CHARACTERIZATION OF gP85^{gag} AS AN ANTIGEN RECOGNIZED BY MOLONEY LEUKEMIA VIRUS–SPECIFIC CYTOLYTIC T CELL CLONES THAT FUNCTION IN VIVO

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Recent advances in tissue culture technology and the derivation of T cell clones have led to the generation and extended culture in vitro of a wide variety of antigen-specific T cell clones (1, 2). Virus-specific murine T cell clones exhibiting either a helper or cytolytic phenotype have been shown to function in vivo in several model systems, including those for cells infected by influenza virus (3), herpes simplex virus (4), and Sendai virus (5), and tumor cells induced by Abelson leukemia virus (6) or Moloney murine sarcoma virus–Moloney murine leukemia virus (M-MSV–M-MLV)[1] (7). In spite of several recently published reports (8–10), it is still unclear what target molecule(s) is recognized by cytolytic T lymphocytes (CTL) directed against M-MSV–M-MLV–infected target cells. Most studies have used mixed populations of CTL and did not allow one to distinguish if the CTL recognizes an antigen encoded by M-MSV–M-MLV or a cellular antigen induced as a result of the infection. In the former case, candidate molecules are the envelope glycoproteins gp70 and p15 (E) and the *gag* membrane proteins gP85^{gag} and gP95^{gag} (gP95^{gag} being the precursor protein of gP85^{gag}; 11, 12). Investigators using purified gp70 or anti-gp70 antibodies to block CTL recognition of target cells have obtained contradictory results (9, 13, 14). Experiments using recombinant DNA clones to transfect target cells and yield the expression of gp70 and p15 (E) demonstrated the existence of a subset of BALB/c (H-2^d) M-MLV-specific CTL able to recognize these molecules (10). In this report we have characterized gP85^{gag} as a target molecule expressed on M-MSV–M-MLV- or M-MLV-infected cells recognized by the Lyt-2$^+$ M-MSV–M-MLV-specific CTL clones under investigation. The in vivo activity of these CTL clones was tested in two systems. (a) CTL clones were used in long-term protection studies in mice injected intraperitoneally with M-MLV-induced tumor cells. 30–60 d after injection of tumor cells and the CTL clone, we observed the

[1] *Abbreviations used in this paper:* CTL, cytolytic T lymphocyte; FSV, Fujinami sarcoma virus; GCSA, Gross cell surface antigen; LTR, long terminal repeat; MCF, mink cell focus-inducing; MEF, murine embryo fibroblasts; MLTC, mixed leukocyte-tumor cell population; M-MLV, Moloney murine leukemia virus; M-MSV, Moloney murine sarcoma virus; PBS, phosphate-buffered saline.

growth of antigen-loss variants in certain mice that were initially protected against tumor formation (7). After cloning in vitro, these tumor cells were no longer lysed by the CTL clones or by mixed leukocyte-tumor cell populations (MLTC). After several months of culture in vitro, two of five of these antigen-loss variants reverted to the original CTL-susceptible phenotype. Both the CTL-nonsusceptible and -susceptible variants were characterized on the molecular level. (b) We obtained long-lasting protection against tumor formation induced by M-MSV–M-MLV injection in syngeneic mice after intravenous injection of the same virus-specific CTL clones.

Materials and Methods

Mice. C57BL/6 (H-2^b), DBA/2 (H-2^d), and BALB/c (H-2^d) female mice were obtained from the animal colony of the Swiss Institute for Experimental Cancer Research. The breeding pairs for this colony originated from The Jackson Laboratory, Bar Harbor, ME.

Cells and Viruses. MBL-2 (H-2^b) and a subclone MBL-2.9, and LSTRA (H-2^d), all M-MLV-induced lymphomas, were maintained by passage as ascites in C57BL/6 and BALB/c mice, respectively (15). They were labeled with ^{51}Cr and used as target cells in ^{51}Cr release assays as described (15), with one lytic unit (LU) equal to that number of effector cells required to give 50% lysis of 10^4 labeled target cells in 3.5 h at 37°C (16). L cells, A-9 cells, (an M-MLV-infected NIH-3T3 cell line), and Ab-X-MLV-infected NIH-3T3 cells (17) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Murine embryo fibroblasts (MEF) were prepared from 15-d-old C57BL/6 embryos and used as a second or third passage. Infection of cells was performed in the presence of 4 μg/ml polybrene. B cell lymphoblasts were prepared (18) and infected with virus as described previously (19).

Cell Labeling and Immunoprecipitations. Cells were labeled with [^{35}S]methionine (100 μCi/ml) for 1 h, washed with phosphate-buffered saline (PBS), and lysed in a buffer containing PBS, 1% Triton X-100, 0.5% deoxycholate, and 0.1% sodium dodecyl sulphate according to Van Zaane et al. (20). Viral proteins were immunoprecipitated from cleared lysates by incubation of portions of the lysates (500,000 cpm) first with antisera directed against Rauscher MLV p30 or gp70 (obtained from the National Cancer Institute) and second with protein A–Sepharose, as described (21). Immunoprecipitates were analyzed by electrophoresis on 10% SDS-polyacrylamide gels and radioactivity was visualized by exposure of EN^{3}HANCE-treated gels to Kodak XAR-5 film.

Generation of M-MSV–M-MLV–specific CTL Clones. M-MSV–M-MLV–specific CTL clones were derived from C57BL/6 regressor mice by micromanipulation techniques as described (22, 7). Lyt-2$^+$ CTL clones that demonstrated vigorous growth and strong specific cytolytic activity when tested on MBL-2.9 target cells were selected for in vivo protection studies (7). MLTC cultures, which served as a source of an M-MSV–M-MLV–specific CTL population, were set up as reported previously (15). C57BL/6 anti-DBA/2 CTL clones were derived by micromanipulation of immune peritoneal exudate lymphocytes obtained from primed C57BL/6 mice as described (7).

In Vivo Protection Studies. The potential for M-MSV–M-MLV–specific CTL clones to protect mice against M-MSV–M-MLV–induced tumors was studied in two ways: (a) 4-wk-old normal or irradiated (650 rad) C57BL/6 female mice were injected in the rear right footpad with 40 μl of a stock preparation of M-MSV–M-MLV (7). At the same time, various numbers of an M-MSV–M-MLV–specific CTL clone were injected intravenously and the mice monitored for tumor growth by measurement of footpad thickness using a Vernier caliper (Etalon, Rolle, Switzerland). (b) In a second approach, 2 × 10^6 ^{131}I-uridine (^{131}I-UdR)-labeled MBL-2.9 tumor cells were injected intraperitoneally with the simultaneous intravenous injection of 20 × 10^6 M-MSV–M-MLV–specific CTL clone cells. Tumor cell destruction was monitored by whole-body counting techniques (23). In additional experiments designed to investigate long-term survival in this tumor model, varying

numbers of nonlabeled MBL-2.9 tumor cells were injected intraperitoneally, followed by 20 × 10^6 cells of an active CTL clone intravenously.

Isolation of Antigen-Loss Tumor Cell Variants. In the long-term protection studies described above, some mice developed ascites 30–60 d after tumor cell and CTL clone injection. Ascites cells were harvested from such mice, washed, and cloned by micromanipulation into single flat-bottom microwells without added filler cells. Those tumor cell lines which grew out after cloning (HEMO-4, 10^5-2, and 10^5-5 derived from MBL-2; and 1/7.1 and 1/7.2 derived from MBL-2.9) were then tested for the ability to serve as target cells for M-MSV–M-MLV–specific CTL populations. In certain cases, the antigen-loss tumor cell clones derived in vitro were readapted to growth as ascites and retested in the I-UdR whole-body counting assay system.

DNA and RNA Analysis. Cellular DNA was isolated and analyzed by Southern hybridizations as described (24). Restriction enzymes (Boehringer Mannheim) were used as described by the manufacturer. The DNA probes used in hybridizations were prepared as follows: DNA of plasmids MS-2 and MS-3 (24) was digested with Pst I; the inserts were isolated and self-ligated, yielding concatamers of up to eight times the insert's size. These products were nick-translated with [^{32}P]dATP (3,000 Ci/mmol) to specific activities of 7 × 10^8 cpm/μg. Cytoplasmic RNA was isolated as described (25). M-MLV-specific RNA was detected in Northern hybridizations using the MS-2 probe (25).

Results

Target Molecule Recognized by M-MSV–M-MLV–specific CTL Clones. The molecule recognized by the Lyt-2$^+$ CTL clone CHM-14 that is specific for antigens induced by the oncogenic virus complex, M-MSV–M-MLV (7), is expressed on the surface of virus-infected but not uninfected cells. To characterize target molecules, we used the Ab-X-MLV mutant virus, which was derived from M-MLV by molecular recombination (17). As shown in Fig. 1, M-MLV harbors three genes, of which the first one, *gag*, codes for the precursor proteins gPr80^{gag} and Pr65 (26). gPr80^{gag} is processed to *gag* membrane proteins gP85^{gag} and gP95^{gag}, while Pr65 is processed to p15, p12, p30, and p10, which are found in the virion core. The Ab-X-MLV mutant, which harbors a point mutation in the *gag* gene, can no longer produce gPr80^{gag} but is able to synthesize all other M-MLV-encoded proteins (17). C57BL/6 "B lymphoblasts" and C57BL/6 murine embryo fibroblasts (MEF) were mock-infected or infected with either M-MLV or Ab-X-MLV. Cell cultures were examined for the synthesis of virus-related proteins and for susceptibility to lysis by CTL clones. As shown in Fig.
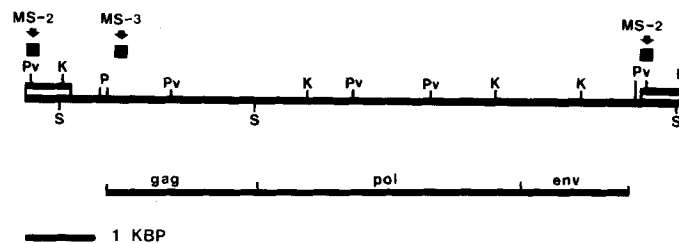

FIGURE 1. Location of the M-MLV-specific DNA probes on the M-MLV restriction map. The locations of the M-MLV-specific DNA probes MS-2 and MS-3 were determined previously (24) and are indicated. Also shown are the recognition sites for the following restriction enzymes: Kpn I (*K*), Pst I (*P*), Pvu II (*Pv*), and Sac I (*S*) (26). The location of the three viral genes *gag*, *pol*, and *env*, with respect to the M-MLV physical map, was according to reference 26.
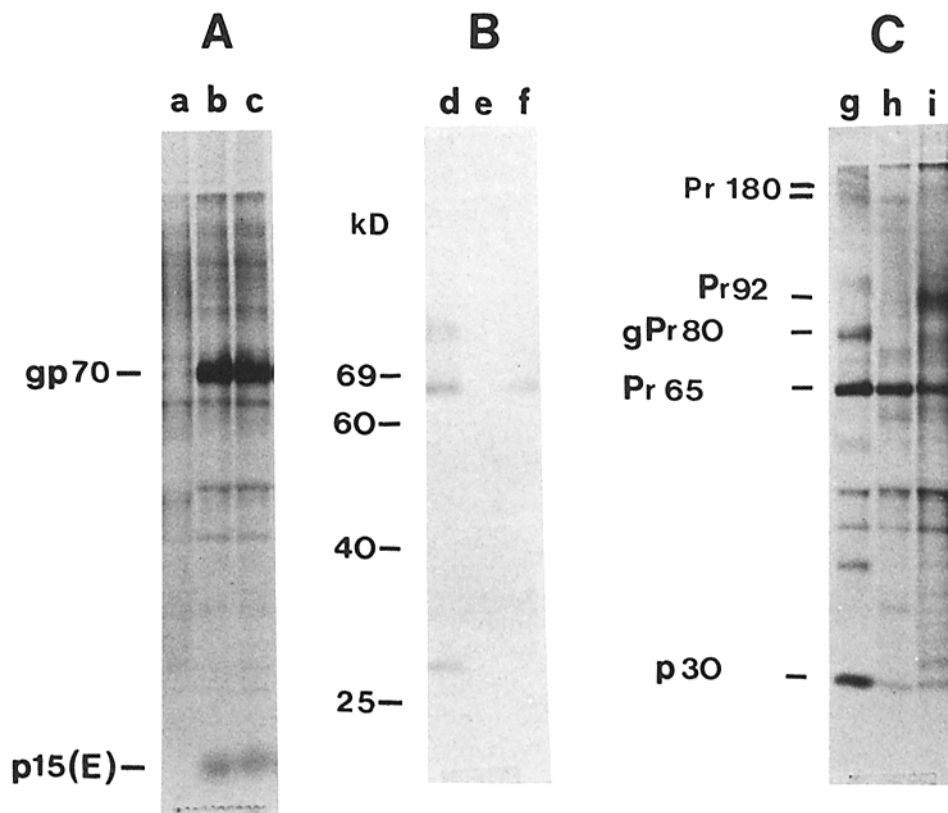
FIGURE 2.   gag Membrane precursor proteins in virus-infected cells. Cells, labeled metabol-
ically with [^{35}S]methionine for 1 hr, were lysed immediately (B and C) or after a chase period
of 4 h (A). Viral proteins were analyzed by immunoprecipitations, using anti-gp70 antiserum
(A) or anti-p30 antiserum (B and C), and electrophoretic separation on 10% SDS-polyacryl-
amide gels. EN^{3}HANCE-treated gels were exposed for 1 wk (A and C) or for 1 d (panel B) to
Kodak XAR-5 film. The precipitated proteins originated from uninfected MEF (lanes a and
e), M-MLV-infected MEF (b, d, and g), Ab-X-MLV-infected MEF (c, f, and h), and 1/7.2R
cells (i).

2, MEF did not synthesize proteins recognized by anti-gp70 or anti-p30 antisera
(A, lane a and B, lane e). In M-MLV-infected MEF, gPr80^{gag}, Pr65, and p30
were detected using anti-p30 antiserum (Fig. 2B, lane d). In contrast, Ab-X-
MLV-infected MEF synthesized Pr65 and low amounts of p30 (Fig. 2, B lane f
and C lane h) but no gPr80^{gag}. As shown in Table I, the M-MSV–M-MLV–
specific CTL clones efficiently lysed M-MLV-infected B lymphoblasts or MEF,
but not mock-infected or Ab-X-MLV-infected cells. The observed difference in
sensitivity to lysis of B lymphoblasts vs. MEF by CTL is most likely due to
differences in inherent susceptibility to lysis (16). Ab-X-MLV- and M-MLV-
infected MEF showed indistinguishable patterns of synthesis of the env gene–
encoded proteins gp70 and p15 (E) (Fig. 2A, lanes c and b). Therefore, although
we cannot rule out an undetected role of gp70 in the recognition of the target
cells by CTL, it is unlikely that gp70 or p15 (E) themselves serve as target
molecules. Thus, these results suggest that the CTL clones specifically recognized

CHARACTERIZATION OF $gP85^{gag}$

TABLE I

C57BL/6 M-MSV–M-MLV–specific CTL Clones Recognize a gag Gene Product on Virus-infected Primary Tissue Culture Target Cells

Exp.	CTL clone/target cell combination	Percent specific ^{51}Cr release								
		Mock-infected			M-MLV-infected			Ab-X-MLV-infected		
		0.3:1*	1:1	3:1	0.3:1	1:1	3:1	0.3:1	1:1	3:1
A	C57BL/6 "B lymphoblasts" with CTL clones:									
	CHM-14‡	1	1	3	36	65	81	8	10	12
	MOM-1.2	1	2	2	58	77	97	3	6	18
	MOM-2.5	1	2	4	57	68	79	4	10	22
B	C57BL/6 embryo fibroblasts with CTL clone									
	CHM-14§	1	1	4	13	29	41	2	5	11

"B lymphoblasts" and murine embryo fibroblasts were prepared and infected with virus as described in Materials and Methods.
* Effector-to-target cell ratio, 4 h incubation.
‡ Clone CHM-14 gave 155 LU/10^6 tested on MBL-2.9 target cells.
§ Clone CHM-14 gave 103 LU/10^6 tested on MBL-2.9 target cells.

the gag-related membrane protein $gP85^{gag}$, encoded by M-MLV, of which $gPr80^{gag}$ is the precursor protein.

Isolation of Antigen-Loss Tumor Cell Variants after Immunotherapy With a CTL Clone. A modest protection against tumor formation was observed in our previous studies (7), in which 2 × 10^6 M-MLV-induced MBL-2.9 tumor cells were injected intraperitoneally, followed by intravenous injection of 20 × 10^6 CTL clone CHM-14. When lower numbers of MBL-2.9 cells were injected, a much more significant long-term survival of mice was observed (up to 150 d after injection). However, after 1–2 mo, certain mice that had been initially protected developed ascites. Tumor cells of such mice were cloned in vitro, labeled with ^{51}Cr, and tested for their ability to serve as target cells for virus-specific CTL clones. As shown in Table II, several of the antigen-loss variant tumor cell clones were no longer lysed by either the virus-specific CTL clones or an M-MSV–M-MLV–specific MLTC population. We determined that these antigen-loss variants expressed unaltered levels of H-2^b surface alloantigens, as tested using monoclonal antibodies and cell sorter analysis (data not shown). Furthermore, we show that they are lysed by allogeneic BALB/c anti-C57BL/6 MLC cells containing CTL directed against C57BL/6 H-2^b alloantigens (Table II).

Transcriptionally Silent M-MLV Genomes in Antigen-Loss Variants. Several of the CTL-resistant antigen-loss tumor cell variants were examined for expression of M-MLV gag-related proteins. All of the variants were negative for M-MLV protein expression compared with the parental tumor cell line (data not shown). This phenotypic change could be due to loss of M-MLV genomes or to absence of expression of M-MLV DNA. Three independent antigen-loss variants, 1/7.1, 1/7.2, and HEMO-4, were examined in detail with the M-MLV-specific DNA

TABLE II

Characterization of M-MLV Antigen-Loss Tumor Cell Variants Using Various CTL
Effector Cells

Effector cell population	Target cell lysis (LU/10⁶)						
	MBL-2	10⁵-5	10⁵-2	HEMO-4	MBL-2.9	1/7.1	1/7.2
M-MSV–M-MLV–specific CTL clone CHM-14	330	300	0.3	0.3	370	0.3	0.3
M-MSV–M-MLV–specific MLTC population	15	10	0.3	0.3	30	0.3	0.3
BALB/c anti-C57BL/6 allogeneic MLC	22	25	22	17	71	42	36

10⁵-5, 10⁵-2, and HEMO-4 are tumor cell clones derived by micromanipulation from the peritoneal cavity of mice initially protected against MBL-2 tumor cells. 10⁵-2 and 10⁵-5 originated from the same mouse. 1/7.1 and 1/7.2 are tumor cell clones derived by micromanipulation from the peritoneal cavity of a mouse initially protected against MBL-2.9, a subclone of MBL-2.
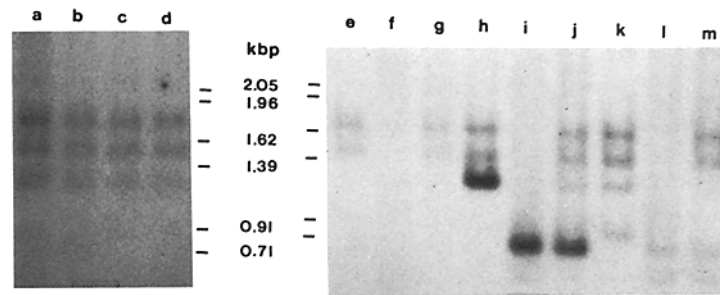

FIGURE 3. Analysis of M-MLV genomes in antigen-loss variants and in a revertant. The presence of M-MLV-specific DNA in antigen-loss variants, parental tumor cell lines, and in the revertant 1/7.2R cell line was examined by hybridizations of Southern blots (25) of gel-separated, Kpn I–digested DNAs using the nick-translated, M-MLV-specific MS-2 probe (24). The methylation patterns of M-MLV genomes were analyzed by comparing Kpn I/Hpa II– and Kpn I/Msp I–restricted DNAs using the same probe. Radioautographs of hybridized filters were made on Kodak XAR-5 film and photographed. The sources of DNA and the restriction enzymes were: Kpn I–digested MBL-2.9 DNA (lane a); Kpn I–digested 1/7.1 DNA (b); Kpn I–digested 1/7.2 DNA (c); Kpn I–digested HEMO-4 DNA (d); L cell DNA digested with either Kpn I (e), Kpn I/Msp I (f), or Kpn I/Hpa II (g); 1/7.2R DNA digested with Kpn I (h), Kpn I/Msp I (i), or Kpn I/Hpa II (j); and MBL-2.9 DNA digested with Kpn I (k), Kpn I/Msp I (l), or Kpn I/Hpa II (m). An Eco RI/Hind III digest of bacteriophage λ DNA served as marker.

probes MS-2 and MS-3, which previously had been used to detect M-MLV-specific DNA restriction fragments (24). The location of these DNA probes with respect to the M-MLV restriction map is indicated in Fig. 1. Cross-hybridization of MS-2 with the long terminal repeats (LTR) of endogenous MLV present in mouse DNA (27) was observed, while no such cross-hybridization was found for MS-3. To distinguish the M-MLV LTR from LTR of endogenous MLV, using MS2 as probe, DNA of the three variants and of MBL-2.9 was digested with Kpn I, which generated a 1.3 kilobasepair (kbp) fragment recognized with MS-2, which is diagnostic for M-MLV (24). The endogenous MLV DNA give rise to fragments of 1.5 and 1.8 kb upon digestion with Kpn I, recognized with MS-2 (27). As shown in Fig. 3, b–d, all three variants retained M-MLV genomes, as indicated by the presence of the 1.3 kbp Kpn I fragment, which was also detected

in Kpn I–digested MBL-2.9 DNA (Fig. 3a). In contrast, M-MLV-negative mouse cells (L cells) only harbor the 1.5 and 1.8 kbp fragments (Fig. 3e). Using MS-3 as a probe, we observed the 2.9 kbp Kpn I M-MLV fragment in the antigen-loss variants (not shown). However, the size analysis of RNA synthesized in MBL-2.9 cells (Fig. 4a) and in the antigen-loss variants (Fig. 4, b–d) revealed M-MLV RNA in the parental tumor cell line only. The 8.2 kb genomic RNA has been previously shown (12) to serve as the mRNA for gag and gag-pol protein products, while the 3.2 kb RNA species is translated into env gene products. The absence of measurable amounts of viral RNA suggests that the antigen-loss variants retained transcriptionally inactive M-MLV copies. The remaining lanes in Figs. 3 and 4 are the results of experiments described below.

Reversion of Antigen-Loss Variants to CTL-susceptible Phenotype. Upon prolonged culture in vitro (3 mo), two of the five cloned antigen-loss tumor cell variants reverted back to their original phenotype, i.e., they were again lysed by the virus-specific CTL clone CHM-14 when tested in a ^{51}Cr release assay, suggesting the reexpression of M-MLV genomes in these cells. One of these CTL-susceptible revertants (1/7.2, tested on February 16, 1984, hereafter referred to as 1/7.2R) (Fig. 5) was analyzed at the molecular level. A preliminary DNA analysis had revealed that the 1/7.2R cells harbored an amplified number of M-MLV genomes, which might be responsible for the expression of M-MLV genomes. To quantitate this result and to examine the state of methylation of the M-MLV genomes, we performed experiments comparing 1/7.2R and MBL-2.9 cells as well as L cells, which do not harbor M-MLV genomes. For the methylation studies we used selective inhibition of the restriction enzyme Hpa II by methylation of the second cytosine in the recognition sequence CCGG. The isoschizomer Msp I cleaves this sequence regardless of the state of methylation (28). DNA of the three cell lines was digested with Kpn I, Kpn I/Hpa II or Kpn I/Msp I and analyzed using the MS-2 probe (Fig. 3). As expected, the 1.3 kbp Kpn I M-MLV fragment detected in 1/7.2R (Fig. 3h) and MBL-2.9 cells (k) is absent from L cells (e). Densitometric scanning of lanes h and k revealed that
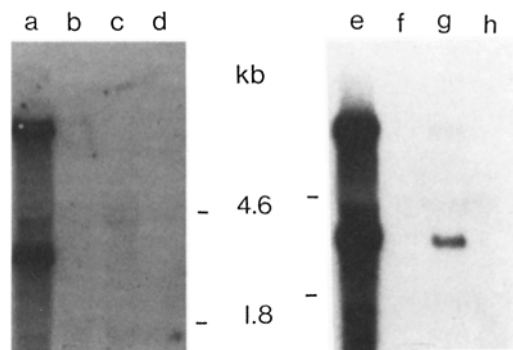

FIGURE 4. Absence of expression of M-MLV-specific RNA in antigen-loss variants. Cytoplasmic RNA was isolated from MBL-2.9 cells (a and e), 1/7.1 cells (b and f), 1/7.2 cells (c), HEMO-4 cells (d and h), and 1/7.2R cells (g). RNA were denatured in glyoxal/dimethyl sulfoxide, separated electrophoretically on agarose gels, transferred to nitrocellulose filters, and hybridized to nick-translated M-MLV-specific MS-2 probe (25). Autoradiographs were prepared and photographed. Ribosomal RNA served as markers.
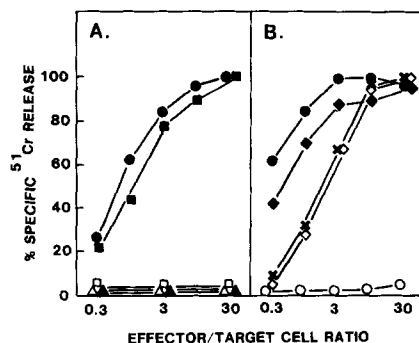
FIGURE 5.   Reversion of antigen-loss variants to a CTL-susceptible phenotype upon passage in vitro. All target cells were tested using CTL clone CHM-14 under standard ⁵¹Cr release assay conditions (16). (A) Antigen-loss tumor cell variants 1/7.1 and 1/7.2 were cloned in vitro by micromanipulation of tumor cells obtained from the peritoneal cavity of a mouse initially protected against MBL-2.9 tumor cell growth by the intravenous injection of CTL clone CHM-14. (△) 1/7.1 tested on 6 September, 1983; (□) 1/7.2 tested on 6 September, 1983; (●) MBL-2.9 tested on 6 September, 1983; (▲) 1/7.1 tested on 16 February, 1984; (■) 1/7.2 tested on 16 February, 1984. (hereafter called 1/7.2R). (B) Antigen-loss tumor cell variant 10⁵-2 and the susceptible tumor 10⁵-5 were cloned in vitro from a mouse initially protected against MBL-2 tumor cells using CTL clone CHM-14. (○) 10⁵-2 tested on 1 June, 1983; (×) MBL-2 tested on 1 June, 1983; (◇) 10⁵-5 tested on 1 June, 1983; (●) 10⁵-2 tested on 6 September, 1983; (◆) 10⁵-5 tested on 6 September, 1983.

1/7.2R yielded approximately eight times more of the 1.3 kbp fragment than did MBL-2.9, in contrast to 1/7.2 cells, which contained as many M-MLV genomes as did MBL-2.9 cells (Fig. 3, a and c). Digestion of 1/7.2R and MBL-2.9 DNA with Kpn I/Msp I produced a 0.7 kbp fragment detectable with MS-2, which was amplified eight times in 1/7.2R cells (Fig. 3, i and l), whereas no such fragment was found in L cell DNA (f). Digestion with Kpn I/Hpa II yielded 0.7 kbp fragments in 1/7.2R and MBL-2.9 DNA (Fig. 3, j and m), but not in L cell DNA (g), indicating the presence of nonmethylated M-MLV genomes in 1/7.2R and MBL-2.9 cells. Upon digestion of 1/7.2R DNA with Kpn I/Hpa II, we also observed the 1.3 kbp Kpn I fragment (Fig. 3j), indicating the presence of some methylated M-MLV genomes in these cells. Consistent with the detection of an amplified number of nonmethylated M-MLV copies in 1/7.2R cells, we observed the expression of the 8.2 and 3.2 kb M-MLV-related RNA species in these cells (Fig. 4g), but not in the antigen-loss variants 1/7.1 and HEMO-4 (Fig. 4, f and h).

Expression of an Aberrant gag Precursor Protein in 1/7.2R Cells.   The susceptibility of the 1/7.2R cells to lysis by CTL clone CHM-14 as well as the expression of M-MLV-specific mRNA suggested that these tumor cells reexpressed *gag*-related membrane proteins. The precursor of these proteins was characterized by metabolic labeling of 1/7.2R cells and immunoprecipitation with anti-p30 antiserum (Fig. 2, panel C). M-MLV- and Ab-X-MLV-infected MEF were included as controls: these cells synthesized, respectively, gPr80ᵍᵃᵍ, Pr65 and p30 (Fig. 2C, lane g) and Pr65 and p30 (lane h). In 1/7.2R cells, Pr65 and p30 as well as large amounts of a new *gag*-related protein, Pr92, were detected (Fig. 2C, lane i); surprisingly, no gPr80ᵍᵃᵍ was observed in these cells. Also, whereas Ab-X-MLV-infected MEF only synthesized the lower molecular weight species

of the Pr180$^{gag\text{-}pol}$ doublet, which is related to Pr65 (Fig. 2C, lane h), M-MLV-infected MEF and 1/7.2R cells expressed both species of Pr180$^{gag\text{-}pol}$ (12) (lanes g and i). Apparently, the M-MLV genomes that are expressed in 1/7.2R cells have undergone a mutational event in vitro, leading to the disappearance of gPr80^{gag} and its replacement by Pr92, but leaving the synthesis of Pr65 unaffected.

gag Region Recombination in 1/7.2R M-MLV Genomes. We attempted to determine the region of the 1/7.2R M-MLV genomes in which the mutation occurred by DNA analysis using MS-3 as a probe. MS-3 is specific for the N-terminal region of the M-MLV *gag* gene (Fig. 1) (24). In preliminary experiments using MS-3 to detect the 2.9 kbp Kpn I M-MLV fragment, we unexpectedly observed the presence of an additional fragment of 3.2 kbp in 1/7.2R DNA, suggesting the insertion of 300 bp of DNA in the 2.9 kbp Kpn I region of some M-MLV genomes in 1/7.2R cells. A restriction enzyme analysis was carried out to identify the site of insertion (Fig. 6). Digestion of 1/7.2R DNA with Kpn I/Sac I, Kpn I/ Pvu II, or Pvu II/Pst I generated fragments that were detected with MS-3 of 2.6 and 2.9 kbp (Fig. 6a), 1.5 and 1.8 kbp (b), and 0.65 kbp (c), respectively. Digestion of L cell DNA with the same combinations of enzymes did not yield fragments detectable with MS-3 (Fig. 6, d–f). Consistent with previous data (24), digestion of MBL-2.9 DNA with Kpn I/Sac I, Kpn I/Pvu II, or Pvu II/Pst I generated fragments of, respectively, 2.6, 1.5, and 0.65 kbp (not shown). Based on the M-MLV restriction map and the location of the MS-3 probe (see Fig. 1), we conclude that the 300 bp insertion event occurred in the *gag* region between the Kpn I site in the 5' LTR and the two Pst I sites. Nucleotide sequence analysis of this fragment of M-MLV DNA has shown (26) that the N-termini of gPr80^{gag} and Pr65 are located in this region, although the precise initiation site of the
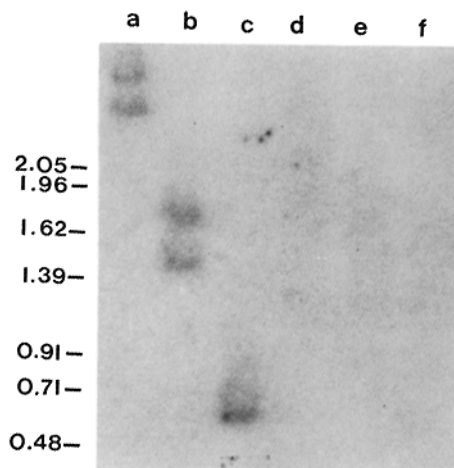

FIGURE 6. Localization of a DNA insertion in M-MLV genomes in 1/7.2R cells. For the localization of the 300 bp DNA insertion in the *gag* region of M-MLV genomes in 1/7.2R cells, which are susceptible to lysis by M-MSV–M-MLV–specific CTL clones, DNA from 1/ 7.2R cells (a, b, and c) and L cells (d, e, and f) was digested with Kpn I/Sac I (a and d), Kpn I/Pvu II (b and e), or Pvu II/Pst I (c and f). Restricted DNA were analyzed as described in Fig. 3, using a nick-translated, M-MLV-specific MS-3 probe (24) and the same marker DNA.

gPr80^{gag} N-terminus remains to be determined. The size difference between Pr92 and gPr80^{gag} can be accounted for by translation of the 300 bp DNA insert during the synthesis of gag-related proteins in 1/7.2R cells. Since the size of Pr65 was not altered in 1/7.2R cells, we conclude that the insertion event took place in a region of the M-MLV genome that codes exclusively for the gag membrane precursor protein.

Inhibition of Virus-induced Tumor Formation by the Intravenous Injection of Virus-specific CTL Clones. To study the potential protective effects in vivo of the CTL clones that specifically recognize gag-related membrane proteins on M-MLV-infected target cells, mice were injected simultaneously with M-MSV–M-MLV in the footpad and injected intravenously with 20 × 10^6 of either virus-specific cloned CTL, syngeneic irradiated spleen cells, or anti-H-2^d-specific cloned CTL (Fig. 7). Mice injected with virus and with spleen cells developed tumors in the footpad and died. In contrast, the intravenous injection of virus-specific CTL clones CHM-14, MOM-1.1, or MOM-2.1 resulted in a complete, long-lived protection against tumor induction in C57BL/6 mice that had been sublethally irradiated (650 rad) 16 h before injection (Fig. 7, A and B). The injection of another group of mice with 20 × 10^6 CTL clone CHA-11, specific for H-2^d alloantigens, had no inhibitory effect on tumor induction (Fig. 7, A and B). This CTL clone has been shown (7) to be active in a specific manner against H-2^d tumor targets in vivo. Thus, the prevention of tumor formation was specific with respect to the CTL clone injected. In additional experiments, we have shown that injection of the virus-specific CTL clone CHM-14 has no effect on the growth of H-2^d allogeneic tumors in the peritoneal cavities of sublethally irradiated mice, which were protected at the same time against M-MSV–M-MLV–induced tumor formation in the footpad (data not shown).
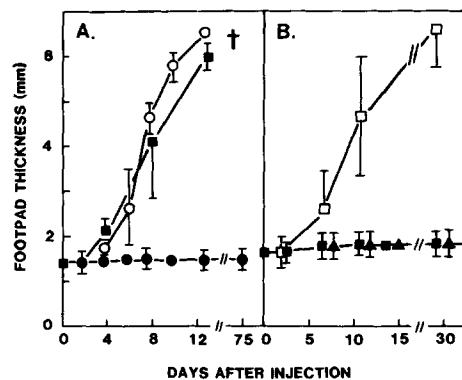

FIGURE 7. Inhibition of virus-induced tumor formation by the intravenous injection of M-MSV–M-MLV–specific CTL clones. Groups of five C57BL/6 female mice were irradiated sublethally (650 rad) and injected in the right rear footpad with 40 μl of an M-MSV–M-MLV stock. Various C57BL/6 effector cells were injected intravenously immediately after virus injection. Experiment A: (○) 20 × 10^6 irradiated (2,000 rad) syngeneic spleen cells; (●) 20 × 10^6 M-MSV–M-MLV–specific CTL clone CHM-14; (■) 20 × 10^6 anti-H-2^d-specific CTL clone CHA-11. Experiment B: (□) 20 × 10^6 irradiated syngeneic spleen cells; (■) 20 × 10^6 M-MSV–M-MLV–specific CTL clone MOM-1.1; (▲) 20 × 10^6 M-MSV–M-MLV–specific CTL clone MOM-2.1.

Discussion

M-MSV–M-MLV–specific CTL Clones.   In this report we describe the protection
of mice against virus-induced tumors by treatment with virus-specific CTL clones.
These same CTL clones had been shown previously to induce the destruction of
M-MLV-induced lymphoma cells after intravenous injection (7). Similar experi-
ments in other systems have used either mixed populations of CTL (8, 29) or
cloned CTL (3, 30). The availability of cloned CTL and specific antisera against
M-MLV and Ab-X-MLV *gag* and *env* polypeptides has allowed us to characterize
the M-MLV-encoded protein gP85^{gag} as a target molecule recognized by the
cloned CTL. Ab-X-MLV-infected cells deficient in the synthesis of gP85^{gag} were
not lysed by the virus-specific CTL clones. The amounts of gp70 and p15 (E)
synthesized in Ab-X-MLV- and M-MLV-infected target cells were shown to be
indistinguishable; therefore, we believe it unlikely that the *env*-related proteins
act as target molecules for the virus-specific CTL clones used in this study.
Several reports have suggested the existence of CTL populations specific for
gag gene products (31–33). Plata et al. (33) investigated the inhibition of CTL
activity by anti-p30 monoclonal antibodies that recognized p30 determinants on
Friend-, Rauscher-, Moloney-, and Gross-MLV–infected cells. However, the
authors detected inhibition of cytolysis only of Gross-MLV–infected cells using
a mixed population of Gross-MLV–specific CTL. Other reports (34, 35) have
suggested that the viral *env* gene products gp70 and p15 (E) could be target
molecules recognized by CTL, but experiments designed to detect blocking of
CTL activity by gp70 or anti-gp70 antibodies have yielded contradictory results
(9, 13, 14). Flyer et al. (10) demonstrated the existence of gp70- or p15 (E)–
specific CTL using a mixed population of CTL and target cells transfected with
and expressing only the M-MLV *env* gene. Therefore, our data suggest that both
gag and *env* gene products can act as target molecules for virus-specific CTL,
depending on the system under investigation. gP85^{gag}, identified in this report
as a target molecule for M-MSV–M-MLV–specific CTL lysis, was previously (36,
37) shown to be identical to the Gross cell surface antigen (GCSA), which is
expressed on cells infected by viruses of the FMR group and by Gross-MLV.
Green (32) showed a correlation between GCSA expression and susceptibility of
Akv-induced tumor cells to CTL lysis.

The two primary *gag* gene products, gPr80^{gag} and Pr65, are synthesized
independently. gPr80^{gag} contains all Pr65 peptides as well as 4–6 kilodaltons of
unique amino-terminal peptides (11, 12). Several viruses, derived from or highly
related to M-MLV, do not synthesize proteins related to M-MLV gPr80^{gag} (38,
39). Since M-MLV only codes for Pr65 (40), the target molecule recognized by
the virus-specific CTL clones must be encoded exclusively by the M-MLV
genome. This suggests that the observed protection of mice against M-MSV–M-
MLV–induced tumor formation by the virus-specific cloned CTL was due to
selective destruction of cells infected with the virus complex, although we cannot
exclude the influence of other factors (41) leading to the elimination of virus-
infected cells.

A Possible Role for CTL in MLV-induced Leukemogenesis.   The identification of
gP85^{gag} as a target molecule recognized by virus-specific CTL suggests a possible
involvement of such CTL in the development of leukemias induced by nonleu-

kemogenic MLV. Their specific cytolytic activity may play a role during the selection of cells infected with recombinant leukemogenic MLV, such as mink cell focus-inducing viruses (MCF) (42), during the preleukemic stage. Analysis of the structure of MCF viruses shows that they arose from multiple recombination events between ecotropic and xenotropic MLV (24, 43). Based on previous studies and the present data, the following model could account for their selection: an endogenous nonleukemogenic MLV can be expressed after birth in certain strains of mice (for instance Akv) (43) or, when it is injected into newborn mice, it can infect several organs. The MLV can recombine either with the LTR region of a xenotropic MLV (44, 45) or with the coding region of other xenotropic MLV. In the latter situation (46, 47), the virally encoded xenotropic cell membrane proteins, such as $gPr85^{gag}$ or gp70, will not be recognized by specific CTL induced as a result of the original MLV infection, leading to appearance of nonleukemogenic class II MCF viruses. In the situation where the original MLV recombines only with specific xenotropic U_3 LTR sequences, the resulting viruses generally will also be nonleukemogenic. Cells infected with such MLV will be eliminated by specific CTL, unless these MLV recombine further with xenotropic MLV in their *gag, pol*, and/or *env* regions. Viruses resulting from these secondary recombination events in the *env* and possibly also the *gag* region possesses the structure of leukemogenic class I MCF viruses (24, 44). Of course, additional factors (e.g., antibody formation) most likely play an important role in these and other steps of MLV-induced leukemogenesis. In addition, Chesebro et al. (48) showed that in some mouse strains and cell types, the expression of MCF viruses may not be required in leukemogenesis. The actual mechanism of transformation of virus-infected target cells is not known. The MCF viral *env* gene product itself has been suggested to play a role in the transformation process (49, 50). It could also stimulate the continuous recruitment of T cells, thereby enhancing the probability of spontaneous transformation (51). Alternatively, the insertion of MLV in the vicinity of oncogenes may lead to the activation of such genes, resulting in the transformation of cells (52, 53). Some aspects of the model presented above can be tested: Are MCF-type viruses generated during MLV-induced leukemogenesis in mice that are unable to generate virus-specific CTL (41)? Do viruses deficient in the synthesis of *gag* membrane proteins induce leukemias in C57BL/6 mice and, if so, what MCF-type viruses, if any, can be isolated from leukemic tissues?

Recombination of MLV Genomes In Vitro. The antigen-loss variants were detected because the variants grew as ascites, while the parental tumor cells were eliminated by virus-specific CTL. Emergence of antigen-loss variants has been demonstrated both in vivo (54) and in vitro (55), but the mechanism for their generation remains to be determined. From methylation experiments we concluded that, in the revertant cell line 1/7.2R, the transcriptionally silent character of the original M-MLV genomes may be due to methylation of these viral genomes in vivo. Methylation events have been described both in vivo (28) and in tissue culture (56) that lead to the loss of expression of the genes under investigation. In the latter study (56) the appearance of flat revertants of Fujinami sarcoma virus (FSV)-transformed cells was shown to be due to hypermethylation of the FSV genome in vitro. The authors observed the reappearance of trans-

formants, again harboring the FSV genome in a nonmethylated state. These retransformants resembled (56) the revertant antigen-loss variant 1/7.2R, which reexpressed a *gag* membrane protein. However, in addition to the nonmethylated state of some M-MLV copies in 1/7.2R cells, the insertion of 300 bp DNA of unknown origin into the *gag* region of these M-MLV genomes was detected. The RNA transcribed from M-MLV genomes harboring this inserted DNA is translated, leading to the addition of ~100 amino acid residues to the gPr80^{gag} protein. Indeed, a Pr92 protein was detected in 1/7.2R cells. Many cases of *gag* region recombinations in vivo have been described (57, 58), but were rarely observed in tissue culture (59). The *gag* recombination event resulting in the expression of Pr92 in 1/7.2R cells most likely occurred in only one or a few cells of the antigen-loss variant 1/7.2R. Recently (Van der Hoorn, unpublished results), we transmitted virus shed by 1/7.2R cells to C3H10T1/2 murine fibroblasts and showed the expression of Pr92 in these cells. Therefore, we believe that this virus, which probably originated in a restricted number of the 1/7.2R cells, spread through the culture. This would explain the CTL-susceptible phenotype of the 1/7.2R cells. A comparison of our data with the M-MLV DNA sequence shows that the insertion event occurred in the region between nucleotides 32 and 621 (26). Molecular cloning of the virus shed by 1/7.2R cells and sequencing of its *gag* region may help to establish the origin of the inserted DNA and identify the initiation codon for gPr80^{gag}.

Summary

The *gag* membrane protein gP85^{gag}, encoded by Moloney murine leukemia virus (M-MLV), was identified as a target molecule recognized by Moloney murine sarcoma virus–M-MLV (M-MSV–M-MLV)–specific cytolytic T lymphocyte (CTL) clones. Target cells infected with Ab-X-MLV, an M-MLV-derived mutant virus not encoding gP85^{gag}, were not lysed by the CTL clones. The same CTL clones were shown previously to induce the destruction of M-MLV-induced tumor cells in the peritoneal cavity. We have now characterized CTL-resistant antigen-loss tumor cell variants that have lost the surface antigen, but which retain transcriptionally silent M-MLV genomes. A cloned antigen-loss variant that reverted in vitro to the CTL-susceptible phenotype reexpressed M-MLV genomes that had undergone an insertion event in the region of the viral DNA coding for the *gag* membrane protein. Intravenous injection of virus-specific CTL clones inhibited tumor formation in mice injected subcutaneously with M-MSV–M-MLV.

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