31. Aliquots of genomic DNA (5 μg) from the five plant species described in Fig. 2C were digested with Xba I, Eco RI, and Sca I, separated by electrophoresis on a 0.8% agarose gel, and blotted onto nitrocellulose. The blot was hybridized as described (*37*) but without formamide. The blot was washed at 55°C in 1× SSC and 0.1% SDS.
32. B. McGurl and C. A. Ryan, unpublished observations.
33. The 747-bp fragment used as an antisense gene was excised from the prosystemin cDNA as a Bam HI–Hind III fragment. The antisense fragment thus duplicated the prosystemin mRNA sequence from the eighth amino acid through 154 bp of the 3′ untranslated region. The antisense construct was transformed into *Agrobacterium* strain LBA 4404 and the recombinant bacteria were used to transform tomato, var. Better Boy, as described (*34*).
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37. Nick-translation was performed according to the manufacturer's instructions (Du Pont Biotechnology Systems). Hybridization was carried out at 42°C in 50% formamide as described [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989), chap. 9]. Unless otherwise stated, blots were washed in 1× SSC and 0.1% SDS at 65°C.
38. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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41. We thank C. Palm and S. Thompson for help with sequence analysis, G. Munske for synthesizing oligonucleotides, and G. Wichelns for growing tomato plants. Supported in part by NSF grants DCB-8702538 and DCB-8608594; Project 1791, College of Agriculture and Home Economics, Washington State University; and the McKnight Foundation.

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Transcription Factor Loading on the MMTV Promoter: A Bimodal Mechanism for Promoter Activation

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The mouse mammary tumor virus (MMTV) promoter attains a phased array of six nucleosomes when introduced into rodent cells. This architecture excludes nuclear factor 1/CCAAT transcription factor (NF1/CTF) from the promoter before glucocorticoid treatment and hormone-dependent access of nucleolytic agents to promoter DNA. In contrast, when the promoter was transiently introduced into cells, NF1/CTF was bound constitutively and nucleolytic attack was hormone-independent. Thus, induction at this promoter was a bimodal process involving receptor-dependent remodeling of chromatin that allows NF1/CTF loading and direct receptor-mediated recruitment of additional transcription factors.

EUKARYOTIC DNA IS PACKAGED into chromatin (*1–3*), which can confer selective access of regulatory factors to their DNA binding sites (*4–6*). Thus, the structure of eukaryotic DNA in chromatin may be important in its interaction with soluble transcription factors (*6–9*). When the MMTV promoter is stably introduced into cells, it acquires a series of six positioned nucleosomes (*3, 10*). One of these nucleosomes (nuc-B) is positioned over binding sites for a group of transcription factors involved in promoter activation, including the glucocorticoid receptor (GR) and NF1. Hormone induction of the promoter results in hypersensitivity around nuc-B to endonucleases, chemical agents, and restriction endonucleases (*3, 6*). In parallel with this structural transition, hormone-dependent loading of a transcription preinitiation complex including NF1 has been demonstrated in vivo (*5*).

The exclusion of NF1 from promoter DNA in noninduced cells was puzzling, given its high affinity for its binding site on purified MMTV DNA (*11*). Further, no alterations in NF1 nuclear concentration or intrinsic binding affinity accompany hormone activation (*12*). Thus, we tested whether NF1 was excluded from the template by nucleoprotein structure before the hormone-dependent remodeling of the chromatin. We compared the interaction of NF1 with MMTV DNA organized in stably replicated chromatin and with an identical promoter introduced into the cell by transient transfection of a nonreplicating plasmid. The MMTV promoter, driving a reporter gene, luciferase (LUC), was transiently introduced into cells that had a stably replicating copy of the identical MMTV long terminal repeat (LTR) driving a second reporter gene, chloramphenicol acetyl transferase (CAT). Expression from each of the two classes of MMTV promoter was examined by use of reporter-specific primers for primer extension and the respective enzyme assays. The assays demonstrated that both promoters are active and strongly hormone inducible (Fig. 1). The degree of induction from the stable promoter was three- to fourfold greater than that from the transient construct (*13*), raising the possibility that the transient promoter may be derepressed (*14, 15*).

To monitor NF1-template interactions, we performed in vivo exonuclease III footprinting in combination with linear amplification by Taq polymerase (*5, 16*). Nuclei were isolated from cells incubated in the presence or absence of dexamethasone and digested with a restriction enzyme whose cleavage site was in a nucleosomal linker (*6*). The nuclei were digested with Exo III, the DNA was purified, and positions at which Exo III digestion was blocked were detected by extension with gene-specific primers that hybridized downstream of the anticipated Exo III boundary. We confirmed the previously reported finding (*5*) that NF1 was excluded from its binding site in the absence of glucocorticoid treatment on stable chromatin templates (Fig. 2, lane 2). Administration of dexamethasone resulted in a hormone-dependent Exo III block at position −82 (Fig. 2, lane 4). Hormone-dependent occupancy of the NF1 site in vivo was confirmed by deoxyribonuclease I footprinting (*15*).

In contrast, NF1 was constitutively present on transient DNA templates (Fig. 2, lanes 5 and 6). Digestion by Exo III was blocked at the NF1 site in cells in the absence of hormone. Furthermore, hormone induction produces no increase in occupancy at the NF1 site. Thus, in nuclei isolated from the same cells, the MMTV promoter was organized into two distinct classes with respect to NF1 loading (Fig. 2, lanes 2 and 4 through 6).

We examined the status of factors downstream of the NF1 site with the use of an Exo III entry point at +107 of the promoter, a site that was specific for the transient template (*6*). Digestion with Exo III revealed a stop at position −56 (Fig. 2, lanes 7 and 8), which corresponded precisely to the 3′ Exo III boundary of NF1 determined in vitro (*5, 12*). Identification of the 3′ boundary of NF1 in vivo confirmed the

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original identification of NF1 as the factor forming the upstream boundary of the hormone-dependent footprint (*5*). As with the 5′ boundary, occupancy of the site was hormone-independent. However, a hormone-dependent stop was observed at −24, a position that corresponds to the 3′ boundary of the TATA binding protein.

These results demonstrate that binding of NF1 to the MMTV promoter occurs without hormonal stimulation on transiently introduced templates. Mutagenesis experiments (with both transiently and stably transfected cells) have demonstrated the importance of the NF1 site in the activation of transcription in response to glucocorticoids (*17, 18*). The observation that transcription of the transient template is hormone-inducible (Fig. 1), but NF1 binding is not (Fig. 2), suggests that NF1 binding was necessary but not sufficient for transcriptional activation.

Constitutive binding of NF1 to its site in transiently transfected DNA suggests that the stable and transient templates are organized differently. To address this possibility, we characterized the micrococcal nuclease sensitivity and restriction enzyme access profile of both templates. Nuclei from cells transfected as in Fig. 1 were treated with micrococcal nuclease and the structure of the MMTV promoter was examined with template-specific probes. The DNA was subjected to electrophoresis, transferred to membranes, and hybridized with probes for luciferase (*19*) (Fig. 3B, transient template) or v-Ha-ras (*3*) (Fig. 3B, stable template). The stably replicating template generated a micrococcal ladder diagnostic of a nucleosome repeat [as reported (*3*)]. Surprisingly, identically treated samples hybridized to the luciferase probe failed to exhibit a specific nucleosome repeat.

To extend this analysis, we characterized the in vivo access of restriction enzymes to each of the templates (Fig. 4). Restriction endonucleases show differential access to their recognition sequences when the MMTV LTR is packaged into chromatin (*6*). Accessibility of sites on nuc-B was enhanced in hormone-treated cells, suggesting a hormone-induced structural transition. An identical pattern of enzyme access was observed when the A-B region of the LTR was reconstituted into a disome in vitro, indicating that histone octamer cores are the basis of the observed in vivo accessibility pattern (*6*).

Digestion of nuclei with the enzyme Sst 1 showed enhanced cleavage in the presence of hormone for the stably transfected template (Fig. 4). In contrast, the transient template was accessible to cleavage in the absence of hormone, and the extent of cleavage was unchanged by hormone treatment. This indicates that the structural transition observed in stable chromatin did not occur with transiently transfected DNA. As a control, we examined digestion by Hae III, an enzyme whose cleavage pattern is independent of hormone induction (*6*). In this case the pattern of Hae III cutting was independent of hormone treatment for both transient and stable templates (Fig. 4).

Although we have not determined what percentage of the transient templates are active, the fact that no hormone-dependent changes in the level of NF1 binding or restriction enzyme digestion are detected suggests that there was little or no hormone-dependent binding of NF1 to the transient template. Although hormone-dependent NF1 binding might occur on a small sub-

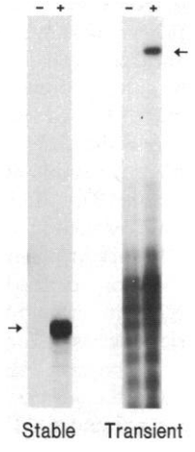

Fig. 1. Hormonal activation of the MMTV promoter. Mouse mammary cells, containing the MMTV promoter mobilized on a bovine papillomavirus (BPV)–based multicopy plasmid (1471.1 and 904.13) as a stable replicating unit, are transfected with the MMTV LTR attached to the luciferase gene, pLTRLuc (*19, 25*). Transcription complex formation and promoter architecture was studied by use of gene-specific probes that distinguish the transient from stable promoters. The hormonal activation of transcription from the MMTV promoter was assayed by primer extension analysis. The results presented were obtained with RNA isolated from cells subjected to hormone treatment for 4 hours either with (+) or without (−) dexamethasone (10^{-7} M), as indicated in the figure. RNA isolation, labeling of the probes, and primer extension were performed as described (*26*). The extension products were purified and then analyzed on 8% sequencing gels before autoradiography at −70°C. The transient template–derived RNA was analyzed with a primer, 5′-TACCAACAGTAC-CGGAATGCT-3′, that corresponds to a portion of the luciferase gene and thus was specific for the transfected copy of the LTR (*19, 25*). The primer used for the stable template–derived RNA, 5′-TTAGCTTCCTTAGCTCCTGAAAAT-3′, corresponds to a portion of the CAT gene (+104–+81) (*24*) and thus was specific for the endogenous copy of the LTR.

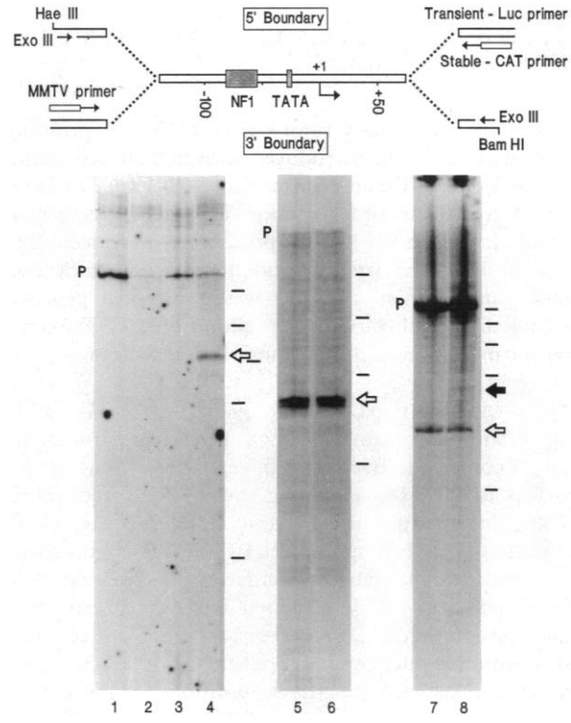

Fig. 2. Hormone-dependent and -independent loading of NF1. 1471.1 cells were transfected with plasmid pLTRLuc and 24 hours later were incubated with or without dexamethasone (10^{-7} M) for 1 hour (*10, 25*). The nuclei were isolated and digested with Hae III ± Exo III [as described (*5, 6*)]. Exo III is a 3′ to 5′ exonuclease, which processively digests DNA until it encounters sequences to which a protein is bound (NF1). Control experiments with Exo III digestion in the absence of the restriction enzyme digestion fail to produce specific Exo III stops. The purified DNA was analyzed by primer extension with Taq polymerase as described (*26, 27*). The primers are specific for either the CAT gene (lanes 1 through 4; stable template) or the luciferase gene (lanes 5 and 6; transiently transfected template). Lanes 1 through 6 were digested with Hae III either with (lanes 2 and 4 through 6) or without added Exo III (lanes 1 and 3). Samples in lanes 1 and 2 represent DNA from untreated cells and lanes 3 through 6 DNA from cells treated with hormone. The presence of an Exo III boundary at sequences shown previously to correspond to an NF1 binding site is indicated by the open arrow. In a separate experiment 1471.1 cells were transiently transfected with plasmid pNBoAf, containing the region −225 to +105 of the MMTV LTR (*6*) (lanes 7 and 8). The nuclei were isolated, digested with Bam HI and Exo III, and the DNA was purified and analyzed by primer extension (*5, 6, 27*). The primer used, 5′-TTAAGTAAGTTTTTGGTTACAAACT-3, corresponds to −200 to −175 of MMTV LTR. Exo III digestion products are specific for the transfected copy of the LTR because the chromatin template was not cleaved by Bam HI and was therefore unable to provide a specific entry site for Exo III. The closed arrow indicates the TATA binding protein site. Positions of φX174 DNA cut with Hae III as a marker are indicated by the hatch marks. The enzymes and probes above the genetic construct determine the 5′ boundary, whereas those below determine the 3′ boundary.

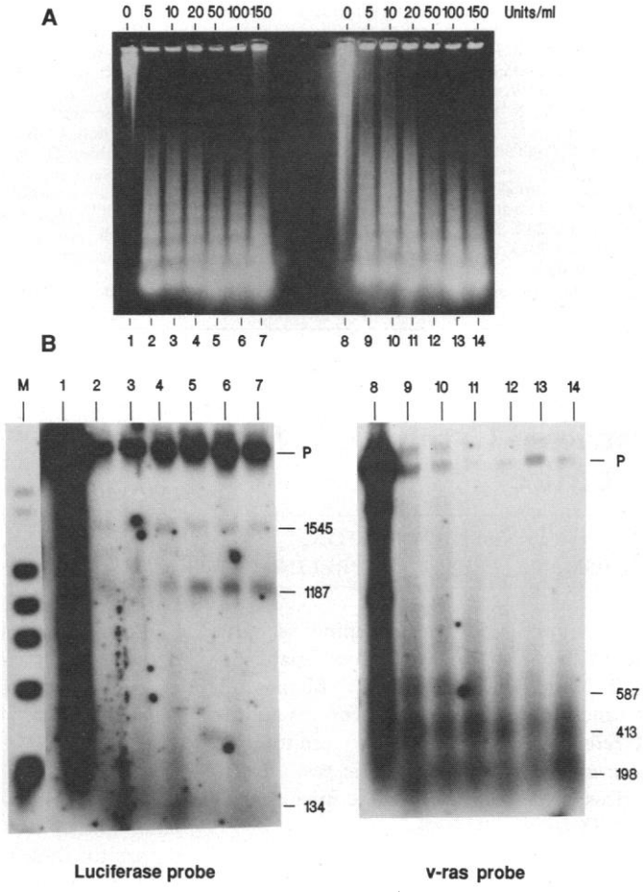

Fig. 3. Micrococcal digestion of chromatin and transfected templates. Cell line 904.13 was transfected with pLTRLuc, and then the cells' nuclei were isolated and digested with micrococcal nuclease (0 to 150 units/ml) (*3*). (**A**) The purified DNA was digested with either Ava I (lanes 1 through 7) or Eco RI (lanes 8 through 14), then analyzed on native agarose gels, and stained with ethidium bromide. (**B**) Gels were transferred to nylon membrane and then hybridized with ^{32}P-labeled DNA probes corresponding to the transfected template (luciferase) (*25*) (lanes 1 through 7) or the endogenous template (v-Ha-ras) (*3*) (lanes 8 through 14) as described (*3*). After hybridization the blots were washed and then subjected to autoradiography at −70°C. The band labeled P was the parental fragment generated by restriction digestion of the DNA. The 1187 and 1545 bands are the result of the micrococcal digestion.

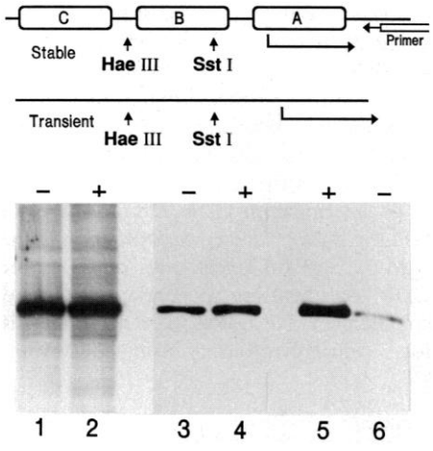

Fig. 4. In vivo restriction enzyme digestion of chromatin and transfected templates. Hae III (lanes 1 and 2) and Sac I (lanes 3 through 6) digestion of transiently transfected DNA (lanes 1 through 4) and stable chromatin (lanes 5 and 6) in the presence (+) or absence (−) of dexamethasone. Cell line 1471.1 was transfected with plasmid pLTRLuc and was treated with hormone for 1 hour. Nuclei were isolated then subjected to in vivo restriction enzyme digest as described (*6*). The DNA was purified and analyzed by Taq polymerase primer extension with reporter-specific primers (*27*).

fraction of the transient templates, we consider this to be an unlikely explanation of our results because we can detect transcription factor IID (TFIID) hormone–dependent loading (Fig. 2). Further, we are unable to detect the nucleosome repeat pattern characteristic of MMTV chromatin on transient DNA, and restriction enzyme access on this template was unchanged by hormone treatment.

We argued previously that a mechanism must exist to exclude NF1 from its site in vivo and that the specific organization of the promoter in stable chromatin may provide such a mechanism. In support of this hypothesis, we (*6, 8*) have demonstrated that NF1 will not bind to the MMTV −82 site when DNA is reconstituted into nucleosomes in vitro. Other mechanisms may be involved in factor exclusion as well, notably the presence of H1 in mature polynucleosome arrays (*14, 15*). Because NF1 binds constitutively if its site is available, but the promoter remains transcriptionally inactive, we propose that receptor activation at the MMTV promoter is a bimodal process. The first step is a structural transition in chromatin that is one consequence of receptor binding, which renders the NF1 site available for occupancy. We suggest that protein-protein contacts between NF1 and steroid receptor (or a bridging protein) (*20*) are not required to establish NF1 in the initiation complex. Rather, once bound, NF1 must interact independently of the receptor with some component of the initiation complex. We suggest that, in a second step, the receptor interacts, directly or indirectly, with some other member of the initiation complex because hormone-mediated activation is still observed in transient assays when NF1 is already loaded (Fig. 1) and TFIID is recruited to the promoter in transient assays (Fig. 2). This interaction is presumably among the activities that have been mapped to the transactivation domains of steroid receptors and may be mediated by protein-protein interactions (*21*). In our model, NF1 serves as an amplifier of the activation process. Its recruitment to the promoter is dependent on a chromatin transition; once bound to the template, it probably enhances transcription by protein-protein contacts because it is a stimulating factor in chromatin-free transcription extracts (*11, 23*).

The bimodal activation mechanism we propose provides obvious opportunities for transcriptional regulation in complex eukaryotic systems. As with NF1, many factors are constitutively present in the nucleus and are components of many promoter or enhancer complexes (*22, 23*). If these proteins can be prevented from binding to their sites except in the presence of more narrowly distributed activating factors, then the amount of background transcription would be reduced. In essence, these promoters would be in a repressed state. The methodology presented here may allow a distinction to be made between activation and derepression of transcription.

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LCMV-Specific, Class II–Restricted Cytotoxic T Cells in β_2-Microglobulin–Deficient Mice

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Intracranial infection of normal mice with lymphocytic choriomeningitis virus (LCMV) causes meningitis and death mediated by $CD8^+$ major histocompatibility complex (MHC) class I–restricted cytotoxic T lymphocytes (CTLs). β_2-Microglobulin–deficient mice ($\beta_2M^{-/-}$) do not express functional MHC class I proteins and do not produce significant numbers of $CD8^+$ T cells. When $\beta_2M^{-/-}$ mice were infected with LCMV, many died from LCMV disease and produced a specific response to LCMV mediated by $CD4^+$ CTLs that were class II–restricted. In these mice, $CD4^+$ CTLs may compensate for the lack of $CD8^+$ CTLs.

LYMPHOCYTIC CHORIOMENINGITIS virus–induced choriomeningitis in normal mice is mediated solely by $CD8^+$ cytotoxic T cells (*1*). Because $\beta_2M^{-/-}$ mice lack this cell population, we expected that they would be resistant to choriomeningitis, as are immunosuppressed or newborn normal mice (*2–6*). The $\beta_2M^{-/-}$ mice are derived from a C57BL/6 (B6) × 129/Ola cross and are *H-2*^{b}. We challenged B6, (B6 × 129/J)F_1, and $\beta_2M^{-/-}$ mice with LCMV (7). All three mouse strains shown (and 129/J mice) succumbed to LCMV disease (Fig. 1). The $\beta_2M^{-/-}$ mice survived longer [median survival time, 14 days; B6, 8 days; (B6 × 129/J)F_1, 7 days], with some surviving more than 3 weeks. The mean titer of LCMV isolated from the brains of $\beta_2M^{-/-}$ mice on day 6 after infection was 1.3×10^6 plaque-forming units (PFU) per gram of tissue ($n = 3$). Intracranial infection was confirmed by the presence of an antibody in all mice tested to date (16 out of 16). Histological examination of infected $\beta_2M^{-/-}$ and 129/J mice revealed similar leptomeningeal infiltrates of mononuclear cells (*8*).

Spleen cells from B6 mice infected with LCMV were cytotoxic when assayed on LCMV-infected targets matched for class I genes (Fig. 2A), including L cells (*H-2*^{k}) transfected with the *D*^{b} gene (L-D^b) and MC57 cells (*H-2*^{b}, class I^+, class II^-) (*9*). In contrast, when $\beta_2M^{-/-}$ mice were infected with LCMV, neither MC57 nor L-D^b targets were killed. Thus, there was no class I–restricted, LCMV-specific cytotoxicity. When $\beta_2M^{-/-}$ cells were tested with a class II^+ B cell lymphoma, CHB2 (*H-2*^{b}), we detected LCMV-specific CTLs (Fig. 2B). This killing was H-2–restricted: the *H-2*^{a} lymphoma CH12.LX was not killed. The B6 effector cells could also lyse CHB2 targets. To investigate the possibility that there was some killing restricted by non–β_2-associated MHC class I heavy chains of $\beta_2M^{-/-}$ mice (*3*), we prepared a fibroblast cell line from $\beta_2M^{-/-}$ embryos. Spleen cells from infected $\beta_2M^{-/-}$ mice did not lyse LCMV-infected $\beta_2M^{-/-}$ fibroblasts, which rules out an appreciable cytotoxic response restricted by the $\beta_2M^{-/-}$ class I heavy chains alone (*10*). To confirm that the killing of CHB2 target cells was restricted by class II molecules, we attempted to inhibit CTLs from $\beta_2M^{-/-}$ mice with monoclonal antibodies (MAbs) to class I or class II (Fig. 3A) (*11*). Only MAbs to class II were effective at inhibiting the killing of LCMV-infected CHB2 cells by $\beta_2M^{-/-}$ CTLs.

We were concerned that there might not be enough class II–expressing cells in $\beta_2M^{-/-}$ brains to serve as targets for the class II–restricted CTLs. Although there are class II^+ cells in normal brains, class II expression is low (*12*). We performed protein immunoblots to determine if up-regulation of class II was important in producing targets for these class II–restricted CTLs in $\beta_2M^{-/-}$ brains. Class II Ab^b ($A_\beta{}^b$) chains were substantially induced in both B6 and $\beta_2M^{-/-}$ brains after infection with the virus (*13*) (Fig. 3B). The infection induced Ab^b expression in B6 brains at a faster rate than in $\beta_2M^{-/-}$ brains, although the maximum expression was similar for both. Thus, it seems likely that there are sufficient targets for class II–restricted CTLs in LCMV-infected $\beta_2M^{-/-}$ brains, although this experiment did not show which cells (either resident or hematogenous) in the infected brains express the Ab^b molecules.

To determine the CD4CD8 phenotype of CTLs in $\beta_2M^{-/-}$ mice, we depleted immune $\beta_2M^{-/-}$ and B6 spleen cells of $CD4^+$ or $CD8^+$ cells by using appropriate MAbs and complement. We tested the remaining cells for residual cytotoxicity using LCMV-in-

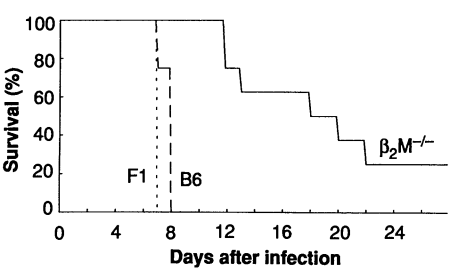

Fig. 1. Survival of $\beta_2M^{-/-}$ mice compared with B6 mice after intracranial infection with LCMV. $\beta_2M^{-/-}$ mice (solid line, $n = 8$), B6 (long dashes, $n = 4$), F1 [(B6 × 129/J)F_1] (short dashes, $n = 4$).

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