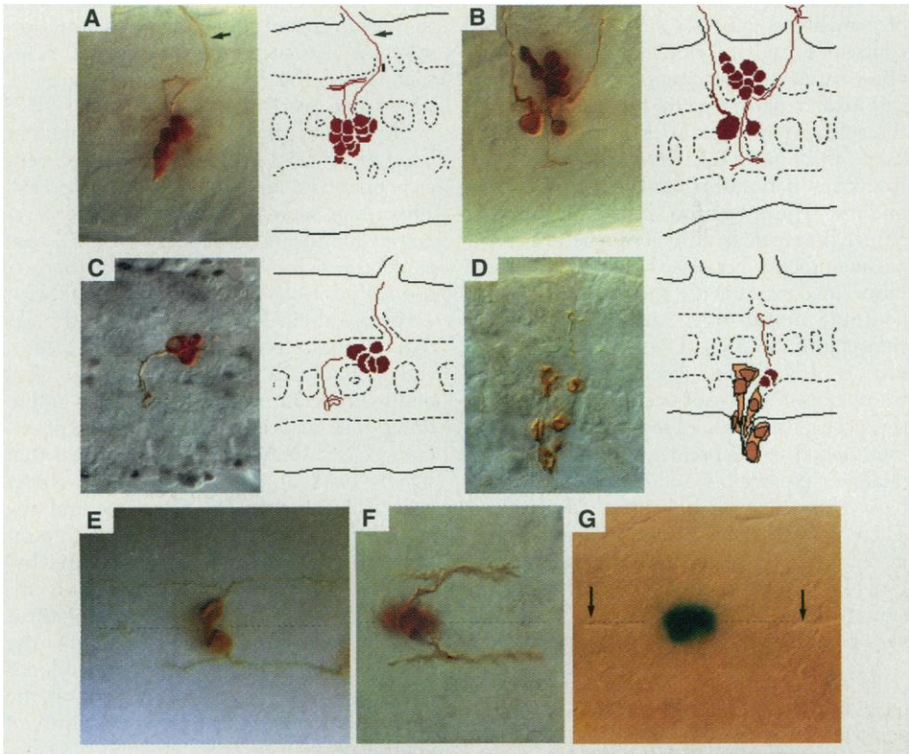

Fig. 1. Examples of CNS lineages at stage 17, which originated from cells of the early gastrula ectoderm (stage 7). Horizontal views; anterior, left. Right panels in (A) to (D) show camera lucida drawings of the clones and the outlines of the neuropil (broken lines) and surface (solid line) of two to three neuromeres of the ventral cord. (**A**) Lineage of the ventral NB3-1 consisting of 7 to 10 interneurons and the 4 motoneurons RP1, 3, 4, and 5 [arrow points to a fascicle leaving the CNS (*14, 31*)]. (**B**) Lineage of the ventral NB4-2 consisting of six to nine interneurons, the RP2 motoneuron (big cell), and one to two further motoneurons (*3, 14*). (**C**) Lineage of a dorsal NB consisting of four to five neurons. (**D**) Lineage of a dorsal NB consisting of two interneurons (dark) as well as six glial cells (light) associated with the nerve roots and peripheral nerve. Clones in (A) and (B) were obtained from single HRP-labeled nPCs upon heterotopic transplantations. The same types of clones were also obtained upon in situ DiI labeling and isotopic transplantations of ventral nPCs. Clones in (C) and (D) were derived from single dorsal nPCs upon DiI labeling [and photoconversion of the labeled progeny (*6*)]. They were also obtained upon isotopic transplantations, but not upon heterotopic transplantations. (**E**) Clone consisting of the two MP1 interneurons (*6*) on either side close to the midline (broken line) derived from a midline progenitor upon isotopic transplantation. (**F**) MP1 clone derived from a midline progenitor upon heterotopic transplantation into the dorsal neuroectoderm. Although the two cell bodies become located on top of each other at the midline, the clone appears basically normal [compare (E)]. (**G**) β-Galactosidase–expressing clone derived from a dorsal nPC of an *X55* transformant donor (expressing *lacZ* only in the midline) upon heterotopic transplantation into the ventral midline (broken line) of a wild-type host (arrows point to dorsoventral channels that demarcate the neuromere border). (**H**) Wild-type midline cells (blue) heterotopically transplanted into a *sim* mutant background are unable to migrate to the midline (compare Fig. 3), instead becoming part of the lateral CNS cortex (*32*). Arrows mark the midline, arrowheads mark segmental nerve roots.

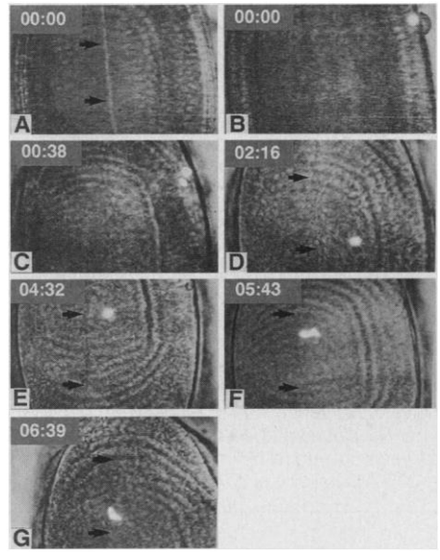

Fig. 3. In vivo tracing of an FITC-dextran–labeled midline progenitor upon heterotopic transplantation into the dorsal neuroectoderm. Ventral view; anterior, top. (**A** and **B**) Two different focal planes of the early gastrula host (stage 7) showing the ventral midline (A) and the lateral site of implantation (B). (**C** to **G**) About 40 min after transplantation, the cell divides (C) and the two progeny are shifted ventrally during germ band elongation [(C) and (D), stages 8, 9 and 10, 11] and germ band retraction [(E) to (G), stages 12 and 13] to finally reach the ventral midline (arrows). Concomitantly, the cells become shifted posteriorly in the course of germ band elongation and back anteriorly during germ band retraction. Insets in the upper left indicate time after transplantation in hours. Arrows mark the ventral midline.

46) (*20*), as judged from division pattern (symmetrical division), numbers and positions of neuronal and glial progeny (in or close to the midline), neuronal fiber projections (6), and the expression of the midline-specific markers *X55* (*18*) and *sim-lacZ* (*21–23*). Although the fiber projection patterns or precise location of cell bodies were often affected, their basic features were typical for midline cells [for the midline precursor 1 cell line (MP1), see Fig. 1, E and F]. The ectopically transplanted ventral nPCs gave rise to clones corresponding to the lineages of ventral NBs (n = 47) (*24*). As judged from neuronal fiber projections, the number and position of neuronal or glial progeny or of both the clones showed no obvious abnormalities. In five cases, the lineage was not identifiable. In contrast to the midline cells, the ventral nPCs also gave rise to epidermal clones (*25*). These data show that mesectodermal cells and ventral nPCs do not respond to dorsal cues. Instead, before their delamination from the ectoderm, mesectodermal cells become committed to give rise to midline lineages, and ventral nPCs to give rise to ventral NB lineages or epidermal clones.

The midline clones and the ventral NB clones occupied almost normal positions in the CNS of late embryos, which suggests that upon ectopic dorsal transplantation, ventral progenitor cells or their progeny migrate ventrally. This result was confirmed by tracing, in vivo, the behavior of midline cells marked with a fluorescent dye. Midline cells from donors labeled with fluorescein isothiocyanate (FITC)–dextran were individually transplanted into the dorsal neuroectoderm of unlabeled hosts. In a typical case (Fig. 3), the cell divides about 40 min after transplantation and the two progeny become shifted posteriorly and about halfway to the ventral midline, which is presumably a passive movement in the course of germ band elongation. In a second phase (after completion of germ band elongation and during germ band retraction), the cells (which remain closely attached to each other) move further ventrally to finally occupy a position close to the ventral midline. The migration during the second phase, however, is unlikely to be a merely passive process, because the cells no longer move in concert with their neighbors. In contrast, dorsal nPCs upon heterotopic transplantation toward ventral sites do not migrate dorsally and upon isotopic dorsal transplantation do not occupy ectopic ventral positions. These observations lend further support to the finding that early gastrula midline progenitors and ventral nPCs have already acquired ventral specificities. These commit them to the occupation and maintenance of specific ventral positions and thus to the production of midline lineages and ventral NB lineages, respectively. Our data also show that the ventral cells are able to distinguish an ectopic dorsal environment and to respond to cues that attract them ventrally.

To assay whether the ventral midline is a possible source for these cues, we ectopically implanted wild-type midline cells into mutant embryos that fail to develop a normal CNS midline. The midline cells were taken from a strain with midline-specific reporter gene expression [*sim-lacZ* (*21*)]; they were then transplanted into the dorsolateral neuroectoderm of mutant hosts that lacked the function of the *sim* gene, which acts as a master regulator of CNS midline development (*21*). In the mutant hosts (n = 23), the ectopic midline cells were unable to reach the midline (Fig. 1H). This result suggests that the midline is required for the establishment of cues that guide the ectopic cells ventrally to their normal positions in the developing CNS.

Before their delamination from the ectoderm, mesectodermal and ventral neuroectodermal cells are committed to form midline progenitors and ventral NBs and to occupy characteristic positions in the developing CNS. Our results further reveal that signals exist at ventral sites that confer ventral specificities on the cells and the ability to migrate back from ectopic positions. Finally, our results support the hypothesis that the ventral midline is involved in the correct positioning of these cells. In vertebrates, the specification of the fate of neural cells and the ventrodorsal patterning of cell types begin early at the neural plate stage (*26*, *27*). The identity of ventral neural cell types and the positions that they occupy in the neural tube appear to depend on signals from the ventral midline (floor plate) and the notochord (*27–30*). Whether in *Drosophila* the ventral midline (mesectoderm) and the mesoderm specify in a similar manner ventral neural cell types remains to be determined.

Table 1. Type of lineages in the late embryo (stage 17) obtained from early gastrula (stage 7) midline progenitors and nPCs. The midline consists of neural progenitors exclusively. Among the nPCs are presumptive neuroblasts as well as epidermoblasts; the proportion of presumptive NBs decreases dorsally. Some nPCs divide in the ectoderm to give rise to a neural as well as an epidermal subclone (*25*) (here, assigned to the NB clones). Epidermal and mixed neural-epidermal clones were not obtained from nPCs upon transplantation into the midline. Each CNS progenitor gives rise to a characteristic lineage that is clearly identifiable by the morphology, number, and position of its neuronal or glial components (Fig. 1) (*5, 6, 14*). Labeling with DiI of individual ectodermal cells in situ (*6*) as well as isotopic transplantations of HRP-labeled cells (*13*) reveal that the different types of progenitors emerge from particular locations along the ventrodorsal axis of the ectoderm. Heterotopic transplantation experiments (I, II, and II) (see Fig. 2) indicate that ventral and dorsal CNS progenitors are deteremined to different degrees; d, dorsal; dorsal NBs, NB clones derived from dorsal nPCs; Epid., epidermal clones; v, ventral; ventral NBs, NB clones derived from ventral nPCs; % VD, percent of ventrodorsal perimeter; 0 to 5% VD, midline progenitors; 0 to 20% VD, midline and ventral neuroectoderm; 30 to 50% VD, dorsal neuroectoderm.

Labeling or transplantation (stage 7)	% VD	Types of lineages (stage 17)			
		Midline	Ventral NBs	Dorsal NBs	Epid.
DiI labeling and isotopic transplantations	0 to 5	+	−	−	−
	0 to 20	+	+	−	+
	30 to 50	−	−	+	+
Heterotopic transplantations					
d → v (I)	30 to 50 ↓ 0 to 20	+	+	−	+
v → d (II)	5 to 20 ↓ 30 to 50	−	+	−	+
v → d (III)	0 to 5 ↓ 30 to 50	+	−	−	−

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16. For example, such lineages included NB1-1 [(*5, 10*), *n* = 8], NB3-1 [(*14*), Fig. 1A, *n* = 4], NB4-2 [(*3, 14*), Fig. 1B, *n* = 11], and MP2 [J. B. Thomas, M. J. Bastiani, M. Bate, C. S. Goodman, *Nature* **310**, 203 (1984), *n* = 9].
17. Such lineages included ventral unpaired median neurons (VUM) (*n* = 8), MP1 (*n* = 7), unpaired median interneurons (UMI) (*n* = 5), median neuroblast (MNB) (*n* = 2), and midline glia (MG) (*n* = 16); for a description of the midline lineages, see (*6*).
18. C. Klämbt, J. R. Jacobs, C. S. Goodman, *Cell* **64**, 801 (991).
19. Dorsal nPCs taken from the midline-specific marker strain P[3.7 *sim-lacZ*] (*21*) did not express the marker when transplanted into ventral midline positions. Expression of *lacZ* in this strain is driven by the *sim* early promotor, which is first activated in the cellular blastoderm (*21*). Thus, upon transplantation into the midline at the gastrula stage the *sim* early promotor does not seem to be sufficiently activated in dorsal nPCs. This result suggests that early *sim* expression (until gastrulation) is dispensable for the generation of morphologically normal midline clones.
20. Such clones included VUM (*n* = 28), MP1 (*n* = 4), UMI (*n* = 7), MNB (*n* = 1), and midline glia (*n* = 6).
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22. As opposed to *X55*, *sim-lacZ* is already expressed in the midline progenitors before gastrulation (*21*). This, and the fact that dorsal nPCs from the same P[3.7*sim-lacZ*] strain do not express the marker upon transplantation into the midline at gastrulation (*19*), suggest that positive staining of the donor cells is the result of cell-autonomous expression or perdurance of the marker.
23. K. Lüer and G. M. Technau, data not shown.
24. For example, such clones included NB1-1 (*n* = 10), NB3-1 (Fig. 1A, *n* = 4), NB4-2 (Fig. 1B, *n* = 2), and MP2 (*n* = 14).
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32. Cells were removed (at stage 7) from the midline of *sim-lacZ* donors and transplanted into the dorsolateral neuroectoderm of mutant hosts. Because of autonomous expression or perdurance of β-galactosidase, the progeny of the donor cell can be unamibiguously identified in late host embryos. Homozygous mutant hosts are characterized by fusion of the longitudinal axon bundles and collapse of the axon scaffold [J. B. Thomas, S. T. Crews, C. S. Goodman, *Cell* **52**, 133 (1988)]. The preparation was double-stained with the glia-specific reversed polarity (repo) antibody 4α3 [D. A. Halter *et al.*, *Development* **121**, 317 (1995)]; the staining showed irregular distribution of many glial cells. In Fig. 1, A to D, left panels, and in Fig. 1, E and F, different focal planes were combined with the use of the Photoshop program on a Macintosh computer.
33. We thank A. Brand, A. Prokop, and J. Urban for comments on the manuscript, C. Klämbt and S. Crews for the *X55*, *sim-lacZ*, and *sim*^{H9} strains, and C. Rickert for assistance with the artwork. Supported by a grant from the Deutsche Forschungsgemeinschaft (G.M.T.).

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A p16^{INK4a}-Insensitive CDK4 Mutant Targeted by Cytolytic T Lymphocytes in a Human Melanoma

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A mutated cyclin-dependent kinase 4 (CDK4) was identified as a tumor-specific antigen recognized by HLA-A2.1–restricted autologous cytolytic T lymphocytes (CTLs) in a human melanoma. The mutated CDK4 allele was present in autologous cultured melanoma cells and metastasis tissue, but not in the patient's lymphocytes. The mutation, an arginine-to-cysteine exchange at residue 24, was part of the CDK4 peptide recognized by CTLs and prevented binding of the CDK4 inhibitor p16^{INK4a}, but not of p21 or of p27^{KIP1}. The same mutation was found in one additional melanoma among 28 melanomas analyzed. These results suggest that mutation of CDK4 can create a tumor-specific antigen and can disrupt the cell-cycle regulation exerted by the tumor suppressor p16^{INK4a}.

Tumor antigens have been demonstrated in murine tumors induced by chemicals and ultraviolet (UV) light (*1*). CTLs mediate the rejection of these experimental tumors, and their target antigens have been identified in some instances (*2*). Human tumors have also been examined for equivalent antigens (*3*). In the human melanoma model of patient SK29(AV), the response of blood-derived lymphocytes to autologous cultured tumor cells has been studied over a long period (*4*, *5*). Some autologous tumor-reactive CTL clones were broadly cross-reactive and were found to recognize melanocyte differentiation antigens such as tyrosinase and Melan-A/MART-1 (*6*). However, HLA-A2–restricted CTLs against a third antigen, called SK29-C, lysed only autologous tumor cells but did not recognize autologous Epstein-Barr virus–transformed B lymphocytes or a panel of allogeneic HLA-A2–positive melanoma cell lines (*5*). Three HLA-A2–restricted CTL clones recognizing SK29-C [CTLs requiring antigen

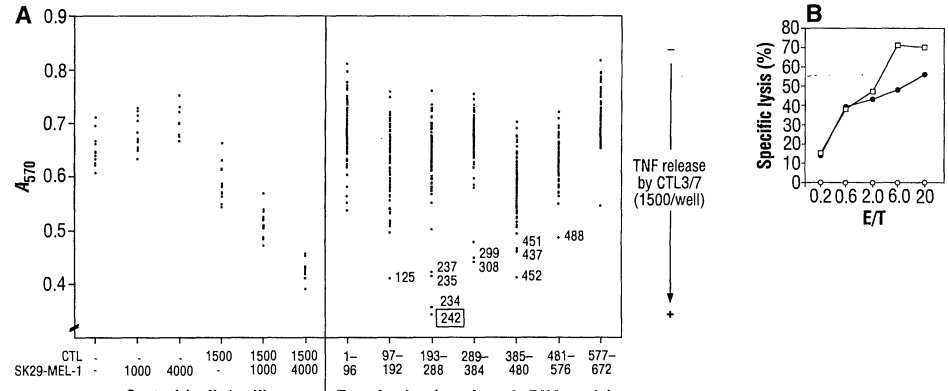

Fig. 1. Identification of cDNAs encoding the melanoma antigen SK29-C. (**A**) A cDNA library from SK29 melanoma cells in vector pcDNAI/Amp (Invitrogen) (*6*) was divided in pools of about 200 bacterial colonies. Plasmid DNA from 672 cDNA pools was cotransfected with HLA-A*0201 (inserted with pcDNAI/ Amp) into COS-7 cells, and transfectants were screened for antigen expression with CTL3/7 (*7*) as described in (*6*). TNF production by CTLs was assessed by measurement of the supernatant cytotoxicity to WEHI 164 clone 13 (W13) cells in a colorimetric assay (*6*). Each point indicates the screening result with a single cDNA pool. Positive pools, confirmed in an independent experiment, are indicated by their number. As a control, production of TNF by CTLs was measured in the presence of increasing numbers of SK29-MEL-1 cells. A_{570}, absorbance at 570 nm. (**B**) The cDNA clone C11.1 was cloned from pool 242. Allogeneic melanoma cells were cotransfected with HLA-A*0201 and C11.1 by electroporation and were tested for susceptibility to lysis by CTL anti-C (CTL5/76) (*7*). Data of a 4-hour, ^{51}Cr release assay are shown. E/T, effector-to-target ratio. Targets were autologous melanoma cells SK29-MEL-1 (open squares), allogeneic melanoma cells MZ2-MEL-2.2–A2.1 (open circles) cotransfected by electroporation with HLA-A*0201 and the hygromycin B resistance gene (*27*), and MZ2-MEL-2.2–A2.1-C11.1 cells (closed circles) additionally cotransfected with C11.1 and the neomycin resistance gene. The ^{51}Cr release assay, electroporation of melanoma cells, and cell culture conditions were as described (*5*).