A Gene Encoding an Antigen Recognized by Cytolytic T Lymphocytes on a Human Melanoma

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Many human melanoma tumors express antigens that are recognized in vitro by cytolytic T lymphocytes (CTLs) derived from the tumor-bearing patient. A gene was identified that directed the expression of antigen MZ2-E on a human melanoma cell line. This gene shows no similarity to known sequences and belongs to a family of at least three genes. It is expressed by the original melanoma cells, other melanoma cell lines, and by some tumor cells of other histological types. No expression was observed in a panel of normal tissues. Antigen MZ2-E appears to be presented by HLA-A1; anti-MZ2-E CTLs of the original patient recognized two melanoma cell lines of other HLA-A1 patients that expressed the gene. Thus, precisely targeted immunotherapy directed against antigen MZ2-E could be provided to individuals identified by HLA typing and analysis of the RNA of a small tumor sample.

MOST MOUSE TUMORS EXPRESS ANtigens that constitute potential targets for rejection responses in syngeneic hosts (*1*). Against some of these tumors, highly active and specific CTLs can be derived from immunized animals by restimulation in vitro with tumor cells (*2*). That the antigens recognized by these CTLs in vitro can be effective tumor-rejection antigens is indicated by the finding that tumor cells that had escaped immune rejection in vivo were found to be resistant to the tumor-specific CTLs (*3*).

For human tumors, autologous mixed cultures of tumor cells and lymphocytes can generate CTLs that lyse the tumor cells (*4*). These anti-tumor CTLs do not lyse targets of natural killer cells and autologous control cells such as fibroblasts or EBV-transformed B lymphocytes. However, it is difficult to evaluate to what extent the antigens recognized on human tumors by autologous CTLs are relevant for tumor rejection.

We have obtained a panel of autologous CTL clones (*5*) that lyse melanoma cell line MZ2-MEL, which was derived from patient MZ2. By selecting clonal sublines of MZ2-MEL that are not killed by these CTL clones, we obtained antigen-loss variants that were resistant to subsets of the CTL clones, demonstrating that autologous CTLs recognize a total of six independent antigens on MZ2-MEL (*6*). We then attempted to identify the gene for one of these antigens, MZ2-E, by an approach that had enabled the isolation of genes for antigens recognized by CTLs on mouse tumors (*7*). This approach is based on the transfection of cosmid libraries prepared with DNA of cells that express the relevant antigen. Transfectants expressing the antigen are identified by their ability to stimulate the appropriate CTLs. As a first step, we tried to obtain transfectants expressing antigen MZ2-E (E) with genomic DNA of an E^+ MZ2-MEL subline. The DNA was transfected into E^- antigen-loss variant MZ2-MEL.2.2, which had been obtained by selection with an anti-E CTL clone. A transfectant was obtained that was lysed by autologous anti-E CTL clone 82/30 (*8*).

A library of 700,000 independent cosmids was prepared with DNA of an E^+ MZ2-MEL subline and groups of 50,000 cosmids were amplified (*9*). DNA from each group of cosmids was cotransfected into E^- line MZ2-MEL.2.2 together with plasmid pSVtkneoβ, that confers resistance to geneticin (*10*). Approximately 5000 geneticin-resistant transfectants were obtained for each group and they were divided into microcultures of 30 independent transfectants. These microcultures were allowed to expand, duplicated, and tested for their ability to stimulate tumor necrosis factor (TNF) release by anti-E CTL clone 82/30 (*11*). This enables identification of those pools that contain one transfectant that expresses antigen E (*8*). Two of the 14 groups of cosmids produced positive microcultures, and the E^+ transfectants were recovered from the duplicates. After additional transfection experiments, a total of five E^+ transfectants out of 29,000 independent geneticin-resistant transfectants were obtained with the first positive group of cosmids and two out of 13,000 with the second group.

By packaging the DNA of cosmid transfectants directly into lambda phage components, it is sometimes possible to retrieve cosmids that contain the sequences of interest (*7, 12*). This procedure was unsuccessful here, so we rescued the transfected sequence by ligating DNA of the transfectant to appropriate restriction fragments of cosmid vector pTL6 (*13*). This was tried with two transfectants and was successful with transfectant 7.4: cosmid B3 was obtained, which transferred the expression of antigen E at high efficiency. Transfectants obtained with this cosmid were as sensitive to lysis by the anti-E CTLs as the original melanoma cell line (Fig. 1).

Cosmid B3 was digested with Sma I and a 12-kb fragment could transfer the expression of the antigen (Fig. 1). After the fragment was digested with Bam HI, a 2.4-kb fragment was found to transfer the expression of antigen E at high efficiency (Fig. 1). This small fragment was used as a hybridization probe on a Southern (DNA) blot prepared with Bam HI–digested DNA of an

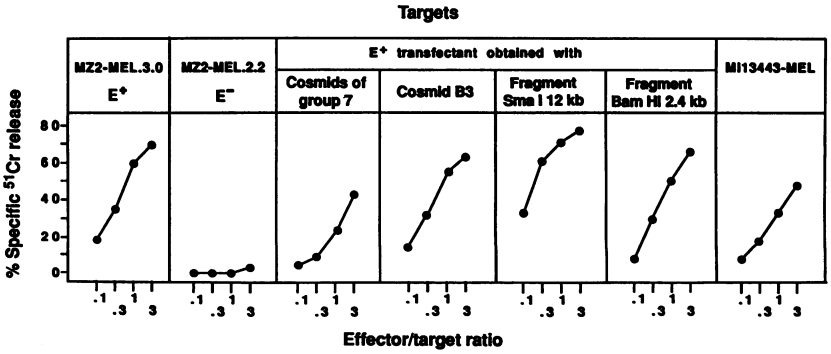

Fig. 1. Sensitivity of ^{51}Cr-labeled target cells to lysis by anti-E CTL clone MZ2-CTL 82/30. Chromium release was measured after 4 hours (*5*). MZ2-MEL.3.0 is a clonal subline of the melanoma cell line of patient MZ2, and MZ2-MEL.2.2 is an antigen-loss variant selected with an anti-E CTL clone (*6*). The E^+ transfectant clones were isolated from MEL.2.2 cell populations transfected with cosmid group 7 (transfectant 7.4); with cosmid B3; with a cloned 12-kb Sma I fragment of B3 (*20*); with a cloned 2.4-kb Bam HI fragment of the 12-kb fragment (*20*). Melanoma cell line MI13443-MEL was derived from another HLA-A1 patient.

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E^+ subline of MZ2-MEL and of the E^- antigen-loss variant. The expected 2.4-kb band was observed only with the DNA of the E^+ melanoma cell (Fig. 2), indicating that the E^- variant had lost the expression of the antigen as a result of a deletion affecting the relevant gene. Partial or complete deletions of tumor-antigen genes also occur in antigen-loss variants of mouse tumor cells (*14*). In addition to the 2.4-kb band, the probe hybridized to several additional bands of different intensities, suggesting that the gene responsible for the production of antigen E has sequence similarities to several other genes.

The sequence of the transfecting 2.4-kb genomic segment showed no significant similarity to any sequence presently recorded in data banks (*15*). Northern (RNA) blots and a cDNA library were prepared with RNA of E^+ subline MZ2-MEL.3.0 (*16*). The 2.4-kb segment hybridized to an mRNA of approximately 1.8 kb on a Northern blot. cDNA clones were obtained whose sequences were identical to parts of the 2.4-kb genomic fragment, thereby identifying two exons in this fragment. The position of one additional exon located upstream was obtained by sequencing segments of cosmid B3 that were located in front of the 2.4-kb Bam HI fragment. The gene extends over approximately 4.5 kb (Fig. 3). The starting point of the transcribed region was confirmed by polymerase chain reaction (PCR) amplification of the 5′ end of the cDNA (*17*). The three exons are 65, 73, and 1551 bp, respectively (Fig. 3). An ATG located in position 66 of exon 3 is followed by an open reading frame of 828 bp.

The ability of the 2.4-kb gene fragment to transfer the expression of antigen E confirms

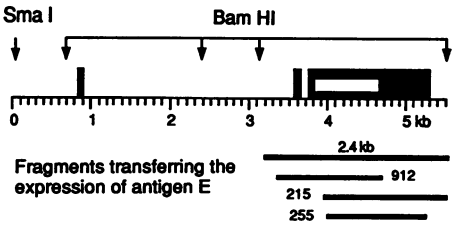

Fig. 3. Structure of the gene of antigen MZ2-E and restriction sites. Exons are indicated as black boxes and the open reading frame in exon 3 is marked in white. Boundaries of transfecting fragments are indicated relative to the first nucleotide of exon 3.

previous observations that truncated genes lacking the promoter can efficiently transfer CTL epitopes (*7*). Smaller regions of the gene corresponding to parts of the 2.4-kb fragment were cloned and tested by transfection into E^- cells. Three segments transferred the expression of the antigen (Fig. 3). Thus, the gene probably encodes the antigenic peptide recognized by the anti-E CTLs, as opposed to producing a protein that activates the encoding gene. The encoding sequence would be where all transfecting fragments overlap, as is the case for the four mouse tumor antigens that we have studied.

Two nonidentical cDNA species were also found when the cDNA library was probed with the 2.4-kb fragment. This confirmed the existence of a gene family suggested by the pattern observed on the Southern blots (Fig. 2). In contrast with the first cDNA, the second and the third types of cDNA were unable to transfer the expression of antigen E in transfection experiments. No significant homology was found by comparing the sequences of the two additional cDNAs to those presently recorded in gene banks (*15*). We propose the name *"MAGE"* (melanoma antigen) for this new gene family, with *MAGE-1* referring to the gene that directs the expression of antigen MZ2-E and *MAGE-2* and *-3* for the two other genes (Fig. 4). Analysis of the third exon showed that the two additional cDNAs are more closely related to each other (12% differences) than to the first (18.1% and 18.9% differences). Out of nine cDNA clones obtained with RNA of MZ2-MEL.3.0, three of each type were obtained, suggesting approximately equal expression of the three genes. It is possible that other closely related genes are expressed in lower amounts in these cells.

Experiments with mouse tumors showed that new antigens recognized by T cells can result from point mutations in the coding region of active genes. New antigens can also arise from the activation of genes that are not expressed in most normal cells (*7*). To clarify this issue for antigen MZ2-E, we compared the *MAGE-1* gene present in the melanoma cells to that present in normal cells of patient MZ2. We amplified by polymerase chain reaction (PCR) the DNA of phytohemagglutinin-activated blood lymphocytes with primers surrounding a 1300-bp stretch covering the first half of the 2.4-kb fragment. A PCR product was obtained, whereas none was obtained with the DNA of the E^- variant. The sequence of this PCR product was identical to the corresponding sequence of the gene carried by the E^+ melanoma cells. Moreover, we found that antigen MZ2-E was expressed by cells transfected with the cloned PCR product. Thus, the activation of a normal gene is responsible for the appearance of antigen MZ2-E. One may wonder how E^- antigen-loss variants could be obtained in these circumstances, because both copies of the gene would have to be inactivated. The same phenomenon has been observed with homozygous mouse tumor P815 (*3*). One explanation would be the existence among the

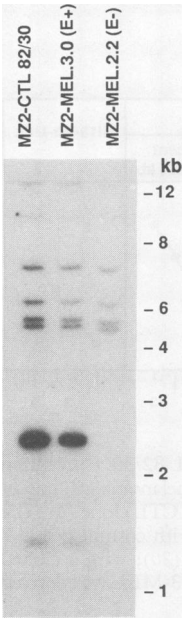

Fig. 2. Identification of a genomic deletion in the E^- antigen-loss variant. The 2.4-kb Bam HI fragment, which transferred the expression of antigen MZ2-E, was labeled with ^{32}P and used as a probe on a Southern blot of Bam HI–digested DNA of E^+ clonal subline MZ2-MEL3.0 and of E^- variant MZ2-MEL.2.2. The 2.4-kb band is absent in the lane of the E^- variant. The DNA of CTL clone 82/30 of patient MZ2 displayed the same bands as the E^+ melanoma cells.

Fig. 4. Part of exon 3 of gene *MAGE-1*, which directs the expression of antigen MZ2-E, and of related genes *MAGE-2* and *MAGE-3*. Lowercase letters in the two latter sequences indicate differences relative to *MAGE-1*. Numbering is relative to the first nucleotide of exon 3. Oligonucleotides CHO-8 and CHO-9 were used to prime the reverse transcriptions and PCR amplification of Fig. 6. Oligonucleotides SEQ-4, CHO-2, and CHO-3 were used as specific hybridization probes discriminating *MAGE-1*, *-2*, and *-3*. The codons of the open reading frame are indicated by points.

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cultured cell populations of a subset of cells that are haploid for the relevant chromosome.

To evaluate the expression of *MAGE-1* by various normal and tumor cells, we hybridized Northern blots with a probe covering most of the third exon. In contrast with the result observed with tumor cell line MZ2-MEL.3.0, no band was observed with RNA isolated from a CTL clone of patient MZ2 and phytohemagglutinin-activated blood lymphocytes of the same patient. Also negative were several normal tissues of other individuals (Fig. 5 and Table 1). Ten of 14 melanoma cell lines of other patients were positive to varying degrees. Two of four samples of melanoma tumor tissue, including a metastasis of patient MZ2, were positive, excluding that the expression of the gene represented a tissue culture artifact. Some tumors of other histological types were also positive (Fig. 5 and Table 1). Thus, the *MAGE* gene family is expressed by many melanomas and also by other tumors. However, because the probe cross-hybridizes, there was no clear indication as to which of the three genes were expressed by these cells. We therefore resorted to PCR amplification and hybridization with specific oligonucleotide probes. cDNAs were obtained and amplified by PCR with oligonucleotide primers that corresponded to sequences of exon 3 that were identical for the three *MAGE* genes. The PCR products were then tested for their ability to hybridize to three other oligonucleotides that showed complete specificity for one of the three genes (Fig. 4). Control experiments that were done by diluting RNA of melanoma MZ2-MEL.3.0 into RNA from negative cells indicated that under our conditions the intensity of the signal decreased proportionally to the dilution and that positive signals could still be detected at a dilution of 1/300. The normal cells that were tested by PCR were confirmed to be negative for the expression of the three *MAGE* genes, suggesting a level of expression of <1/300 that of the MZ2 melanoma cell line (Fig. 6). Some melanomas expressed *MAGE* genes *1, 2*, and *3* whereas others expressed only *MAGE-2* and *-3* (Fig. 6 and Table 1). Some of the other tumors also expressed all three genes, whereas others expressed only *MAGE-2* and *-3* or only *MAGE-3*. The *MAGE* gene family, thus, is expressed by a large array of different tumors and not by most normal cells. The *MAGE* genes may participate in tumor transformation or in aspects of tumor progression such as the ability to metastasize. The observation that *MAGE-1* is expressed in several melanomas shows that tumors of different patients can express the same tumor rejection antigen recognized by autologous CTLs.

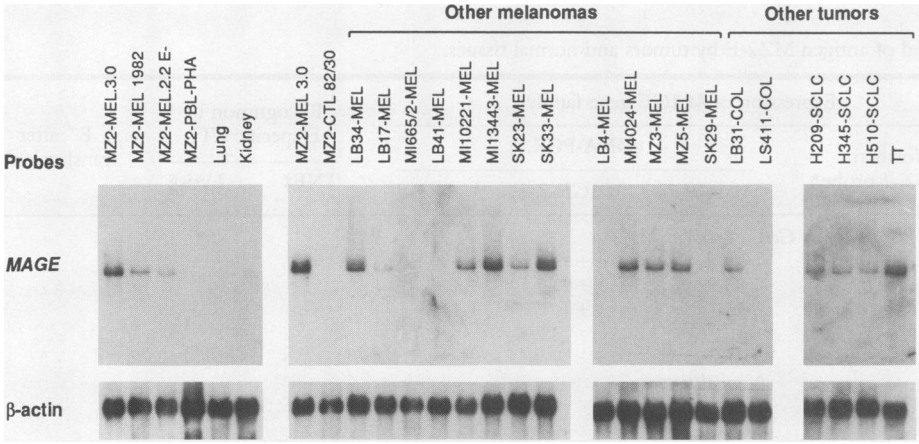

Fig. 5. Northern blot analysis of the expression of *MAGE-1* in tumor cell lines, tumor samples, and normal human tissues. All lanes contained 20 μg of total RNA and were hybridized with a 1.3-kb DNA probe extending from positions 255 to 1544 of exon 3 of *MAGE-1* and with a β-actin probe (*21*). Hybridization with both probes was performed successively on the same blot. Hybridization and washing conditions were the same for all the experiments.

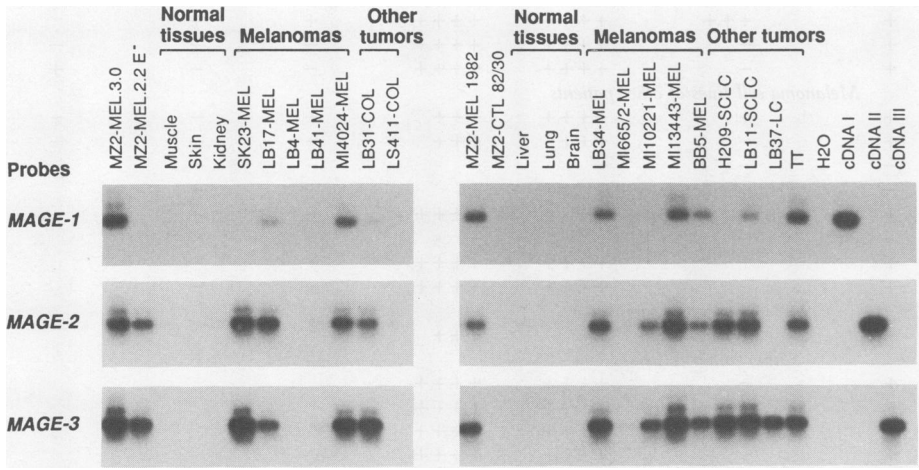

Fig. 6. Detection of the expression of *MAGE-1* and of related genes *MAGE-2* and -3 by reverse transcription and PCR amplification. Total RNA was extracted from the tumor cell lines, tumor samples, and normal human tissues. Oligonucleotides CHO-8 and CHO-9 (Fig. 4), which correspond to identical regions in *MAGE-1*, *-2*, and *-3* were used to prime cDNA synthesis and PCR amplification (*22*). The PCR products were fractionated by size in agarose gels and blotted on nitrocellulose. The blots were hybridized with oligonucleotides probes SEQ-4, CHO-2, and CHO-3, which are highly specific for sequences of *MAGE-1*, *-2*, and *-3*, respectively (Fig. 4). This specificity is demonstrated in the right part of the figure showing hybridization with these three probes of PCR-amplified *MAGE-1*, *-2*, and *-3* cDNA clones. The nitrocellulose filters corresponding to both panels were hybridized, washed, and autoradiographed together.

We also determined the associated major histocompatibility complex (MHC) class I molecule. The class I specificities of patient MZ2 are HLA-A1, A29, B37, B44, and C6. Four other melanomas of patients that had A1 in common with MZ2 were cotransfected with the 2.4-kb fragment and pSVt-kneoβ. Three of them yielded neo^r transfectants that stimulated TNF release by anti-E CTL clone 82/30, which is CD8$^+$ (Table 1). No E$^+$ transfectant was obtained with four other melanomas, some of which shared A29, B44, or C6 with MZ2. Thus, the presenting molecule for antigen MZ2-E is probably HLA-A1. Eight melanomas of patients with HLA haplotypes that did not include A1 were examined for their sensitivity to lysis and for their ability to stimulate TNF release by the CTLs. None were positive even though three expressed *MAGE-1* (Table 1). Out of six melanoma cell lines derived from tumors of HLA-A1 patients, the two lines that expressed *MAGE-1* also stimulated TNF release by anti-E CTL clone 82/30 of patient MZ2. One of these tumor cell lines, MI13443-MEL, also showed high sensitivity to lysis by these anti-E CTLs (Fig. 1). The ability of some human anti-tumor CTLs to lyse allogeneic tumors that share an appropriate HLA specificity with the original tumor has been reported (*18*).

It is difficult to definitively establish that antigens recognized in vitro on human tu-

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mor cells by autologous CTLs constitute potential targets for immune rejection in vivo. This relevance has been demonstrated for mouse tumor antigen P815A (*3*), and we find a striking similarity between this mouse tumor rejection antigen and antigen E of human melanoma MZ2-MEL. Both antigens are on tumors that express four to six antigens recognized by autologous CTLs (*3, 6*). The genes coding for both antigens appear to be silent or quasi-silent on most normal tissues and are activated in the tumors. The sequences of both genes appear to be identical in normal tissues and in the tumors. Finally, both genes are expressed in several independent tumors, resulting in the expression of the antigen in those tumors that carry the appropriate class I MHC molecule.

The finding that a potential tumor rejection antigen is shared by a significant proportion of human tumors and the ability to identify these tumors readily on the basis of their expression of the relevant gene may have important implications for cancer immunotherapy. Small tumor samples of HLA-A1 individuals [26% of total in Caucasian populations (*19*)] could be frozen and the RNA tested by reverse transcription and PCR amplification to identify the tumors that express *MAGE-1*. These tumors should express antigen E and may therefore be sensitive to an anti-E T lymphocyte response. Additional study will be required to assess how much *MAGE-1* expression is needed for effective production of antigenic peptides as well as the critical density of class I molecules and adhesion molecules required at the cell surface. But eventually this should lead to precisely targeted strategies of active or passive immunization. A promising possibility for active immunization involves the use of cells engineered for high expression of *MAGE-1, HLA-A1*, and possibly genes coding for some adhesion molecules and interleukins.

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Table 1. Expression of genes *MAGE-1*, *-2*, and *-3* and of antigen MZ2-E by tumors and normal tissues.

Sample	Expression of *MAGE* gene family: Northern *MAGE-1* probe*	cDNA-PCR⁺† *MAGE-1*	cDNA-PCR⁺† *MAGE-2*	cDNA-PCR⁺† *MAGE-3*	Recognition by E-specific CTL: TNF‡	Recognition by E-specific CTL: Lysis§	E⁺ after transfection¶
Cells of patient MZ2							
Melanoma cell line MZ2-MEL.3.0	+	++++	++++	++++	+	+	
Tumor sample MZ2 (1982)	+	+++	+++	+++			
Antigen-loss variant MZ2-MEL.2.2	+	−	+++	+++	−	−	
CTL clone MZ2-CTL.82/30	−	−	−	−			
PHA-activated blood lymphocytes	−	−	−	−			
Normal tissues							
Liver	−	−	−	−			
Muscle	−	−	−	−			
Skin	−	−	−	−			
Lung	−	−	−	−			
Brain	−	−	−	−			
Kidney	−	−	−	−			
Melanoma cell lines of HLA-A1 patients							
LB34-MEL	+	++	++++	++++	+	±	
MI665/2-MEL	−	−	−	−	−	−	+
MI10221-MEL	+	−	++	+++	−	−	+
MI13443-MEL	+	+++	++++	++++	+	+	
SK33-MEL	+	−	++++	++++	−	−	−
SK23-MEL	+	−	++++	++++	−	−	+
Melanoma cell lines of other patients							
LB17-MEL	+	+	++++	+++	−	−	−
LB33-MEL	+	−	+++	+++	−	−	−
LB4-MEL	−	−	−	−	−	−	
LB41-MEL	−	−	−	−	−	−	
MI4024-MEL	+	+++	++++	++++	−	−	
SK29-MEL	−	−	−	−	−	−	
MZ3-MEL	+	+	++++	++++	−	−	
MZ5-MEL	+	−	++++	++++	−	−	
Melanoma tumor sample							
BB5-MEL	+	+++	++	+++			
Other tumor cell lines							
Small cell lung cancer H209	+	−	++++	++++			
Small cell lung cancer H345	+	−	++++	++++			
Small cell lung cancer H510	+	−	++++	++++			
Small cell lung cancer LB11	+	+	++++	++++			
Bronchial squamous cell carcinoma LB37	+	−	−	+++			
Thyroid medullary carcinoma TT	+	++++	+++	++++			
Colon carcinoma LB31	+	−	+++	++++	−		
Colon carcinoma LS411	−	−	−	−			
Other tumor samples							
Chronic myeloid leukemia LLC5	−	−	−	−			
Acute myeloid leukemia TA	−	−	−	−			

*Data obtained in the conditions of Fig. 5 with a cross-reactive *MAGE-1* probe. †Data obtained as described in Fig. 6. ‡TNF release by CTL 82/30 after stimulation with the tumor cells as described in (*11*). §Lysis of ^{51}Cr-labeled target by CTL 82/30 in the conditions of Fig. 1. ¶Cells transfected with the 2.4-kb fragment of gene *MAGE-1* were tested for their ability to stimulate TNF release by CTL 82/30.

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9. The genomic library was constructed with DNA from MZ2-MEL.43 as described in E. De Plaen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2274 (1988).
10. We used the transfection procedure described in (*8*) with the following modification: 4.5×10^6 cells attached to 600 cm^2 tissue culture flasks (Singletray Unit, Nunc) containing 180 ml of medium were treated with a 20-ml calcium phosphate–DNA precipitate of 240 μg of cosmid DNA and 24 μg of pSVtkneoβ.
11. As described in (*8*). Briefly, 1,500 CTL specific for E were added to 4×10^4 transfected cells in a microwell. After 24 hours, 50 μl of the supernatant were added to 3×10^4 cells of cell line WEHI 164 clone 13. This TNF-sensitive cell line was developed by T. Espevik and J. Nissen [*J. Immunol. Methods* **95**, 99 (1986)]. The mortality of WEHI cells was estimated 24 hours later by a colorimetric assay described by M. B. Hansen, S. E. Nielsen, and K. Berg [*ibid.* **119**, 203 (1989)].
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15. The computer research for sequence homology was done with GenBank release 68 and the FASTA program, described by W. R. Pearson and D. J. Lipman [*Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444 (1988)]. The accession number of *MAGE-1* in GenBank is M77481.
16. cDNA libraries in bacteriophage λgt10 were prepared with the Amersham cDNA synthesis and cloning kits.
17. Amplification of the 5′ end of the cDNA by PCR as described in M. Frohman, M. Dush, G. Martin, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8998 (1988). The primer for the synthesis of the cDNA was 5′-TTGCCGAAGATCTCAGGAA-3′. For the amplification, we used as 3′ primer the oligonucleotide 5′-CTTGCCTCCTCACAGAG-3′ and the 5′ primers described by Frohman *et al.*
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20. pTZ18R and pTZ19R were used as cloning vectors in order to produce double-stranded DNA for transfection and single-stranded DNA for sequencing.
21. RNA isolation was performed as in L. G. Davis, M. D. Dibner, J. F. Battey, *Basic Methods in Molecular Biology* (Elsevier, New York, 1986), pp. 130–135. Northern blot analysis was performed as by B. Van den Eynde *et al.* in (7).
22. For cDNA synthesis, total RNA (1 μg) was diluted to a total volume of 20 μl with 2 μl of 10× buffer from the GENEAmp kit (Perkin Elmer-Cetus), 2 μl each of 10 mM dNTP, 1.2 μl of 25 mM $MgCl_2$, 1 μl of a 80-μM solution of oligonucleotide primer CHO-9, 20 units of RNAsin (Promega), and 200 units of MoMLV reverse transcriptase (BRL). This mixture was incubated at 42°C for 40 min. For PCR amplification, 8 μl of 10× buffer, 4.8 μl of 25 mM $MgCl_2$, 1-μl of a 80-μM solution of primer CHO-8, 2.5 units of Taq polymerase, and water were added to a total volume of 100 μl. Amplification was performed for 30 cycles (1 min at 94°C, 2 min at 52°C, and 3 min at 72°C). Each reaction (10 μl) was size-fractionated in agarose gels, blotted on nitrocellulose, and hybridized with ^{32}P-labeled oligonucleotides. Hybridization and washing conditions were as in (*14*).
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Cell-Free, De Novo Synthesis of Poliovirus

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Cell-free translation of poliovirus RNA in an extract of uninfected human (HeLa) cells yielded viral proteins through proteolysis of the polyprotein. In the extract, newly synthesized proteins catalyzed poliovirus-specific RNA synthesis, and formed infectious poliovirus de novo. Newly formed virions were neutralized by type-specific antiserum, and infection of human cells with them was prevented by poliovirus receptor-specific antibodies. Poliovirus synthesis was increased nearly 70-fold when nucleoside triphosphates were added, but it was abolished in the presence of inhibitors of translation or viral genome replication. The ability to conduct cell-free synthesis of poliovirus will aid in the study of picornavirus proliferation and in the search for the control of picornaviral disease.

VIRUSES ARE COMPLEX AGGREGATES of organic macromolecules that assemble from multiples of either a few or many distinct building blocks (*1*). Cell-free morphogenesis of infectious virus particles, in contrast to de novo synthesis, has been reported with some plant viruses and bacteriophages (*2*). In these cases, the viral structural components were isolated from virions or from infected cells. All previral attempts to assemble animal viruses, including members of the Picornaviridae family, have been unsuccessful.

The prototype picornavirus, poliovirus, is a non-enveloped, icosahedral particle consisting of a single-stranded RNA genome that is surrounded by 60 copies each of capsid polypeptides VP1 and VP3, 58 to 59 copies each of VP2 and VP4, and one to two copies of VP0, the precursor to VP2 and VP4 (*3–5*). Poliovirion formation involves the proteolysis of the capsid polypeptide precursor P1 and the formation of "immature" protomers [(VP0,VP1, and $VP3)_5$], procapsids (an empty shell consisting of 12 immature protomers) and, possibly, "provirions" (*3*). Provirions are noninfectious RNA-containing particles whose VP0 polypeptides have not been cleaved to yield the mature virion (*6*). The formation of provirions in a cell-free extract of poliovirus-infected HeLa cells has been described but, for unknown reasons, these particles did not "mature" to virions (*7*). Palmenberg (*8*) and Grubman *et al.* (*9*) have succeeded in assembling capsid intermediate structures by translating, in rabbit reticulocyte lysates, RNAs of the picornaviruses encephalomyocarditis virus and foot-and-mouth disease virus. Formation of infectious particles in these lysates was not reported, and it is conceivable that the rabbit reticulocyte lysate lacks components essential for morphogenesis (*10*).

Numerous problems concerning the structure and replication of picornaviruses remain unsolved. These include the function of virus-encoded polypeptides, the mechanism of initiation of polyprotein synthesis, the mechanism of genome replication, specific steps in morphogenesis, and the involvement of cellular components in viral proliferation. Here we describe a procedure for de novo synthesis of infectious poliovirus in a cell-free system. We provide evidence not only for the formation and proteolytic processing of the polioviral polyprotein in vitro, a result reported previously (*11*), but also for viral RNA synthesis and assembly.

We have prepared a cell-free extract from uninfected HeLa cells (*12*) capable of translating RNA of poliovirus type 1 (Mahoney) [PVI(M)] with great accuracy (Fig. 1). All viral proteins were visible, with the exception of capsid proteins VP2 and VP4. On longer exposure of gels (approximately 96 hours) a band migrating to the position of VP2 emerged (*13*). However, there was too little material to confirm the identity of the polypeptide by immunoprecipitation with

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