current measurements. However, because of the crystallographic dependence of the twin planes, such pinning must also be anisotropic.

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Downregulation of L3T4$^+$ Cytotoxic T Lymphocytes by Interleukin-2

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Proliferation of activated cytotoxic T lymphocytes (CTLs) that recognize foreign histocompatibility antigens is induced by interleukin-2, a potent immunoregulatory molecule originally described as T cell growth factor. Interleukin-2 (IL-2) is widely used to isolate and induce clonal expansion of CTLs for functional studies in vitro and in vivo. However, in studies with CTLs specific for class I and class II histocompatibility antigens, IL-2 rapidly downregulated the lytic activity of some class II–specific CTLs in a time- and dose-dependent manner. Lytic activity of L3T4$^+$ CTLs specific for the murine class II antigen I-E^k was repeatedly up- and downregulated in vitro by alternate exposure to specific (alloantigen) and nonspecific (recombinant IL-2) signals, respectively. These results demonstrate that some CTLs modulate their functional property (cytolysis) while undergoing IL-2–driven cell proliferation without loss of antigen specificity or ability to revert to a lytic phenotype.

INTERLEUKIN-2 (IL-2) IS A SOLUBLE glycoprotein that causes cell proliferation when it binds to cells via a specific membrane receptor (*1–3*). Various immunoenhancing activities have been ascribed to IL-2 (*2–5*), including clonal expansion of activated cytotoxic T lymphocytes (CTLs) directed against alloantigens encoded by the major histocompatibility complex (MHC). We now report that IL-2 can also rapidly downregulate, in a dose-dependent manner, the lytic activity of at least some class II antigen–specific CTLs without affecting their ability to undergo antigen-driven proliferation in the absence of IL-2. Downregulation of lytic activity occurred when murine CTLs specific for the MHC molecule encoded by I-E^k were exposed to IL-2 for at least 6 hours. These class II antigen–specific CTLs could be repeatedly induced to gain and lose lytic function in a cyclical fashion by alternate exposure to specific (alloantigen) and nonspecific (IL-2) environmental signals.

The CTL cell line used in these studies is designated SATC. SATC cells were derived from the wells of a limiting-dilution microcytotoxicity assay (*6*) in which the responder cells were splenocytes obtained from an allogeneic bone marrow chimera (SJL → AKR) that had been given SJL (H-2^s) bone marrow cells together with a noncytotoxic class II antigen–specific SJL T cell clone designated SAC-9.12 [see (*7*) for description of clone] 4 weeks earlier. After 7 days of coculture with irradiated AKR (H-2^k) stimulator cells in the presence of IL-2–conditioned medium obtained from a secondary mixed leukocyte culture, a portion of the cells from each well were tested for cytotoxicity against E8/AK.D1, a leukemic AKR cell line. The cells taken from several cytolytic microwells were subsequently pooled and maintained thereafter in culture wells (35 mm) or tissue culture flasks (25 cm^2) containing complete Dulbecco's modified Eagles medium (C-DMEM) (*6*) plus recombinant IL-2 (rIL-2) at 2.5 U/ml [*Escherichia coli*–derived human IL-2 (Ala-125); Amgen Biologicals, Thousand Oaks, California] without further antigen stimulation. Cell cultures were split every 3 or 4 days to attain a concentration of 1×10^5 cells per milliliter. Clones and subclones were established from the parent SATC culture by standard limiting-dilution techniques (*8*).

Flow cytometric analysis (*9*) showed the original SATC cells to be T lymphocytes of SJL (Thy-1.2) and not AKR (Thy-1.1) origin. More than 99% of the cells were Thy-1.2$^+$, L3T4$^+$, Ly-1$^-$, and Ly-2$^-$. All clones and subclones isolated from the SATC line had the same cell-surface phenotype as the parent line.

SATC cells showed rIL-2–driven cell proliferation in the absence of alloantigen (Table 1). In addition, they proliferated in the absence of rIL-2 when stimulated with cells that expressed I-E^k–encoded MHC molecules [for example, cells from B10.BR, B10.A, and B10.A(5R), but not B10.A(4R) mice] (Table 1). The response of SATC clones and subclones was identical to that of the parent line (*9*).

SATC cells were found to be highly cytolytic for H-2^k targets when tested in the original limiting-dilution microcytotoxicity assay immediately after isolation from the SJL → AKR chimera, but they were not lytic when retested in standard 3.5-hour cell-mediated lympholysis (CML) assays after 2 weeks of continuous culture in rIL-2. However, SATC cells obtained from antigen-driven proliferation assays (in which the rIL-2 had been removed 72 hours earlier) reexpressed cytolytic activity (Table 1). Their lytic activity was directed specifically against I-E^k–bearing target cells.

The absence of lysis by SATC cells cultured in rIL-2 suggested to us that IL-2 may have downregulated lytic activity. To test this, we washed SATC cells free of rIL-2–supplemented maintenance medium and recultured them for 2 to 72 hours in medium alone, medium plus rIL-2, or medium plus alloantigen (no added IL-2) (Fig. 1A). In cultures containing rIL-2, no significant lysis was observed at any of the times tested. In contrast, cytolytic activity reappeared be-

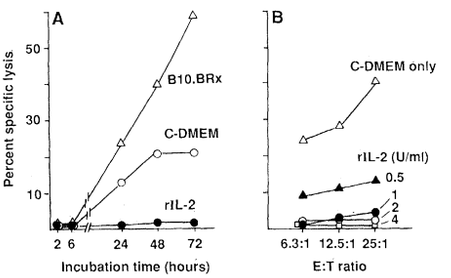

Fig. 1. (**A**) Nonlytic SATC cells were washed free of rIL-2 maintenance medium and placed in multiple culture wells (35 mm) containing 2×10^6 SATC cells per 8 ml of C-DMEM plus rIL-2 (4 U/ml) (●), 5×10^6 cells per 8 ml of C-DMEM alone (○), or 5×10^6 cells per 8 ml of C-DMEM plus 20×10^6 BRx cells per well (△). After incubation for the times indicated, effector cells were collected, washed, and tested in CML assays (see Table 1) with E8/AK.D1 target cells. Data are shown as percent specific lysis at an E:T ratio of 25:1 for each time point. (**B**) SATC cells (5×10^6) were washed free of rIL-2 and cocultured with BRx stimulator cells (20×10^6) in 8 ml of C-DMEM alone (△) or C-DMEM supplemented with rIL-2 at a final concentration of 0.5 (▲), 1.0 (●), 2.0 (○), or 4.0 (□) U/ml. After 72 hours of culture, CML assays were done at the E:T ratios shown with E8/AK.D1 target cells. Representative results from one of three replicate experiments are shown.

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tween 6 and 24 hours in cultures that contained medium alone or medium plus alloantigen without rIL-2. This negative regulatory effect of rIL-2 on lytic SATC cells was dose-dependent and was not reversed by stimulation with alloantigen in the presence of rIL-2 (Fig. 1B). Significant loss of activity was still seen after 72 hours in alloantigen-activated cultures that contained rIL-2 at a concentration of at least 0.5 U/ml (Fig. 1B).

Using limiting-dilution techniques (*8*), we isolated clones from the parent SATC culture and expanded them in rIL-2 without alloantigen. Subclones were derived from selected clones in a similar manner. Cells from the parent SATC line as well as its clones and subclones were washed and recultured for 48 hours in medium alone or in medium plus rIL-2 and then tested for cytotoxicity. Thirteen of 14 proliferating clones lysed the target cells by at least three standard deviations above the background level (spontaneous chromium release). Those subclones derived from clones with high lytic activity (in the absence of rIL-2) tended to have high lytic activity, while subclones derived from clones with moderate lytic activity tended to have moderate lytic activity. However, repeated selection for clones (or subclones) with low levels of activity eventually resulted in cells that had completely lost their lytic activity. All clones and subclones cultured in the presence of rIL-2 (2.5 U/ml) during the previous 48 hours were nonlytic [0% lysis even when tested at effector-to-target (E:T) ratios up to 80:1]. Thus, all lytic clones and subclones derived from the SATC cell line behaved exactly like the parent culture and were downregulated by rIL-2.

Although SATC cells stimulated with alloantigen regained lytic function and proliferated in the absence of added rIL-2 (the average recovery of viable cells after 72 hours was 141% in four experiments), antigen-driven cell proliferation was not necessary for reexpression of lytic activity. Cells cultured in medium without rIL-2 or alloantigen also regained lytic activity (Fig. 1A), but they did not proliferate (Table 1); the average cell recovery was 38% after 72 hours of culture in medium alone. We have not been able to detect measurable amounts of IL-2 or IL-4 activity in supernatant taken from antigen-activated SATC by using murine indicator T cells that are responsive to both IL-2 (*10*) and IL-4 (*9*).

To determine the duration of rIL-2 exposure required to downregulate activity, we treated lytic SATC cells for 2, 6, or 24 hours with medium containing rIL-2, then washed them free of rIL-2 and tested them in CML assays (Fig. 2). Cultures that had been treated with rIL-2 for only 2 hours showed enhanced lysis, whereas lytic CTLs that were treated for at least 6 hours showed decreased activity. Exposure to rIL-2 for 24 hours resulted in complete loss of lytic function.

The transient increase in lytic activity after 2 hours of treatment with rIL-2 (Fig. 2) was reproducible and also was observed when rIL-2 was added directly to 3.5-hour CML assays. However, increased killing was detected only when the cultures had measurable lytic activity; nonlytic cells were unaffected. Kinetic analysis of lysis (*11*) shows that the enhanced activity was due to an increase in the rate of target cell lysis (*12*).

We next sought to determine whether lytic activity could be alternately up- and downregulated (that is, cycled) by repeated manipulation of environmental (culture) conditions. SATC cells were washed free of rIL-2 and recultured in fresh medium containing either rIL-2 or alloantigen alone (Fig. 3). Cytolytic activity returned within 24 hours after stimulation with alloantigen in the absence of rIL-2, but remained downregulated in the presence of rIL-2. Without rIL-2 present, activity steadily increased over a 72-hour period. Addition of rIL-2 to the culture at 48 hours resulted in complete downregulation of activity within the next 24 hours. This cycle of up- and downregulation was repeated three times in a continuous culture by alternate stimulation with alloantigen in the absence of rIL-2 or addition of rIL-2 without alloantigen (Fig. 3).

The ability of SATC cells to cycle between their lytic and nonlytic phenotypes was reproducible. Over a 10-month period, we repeatedly induced SATC cells maintained without alloantigen stimulation to become lytic by simply washing away the rIL-2 or allowing it to be exhausted (that is, by not feeding the cells with fresh rIL-2). These lytic cultures were readily returned to a nonlytic state when fresh rIL-2 was added to the medium. Clones and subclones derived from the SATC line also showed cyclic loss and gain of cytolytic activity in the presence and absence of rIL-2, respectively (*9*).

The mechanism by which IL-2 downregulates lytic function is not known. IL-2–driven cell proliferation may turn off the lytic machinery, modulate some as yet unidentified molecule (or gene) that is essential for lysis, or do both. However, alloantigen-activated SATC cells also proliferate (Table 1), yet they retain lytic activity (Fig. 1A). Preliminary studies suggest a direct correla-

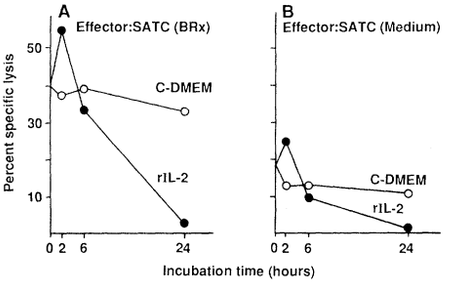

Fig. 2. (**A**) SATC cells were washed free of rIL-2 and cultured for 72 hours with BRx cells (as described in Fig. 1A) until they reexpressed lytic activity (0 hour). The cells were then distributed to macroculture wells (2×10^6 cells per 2 ml) containing C-DMEM alone (○) or C-DMEM plus rIL-2 (4 U/ml) (●). Cells from each group were collected after 2, 6, or 24 hours of incubation, washed, and tested in a 3.5-hour CML assay with E8/AK.D1 target cells. Data shown are percent specific lysis at an E:T ratio of 25:1. (**B**) Same as (A) except that the initial lytic effector cells (0 hour) were SATC cells that had been cultured in C-DMEM alone (that is, without rIL-2 or alloantigen) for the previous 48 hours. The results shown are from a single representative experiment.

Table 1. Functional characteristics of L3T4$^+$ SATC cells. For the proliferation assays, SATC cells (25,000 per well) were washed free of rIL-2 and cultured for 72 hours (37°C, 10% CO_2) in microwells (0.2 ml per well) containing C-DMEM alone or together with rIL-2 (1.2 U/ml) or irradiated (3000 R) splenic stimulator cells (125,000 per well) as shown. [^{3}H]Thymidine (1 μCi per well) was added for the final 24 hours. Data are mean counts per minute ± SD for triplicate wells. For the lytic assays, ^{51}Cr release CML assays were done as described in (*6*). Target cells were lipopolysaccharide-induced lymphoblasts from the mice shown or E8/AK.D1, a cloned AKR leukemia cell line. Spontaneous ^{51}Cr release was 10% to 20%. Effector cells were prepared by washing SATC cells free of rIL-2 and coculturing them for 72 hours with irradiated B10.BR (BRx) stimulator cells (5×10^6 SATC to 20×10^6 BRx) in culture wells (35 mm) containing 8 ml of rIL-2–free C-DMEM. Mean percent specific lysis ± SD in triplicate wells at an E:T ratio of 25:1 is shown.

Stimulator or target cell	H-2				[^{3}H]thymidine uptake (count/min)	Specific lysis of target (%)
	K	I-A	I-E	D		
C-DMEM					702 (± 120)	
rIL-2					49,775 (± 2844)	
B10.BR	k	k	k	k	51,507 (± 6877)	47 (± 2.7)
B10.A	k	k	k	d	41,934 (± 3723)	33 (± 6.2)
B10.A(4R)*	k	k		b	8,434 (± 2744)	1 (± 1.7)
B10.A(5R)*	b	b	b/k	d	23,528 (± 2072)	32 (± 3.9)
E8/AK.D1	k	k	k	k	Not done	32 (± 1.3)

*Cells from B10.A(4R) mice do not express I-E molecules (*19*). B10.A(5R) has had a recombination within the E_β locus resulting in I-E molecules of mixed origin (*19*).

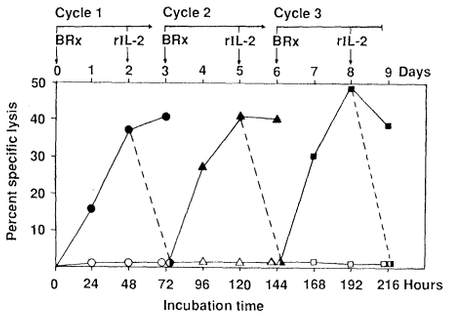

Fig. 3. SATC were induced to gain or lose lytic activity by manipulating environmental signals. Nonlytic SATC cells washed free of rIL-2 were distributed to multiple macroculture wells (2×10^6 cells per 2 ml) containing medium plus alloantigen (5×10^6 BRx) (●) or medium plus rIL-2 (4 U/ml) (○) on day 0. A sample of cells from each group was collected, washed, and tested for lysis of E8/AK.D1 cells in CML assays after 24, 48, and 72 hours of incubation. The rest of the alloantigen-stimulated (and now lytic) SATC cells were pooled on day 2, washed, and resuspended in fresh C-DMEM containing rIL-2 (4 U/ml). Twenty-four hours later (that is, on day 3), the cells were recollected and washed free of rIL-2. An aliquot was tested for lysis of E8/AK.D1 in CML assays (◑); the rest of the cells were placed in macrowells for a second cycle of alloantigen stimulation (▲) or cultured in rIL-2 alone (△). The cells were again tested for lytic activity at 24-hour intervals (that is, at 96, 120, and 144 hours). The cycle was repeated a third time starting on day 6 and concluding on day 9 (■ ◨). The data are expressed as percent specific lysis of E8/AK.D1 target cells at an E:T ratio of 25:1 for each time point and are from a single three-cycle experiment. Closed symbols represent lysis by cells taken from cultures that contained alloantigen-stimulated SATC incubated without rIL-2; open symbols indicate cultures containing rIL-2; and split symbols indicate lysis by alloactivated cells reexposed to rIL-2 for 24 hours. Symbol changes indicate a new cycle (● ◑, cycle 1; ▲ ◭, cycle 2; ■ ◨, cycle 3). The days on which SATC cells were stimulated with alloantigen (↓ BRx) or recultured in rIL-2 (↓ rIL-2) are indicated along the top of the figure.

tion between the proportion of cells in the G_0/G_1 phase of the cell cycle and the level of lytic activity (*9*).

We did not observe any quantitative or qualitative changes in the cell-surface phenotype of SATC cells that were lytic or nonlytic. Expression of L3T4 and Ly-2, which are thought to be involved in recognition of class II and class I MHC molecules, respectively (*13*), did not fluctuate. Expression of Thy-1.2, which has been linked to the murine T cell receptor (*14*), also did not change. Lytic and nonlytic SATC cells appeared to have similar numbers of T cell receptors based on their equivalent proliferation in response to I-E^k alloantigen stimulation in the absence of added IL-2 (*9*); however, we cannot rule out quantitative differences too subtle to be detected by these functional assays.

Culture conditions have been shown to influence the reactivity of cloned T lymphocytes. For example, Wilde and Fitch (*15*) report that ovalbumin-specific helper T lymphocytes become profoundly unresponsive to antigen restimulation for up to 9 days after exposure to antigen or a high concentration of IL-2. Our results with alloreactive CTLs differ from theirs in that (i) antigen recognition was not lost even though lytic function was downregulated, (ii) lytic activity recovered within 24 hours after removal of IL-2, and (iii) downregulation occurred even at relatively low concentrations of rIL-2.

Cyclic changes in lytic activity have been reported by others. Howe and his colleagues (*16, 17*) found that lytic activity was upregulated in quiescent class I MHC antigen–specific CTL clones after exposure to IL-2, but active downregulation of lytic activity was not reported.

It is possible that downregulation of cytolytic activity by IL-2 is confined to subpopulations of CTLs that are similar to those described here. To our knowledge, it has not been reported for other CTLs specific for either class I or class II MHC molecules. However, downregulation of lytic activity by IL-2 may occur but go unrecognized in some T cell cultures. This raises the possibility that T lymphocytes that appear to be nonlytic when expanded ex vivo in IL-2 may revert to a lytic phenotype in vivo or under certain experimental conditions in vitro. The physiologic levels of IL-2 (systemic or localized) may have a similar regulatory effect on such CTLs in vivo.

Vidovic *et al.* (*18*) speculate that the generally reported absence of class II MHC antigen–specific CTLs in vitro may result from regulatory cell interactions. The IL-2–mediated regulatory pathway described here might explain their observation that $Ly\text{-}2^-$ class II–specific CTLs can be detected only when this subset is kept separate from other T cell subsets (*18*). That is to say, production of IL-2 by other T cells could downregulate the activity of some $Ly\text{-}2^-$ CTLs in unselected cell populations, thus decreasing the likelihood of their detection. Whether IL-2–mediated downregulation of lytic cells has an important biological role is not known at this time.

The biological role of SATC or similar CTLs also is not known. SATC cells have the same cell-surface phenotype and antigen specificity as the nonlytic SAC-9.12 cells (*7*) that were injected into the SJL → AKR chimera from which the lytic SATC cells were derived, but we do not know whether the SATC cells represent the progeny of SAC-9.12 cells or derive from T cells present in the SJL marrow inoculum. However, we have isolated T cells with surface phenotype, antigen specificity, and lytic activity identical to that of the SATC cell line from an SJL → AKR chimera that was not given SAC-9.12 cells (*9*). These cells were found at a later time after bone marrow transplantation and were present at a much lower frequency, but like the SATC cells, their lytic activity was downregulated by IL-2 (*9*). Successful isolation de novo of additional class II antigen–specific CTLs from a bone marrow transplant recipient not given the highly manipulated SAC-9.12 T cell clone suggests that SATC and similar CTLs are not artifacts of the experimental model.

We have speculated that SAC-9.12 cells play a role in maintaining donor-host tolerance after allogeneic bone marrow transplantation (*6, 7*). SAC-9.12 cells suppress the generation of antigen-specific CTLs both in vitro and in vivo (*7*), but cytotoxic activity in cultured SAC-9.12 cells has not been detected even when IL-2 was removed from the culture medium (*9*). Defining the relationship between nonlytic SAC-9.12 cells and the lytic SATC cells described here awaits further critical experimentation.

In summary, this study demonstrates unique $L3T4^+$ CTLs that repeatedly alter their function in response to environmental signals and do so without loss of antigen specificity. This could help to explain some anomalous observations with T cell clones that appear to lose or change their function in culture or, conversely, to gain lytic activity in vivo. But, regardless of whether SATC cells and downregulation of lytic activity by IL-2 have biological relevance, the system will provide a valuable research tool for gaining insights into the mechanisms of regulatory processes that control T cell–mediated cytolysis.

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Conservation of the Duchenne Muscular Dystrophy Gene in Mice and Humans

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A portion of the Duchenne muscular dystrophy (DMD) gene transcript from human fetal skeletal muscle and mouse adult heart was sequenced, representing approximately 25 percent of the total, 14-kb DMD transcript. The nucleic acid and predicted amino acid sequences from the two species are nearly 90 percent homologous. The amino acid sequence that is predicted from this portion of the DMD gene indicates that the protein product might serve a structural role in muscle, but the abundance and tissue distribution of the messenger RNA suggests that the DMD protein is not nebulin.

DUCHENNE MUSCULAR DYSTROPHY (DMD) is an X-linked, recessive, human genetic disorder that affects approximately 1 in every 3500 males in all populations that have been studied (*1*). Clinical onset of Duchenne muscular dystrophy is first observed in affected children when they are about 2 to 3 years old, the first evidence being proximal muscle weakness. Biochemical onset of this disease occurs much earlier as seen by histological examination of affected fetal muscle (*2*). Target tissue differences are also observed with regard to clinical versus histological phenotype. Specifically, heart function is only minimally affected, even though heart muscle appears very similar histologically to affected skeletal muscle (*3*). The rapid and progressive wasting of striated muscle, characteristic of DMD, leads to death by the end of the second decade.

Despite nearly 100 years of intensive study, little is known about the primary biochemical defect responsible for the destruction of muscle in individuals with DMD. We have previously described the isolation of genomic DNA that is closely linked to the DMD gene and the delineation of two small putative exons of the DMD gene within the cloned genomic regions (*4–7*). One of these exons was used to isolate a 1-kb segment of human fetal skeletal muscle complementary DNA (cDNA) (FSM 5-1) that spans a portion of the Xp21 DMD locus (*6*). We now report the isolation and characterization of cDNAs corresponding to the homologous locus in the mouse, a comparison of the human and mouse DMD gene expression patterns, and the DNA and predicted protein sequence for about 25% of the total DMD transcript.

Previous work on the identification of human DMD coding sequences was based primarily on the high degree of conservation of two small putative exons from mouse and man (*6*). Since the expression pattern of the 14-kb human DMD gene transcript (*8*) had been studied only in fetal tissues, and in view of the difficulties in obtaining large amounts of various human tissues, we extended our transcriptional studies by using the human cDNA as a probe for Northern blots of mouse tissue RNA (*9*). The human cDNA detected a 14-kb mouse mRNA species, which was present in very low levels in tissue extracts of mouse newborn leg and gravid (15-day) combined uterus and placenta and in higher levels in newborn heart, adult heart, and adult skeletal muscle (Fig. 1). The abundance of the mDMD (mouse Duchenne muscular dystrophy) transcript is roughly one-thousandth that of the mouse α tubulin transcript (Fig. 1). We detected DMD transcription in all of the striated muscle that we tested except for fetal mouse tissues containing skeletal muscle. Our inability to detect fetal transcription was most likely due to the heterogeneity of the fetal mouse tissues, the lack of differentiation in the muscle fibers at early stages of mouse development, or both (*10*).

The smooth muscle cell layers of gravid mammalian uteri undergo massive hypertrophy in preparation for parturition. The gravid uteri of E15-bearing mice (3 days before parturition) are, therefore, an excellent source of mitotically and metabolically active smooth muscle. Our finding of a very low level of mouse DMD gene transcription in E15 combined uterus and placenta is

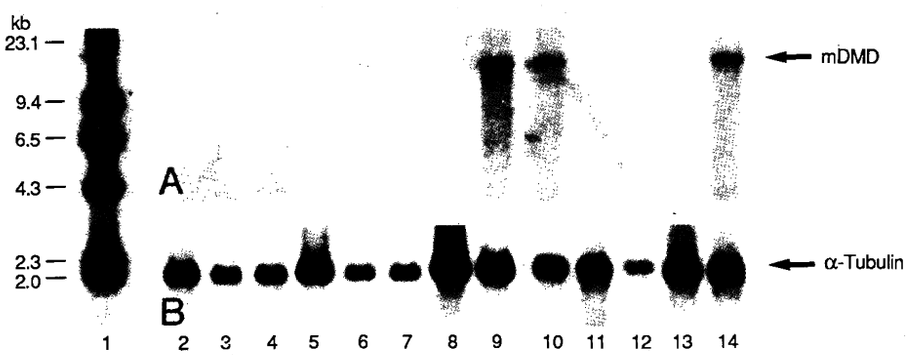

Fig. 1. Expression of the mouse DMD locus. Polyadenylated RNA (7 μg) was separated by electrophoresis in a 1% agarose-formaldehyde gel. The fragments were transferred to a nylon membrane and hybridized with a ^{32}P-labeled (*23*) human fetal skeletal muscle cDNA, corresponding to the human DMD locus (*7, 9*). The 14-kb mRNA species, corresponding to the mouse DMD gene is shown. As a control for the amount and efficacy of the RNA that was loaded, the nylon membranes were dehybridized and rehybridized with a labeled cDNA clone, corresponding to the mouse α-tubulin gene. The 2-kb mRNA, corresponding to α-tubulin, is shown for each lane. In (**A**) (mDMD) autoradiographic exposure was for 10 days; in (**B**) (tubulin) exposure was for 2 hours, that is, one-hundredth the time used for (A). All autoradiography was done with an intensifying screen at −80°C, and each probe was labeled to similar specific activities. The same blots used with the human cDNA probe were then dehybridized and again probed with the mouse cDNA, MC2-6. The results with the mouse probe were identical to those with the human probe. Lanes are as follows: 1, λ DNA Hind III–digest markers; 2, E13 (13-day-old embryos); 3, E13 uterus and placenta; 4, E15 carcass; 5, E15 brain; 6, E15 uterus and placenta; 7, E15 viscera; 8, newborn brain; 9, newborn heart and lung; 10, adult heart; 11, adult kidney; 12, adult liver; 13, adult lung; 14, adult skeletal muscle. Additional tissues that were tested but not shown were as follows: mouse gravid uteri of embryonic day 5 (E5), E7, and E9; isolated mouse embryos of E10 and E11; adult brain, spleen, small intestine, stomach, and testes. All of these tissues tested negative for the mDMD transcript.

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