the *elav* locus on the Y chromosome. Male progeny of this cross should behave (because of dosage compensation) as though four haploid *elav* copies were present, and female progeny should be haploid. Male and female larval CNS and adult heads were dissected separately, and the amount of Mab44C11 antigen was assayed by protein immunoblotting of serial dilutions of the protein extracts. We found that males and females have equal amounts of the Mab44C11 antigen. Thus, at present no conclusive evidence exists to suggest that *elav* is the structural gene for the Mab44C11 antigen.

17. J. Thomas, personal communication.

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HTLV-II Transactivation Is Regulated by the Overlapping *tax/rex* Nonstructural Genes

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The human T-cell leukemia virus (HTLV) types I and II have two nonstructural genes that are encoded in overlapping reading frames. One of these genes, known as *tax*, has been shown to encode a protein responsible for enhanced transcription (transactivation) from the viral long terminal repeats (LTRs). Genetic evidence indicates that the second nonstructural gene of HTLV-II, here designated *rex*, acts in trans to modulate *tax* gene–mediated transactivation in a concentration-dependent fashion. The *rex* gene may regulate the process of transactivation during the viral life cycle.

HTLV-I AND HTLV-II ARE ASSOCIATED with specific T-cell disorders in humans (*1–3*). HTLV-I, HTLV-II, and the related bovine leukemia virus (BLV) each have two genes located in the 3′ region of the genome that encode nonstructural proteins (Fig. 1) (*4–6*). One of these genes, called *tax* (which has also been referred to as *x-lor*, *tat*-1, *tat*-2, and *x*), encodes a protein, designated $p40^{xI}$ in HTLV-I and $p37^{xII}$ in HTLV-II (*4*). The *tax* gene product is required for HTLV replication (*7*), and acts in trans to increase the rate of viral transcription from the viral LTR (*8–11*). The second gene is encoded in an alternate open reading frame that partially overlaps with the *tax* gene on the same subgenomic mRNA (Fig. 1A) (*5, 12, 13*). For HTLV-I, the second nonstructural gene, called *tel* (for trigger for expression of late genes) (*14*) or *rex* (for regulator of expression) (*15*) encodes a major protein species of 27 kD, $p27^{xIII}$, which is localized to the nucleus of HTLV-I–infected cells, as well as a 21-kD protein species $p21^{xIII}$ (*5, 12, 14*). The $p27^{xIII}$ protein of HTLV-I is required for efficient *gag* expression (*14*).

The corresponding *rex* gene of HTLV-II encodes protein species of 26 and 24 kD (*13*). The presumed Met initiation codon for HTLV-II *rex* is located on the middle exon of the 2.1-kb subgenomic mRNA at nucleotide (nt) position 5121 (Fig. 1A) (*13*). The Met initiation codon for $p37^{xII}$ (AUG) is located downstream in the same middle exon at nt 5180 (*16*). The fact that the *rex* gene product is encoded by the same subgenomic mRNA as the trans-acting *tax* gene product, $p37^{xII}$, suggested that, like *tax*, the *rex* gene might also play a regulatory role in the viral life cycle.

To express, HTLV-II viral genes in vitro, we constructed the expression vector, SV-HTLV (Fig. 1B) which contains the entire HTLV-II coding region (nt 361 to 8550) under the control of the SV40 promoter (*8*). Co-transfection of SV-HTLV with the HTLV-II LTR into COS cells results in transactivation of the HTLV-II LTR, as measured by enhanced expression of LTR-linked indicator genes. LTR function was assayed with the LTR-II-CAT construct, in which the bacterial chloramphenicol acetyltransferase (CAT) gene is linked to the HTLV-II LTR (*8*).

Two mutations were introduced into SV-HTLV that specifically prevent wild-type *rex* expression (Fig. 1B). Mutant SV-HTLV-Sph has a 4-bp deletion that eliminates the Met initiation condon for *rex*, but does not affect the *tax* initiation codon, which is located further downstream (*17*). SV-HTLV-rex^{term} contains a single nucleotide substitution that introduces a stop codon in the *rex* coding sequences, resulting in a truncated *rex* gene product of 79 amino acids instead of the wild-type product, which is 170 amino acids (*17*) (Fig. 1B). Co-transfection of LTR-II-CAT into COS cells with either the SV-HTLV-Sph or SV-HTLV-rex^{term} mutants defective for *rex* expression results in a five- to tenfold decrease in the level of transactivation of the HTLV-II LTR when compared to an equimolar amount of transfected SV-HTLV (Table 1). Transfection of a construct designated SV-HTLV-Cla (Fig. 1B), which is deficient for both *tax* and *rex* because of a frameshift mutation, results in no transactivation above baseline levels, as previously reported (*8*).

To determine whether the mutations affected a trans-acting function of the *rex* gene, a recombinant construct 91023-pX-b, which independently expresses the *rex* gene by means of the eukaryotic expression vector p91023-B, was used (*18*). The sequences surrounding the initiator Met codon for *rex*, GCTGC*ATG*C, match poorly to the consensus initiator sequences identified by Kozak (*19*). By contrast, the sequences surrounding the downstream *tax* gene Met

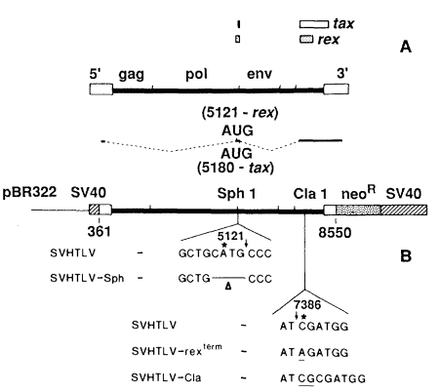

Fig. 1. (**A**) Schematic representation of the HTLV-II genome and coding sequences for nonstructural proteins. The *gag*, *pol*, and *env* coding regions are delineated on the provirus. The boxes above the genome designate coding regions for *tax* and *rex*. The 2.1-kb mRNA species schematically illustrated below the HTLV-II genome contains both *tax* gene sequences encoding $p37^{xII}$, and *rex* gene sequences encoding the other nonstructural protein(s) (*4, 13*). The 5′ leader exon (nt 313 to 449), middle exon (nt 5044 to 5183), and third exon (nt 7214 to 8751) are represented by thick lines. (**B**) Schematic representation of the SV-HTLV expression vector and *rex* gene mutants. The mutations and resultant sequences in SV-HTLV-Sph, SV-HTLV-rex^{term}, and SV-HTLV-Cla are shown below the genome. An arrow designates the Sph I cleavage site at nt 5124 in the SV-HTLV sequence. The Cla I cleavage in SV-HTLV-Cla site is designated by an arrow at nt 7385 in the SV-HTLV sequence.

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initiation codon match well to Kozak sequences, favoring expression of the *tax* gene product. Therefore, in 91023-pX-b, we substituted the sequences ACACC*ATG*C about the *rex* initiator Met codon to favor its utilization. We transfected 91023-pX-b into COS cells, and verified synthesis of the 26-kD and 24-kD *rex* gene products in transfected cells by performing radioimmunoprecipitation with antibody directed at a COOH-terminal tridecapeptide sequence encoded by *rex* (*13*) (Fig. 2). No detectable *tax* gene product $p37^{xII}$ was made by 91023-pX-b in COS cells, as assayed by radioimmunoprecipitation with antibody to $p37^{xII}$ (*4*).

Table 1. Transactivation by constructs. The indicated amounts (5 μg or 2.5 μg) of the specified plasmid was co-transfected with 5 μg of LTR-II-CAT into COS cells and assayed for CAT activity as described (*8, 20*). The total amount of DNA transfected was kept constant at 10 μg by addition of vector (p91023-B or pSV2neo). Two representative experiments are shown. ND, not determined.

Co-transfected plasmids	Acetylation (%) at	
	5 μg	2.5 μg
SV-HTLV	25	34
SV-HTLV-Sph	8	3
SV-HTLV-*rex*^{term}	6	1
pSV2neo	<1	ND
SV-HTLV	64	85
91023*x*II	20	34
91023-pX-b	3	1
p91023-B	<1	ND

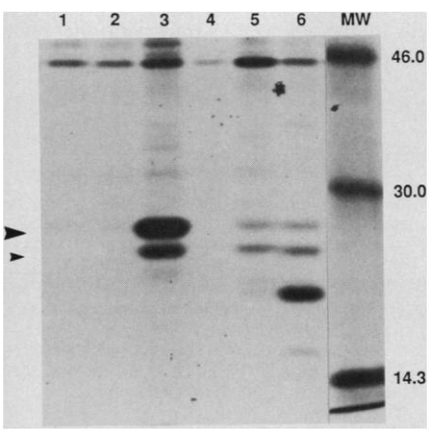

Fig. 2. Radioimmunoprecipitation of *rex* gene products in COS cells transfected with 91023-pX-b (*18*). COS cells (4×10^6) were transfected with 10 μg of plasmid (indicated below), and labeled with [^{35}S]methionine 48 hours after transfection (*4*). [^{35}S]methionine-labeled cell lysates were also prepared from the 729-6 B cell line, and from the HTLV-II–infected cell lines, Mo-T and 729 pH6neo (*7*). Radioimmunoprecipitation was performed with antibody directed against the COOH-terminus of the *rex* gene product (*13*). (Lane 1) COS cells transfected with 91023*x*II, a plasmid which makes $p37^{xII}$, but lacks 5′ *rex* coding sequences; (lane 2) COS cells transfected with p91023-B; (lane 3) COS cells transfected with 91023-pX-b; (lane 4) 729-6 B cell line; (lane 5) 729pH6neo cell line; (lane 6) Mo-T cell line. MW, molecular size markers (kilodaltons shown at right). Two bands corresponding to the 26-kD (large arrowhead) and 24-kD (smaller arrowhead) *rex* gene products are recognized in COS cells transfected with 91023-pX-b, and in the HTLV-II–infected cell lysates.

To determine whether the *rex* gene product could directly transactivate the HTLV-II LTR in the absence of $p37^{xII}$, we co-transfected 91023-pX-b with the LTR-II-CAT construct. There was no enhancement of CAT activity by 91023-pX-b above baseline levels, demonstrating that the *rex* gene itself does not directly transactivate the LTR (Table 1 and Fig. 3A). In contrast, transfection of 91023*x*II, a construct which contains all *tax* coding sequences but which lacks the 5′ *rex* coding sequences, results in transactivation of LTR-II-CAT, as described (*20*). The decreased transactivation by the *rex*-deficient SV-HTLV mutants described above is therefore not likely to be due to loss of a direct transactivation function of the *rex* gene.

To determine whether the *rex* gene product acts in trans, we performed complementation studies of the *rex*-deficient SV-HTLV mutants by means of the *rex* expression plasmid, 91023-pX-b. The 91023-pX-b *rex* expression construct was co-transfected with LTR-II-CAT and either SV-HTLV-Sph or SV-HTLV-*rex*^{term}. Provision of the *rex* gene product in trans by co-transfection of the 91023-pX-b expression construct with either SV-HTLV-Sph or SV-HTLV-*rex*^{term} increased the level of transactivation in a dose-dependent fashion, at doses of 0.1 and 0.5 μg of 91023-pX-b (Fig. 3A). The increase in the level of transactivation seen with the addition of *rex* resulted in a level of transactivation comparable to that seen with wild-type SV-HTLV. A control co-transfection of 91023-pX-b with either the vector pSV2neo (Fig. 3A) or the SV-HTLV-Cla construct deficient in both the *rex* and *tax* gene products did not result in transactivation above basal levels (*21*). These results confirm that the *rex* gene is required for optimal transactivation by the *tax* gene product.

While the addition of 0.1 to 0.5 μg of the 91023-pX-b *rex* construct restored transactivation to optimal levels, further increases in the amount of co-transfected 91023-pX-b caused a decrease in transactivation to levels lower than seen in the absence of *rex* (Fig. 3A). This inhibitory effect of *rex* was further investigated by testing the effect of high levels of the *rex* gene product upon transactivation by the wild-type SV-HTLV construct. As the level of transfected 91023-pX-b was increased, marked inhibition of LTR-II-CAT transactivation by SV-HTLV was observed (Table 2). To exclude a nonspecific effect of *rex* expression on COS cell viability, CAT expression, transfection efficiency, or transactivation, we tested the effect of *rex* on transactivation of the human immunodeficiency virus (HIV) LTR by the HIV *tat* protein and on the expression of the CAT gene from other viral promoters in COS cells (Table 2) (*22–24*). Co-transfection of SV-*tat*, in which the HIV *tat* gene is expressed by the SV40 early promoter, and HIV-CAT, in which CAT sequences are linked to the HIV LTR, results in significant transactivation (*22*). When 91023-pX-b was co-transfected with SV-*tat* and HIV-CAT, no significant inhibition of HIV LTR–directed CAT expression was noted. In addition, co-transfection of 91023-pX-b with either RSV-CAT, in which the CAT gene is directly expressed from the Rous sarcoma virus promoter (*23*), or pSV-CAT, in which CAT is expressed from the SV40 early promoter (*24*), did not result in decreased CAT activity (Fig. 3B). These results further show that high levels of *rex* gene expression are not inhibitory to the SV40 early promoter used in SV-*tat* to express the HIV *tat* gene product, in pSV-CAT to express the CAT gene product, or in SV-HTLV to express HTLV-II gene prod-

Table 2. Effects of the *rex* gene product on expression from other promoters. The amount of 91023-pX-b added to the co-transfected plasmids is shown above each column. Total transfected DNA was kept constant at 11 μg by addition of p91023-B. The data are expressed as the percentage of acetylated [^{14}C]chloramphenicol. The indicated transfections were performed in parallel using 2×10^6 COS cells, as in Table 1. The amount of cellular extract used for CAT assays from each co-transfection was experimentally adjusted to yield comparable baseline levels of acetylation.

Co-transfected plasmids*	Acetylation (%) in the presence of 91023-pX-b (μg)			
	0	0.5	2.5	5
SV-HTLV/LTR-II-CAT	89	92	17	15
SV-*tat*/HIV-CAT	78	75	ND	86
pSV2neo/HIV-CAT	<1	ND	1.0	2.4
pSV-CAT	66	73	49	55
RSV-CAT	54	60	48	62

*Co-transfected plasmids were each present at 3 μg, except for pSV-CAT and RSV-CAT, which were at 5 μg.

ucts. However, we did observe an inhibitory effect of *rex* on transactivation of both the HTLV-II LTR (Table 2), and the HTLV-I LTR (*25*) in response to SV-HTLV. In addition, we have recently confirmed the dose-dependent effects of *rex* expression on transactivation in transient transfection experiments into human lymphoid cell lines (*26*). Therefore, in addition to a positive effect on *tax* gene–mediated transactivation seen with low levels of *rex*, a specific inhibitory effect of higher levels of the HTLV-II *rex* expression is observed on transactivation of the HTLV-I and HTLV-II LTRs.

Transactivation by the HTLV-II *tax* gene product results in increased viral RNA transcription, and hence, higher steady state levels of viral mRNA (*8–11*). We determined that the inhibitory effect of enhanced *rex* expression on levels of CAT activity was due to a decrease in the amount of LTR-II-CAT mRNA by means of S_1 nuclease analysis of RNA synthesized from the cap site of the HTLV-II LTR (Fig. 3B). Transient co-transfection of SV-HTLV and LTR-II-CAT results in transactivation (Fig. 3B, lane 3) and readily detectable levels of LTR-II-CAT mRNA. In contrast, when 91023-pX-b was added to the co-transfection at a level known to inhibit transactivation (Fig. 3B, lane 1), LTR-II-CAT mRNA levels were markedly decreased. Therefore, the inhibition of LTR-linked CAT expression is primarily due to a decrease in the level of correctly initiated mRNA from the HTLV-II LTR cap site.

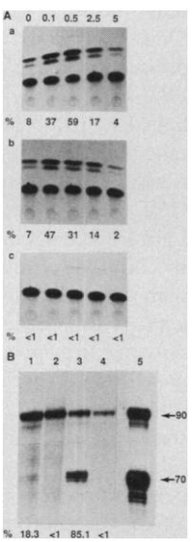

Fig. 3. (**A**) Complementation of *rex*-deficient constructs by provision of *rex* gene products in trans. A fixed amount (3 μg) of either SV-HTLV-Sph (panel a), SV-HTLV-*rex*^{term} (panel b), or pSV2neo (panel c), and LTR-II-CAT (3 μg) was transfected into COS cells, and *rex* was provided in trans by co-transfection of the indicated amount of 91023-pX-b (shown at the top, in micrograms). Total transfected DNA was kept constant at 11 μg by addition of the vector p91023-B. CAT activity was assayed 48 hours following transfection. The absolute percentage of acetylated [^{14}C]chloramphenicol is indicated below each assay. Similar results were obtained in four experiments. (**B**) Effect of the *rex* gene product on LTR-directed mRNA levels. Either SV-HTLV (5 μg; lanes 1 and 3) or pSV2neo (5 μg; lanes 2 and 4) was co-transfected with LTR-II-CAT (5 μg; lanes 1 to 4) and either 91023-pX-b (5 μg; lanes 1 and 2) or vector p91023-B (5 μg; lanes 3 and 4), into COS cells (2×10^6 per plate). Total cellular RNA was prepared from three pooled plates 48 hours following transfection. CAT activity, as indicated by percent acetylation, was assayed in a separate plate transfected in parallel, and is shown below each lane. Lane 5 contains 2.5 μg of total cellular RNA from the HTLV-II–infected Mo-T cell line. S_1 nuclease analysis was performed with a γ-^{32}P-dATP–labeled 90-nt oligonucleotide probe (nt 294 to 383) for the cap site of HTLV-II, as described (*28*). A 70-nt fragment is protected by mRNA synthesized from the HTLV-II cap site. Similar results were obtained in multiple experiments.

Our results indicate that the *rex* gene exerts a concentration-dependent regulatory effect upon transactivation. The *rex* gene product may act upon the *tax* protein p37^{xII} directly, or it may affect cellular or viral proteins involved in transactivation. Alternatively, *rex* may affect the expression of p37^{xII} by altering the pattern of viral mRNA splicing, stability of viral mRNA, or translation of p37^{xII} from the subgenomic mRNA. Finally, it is also possible that the *rex* gene product directly binds to sequences in the LTR, thereby affecting the binding of transcription factors which interact with p37^{xII} and the LTR.

Recently, Inoue and co-workers (*14*) observed that the HTLV-I *rex* products p27^{xIII} is required for efficient expression of *gag* mRNA, which is consistent with the positive regulatory effects we observe at lower concentrations of *rex*. In addition, when the SV-HTLV–based *rex*-deficient mutants are introduced into an infectious HTLV-II proviral clone, the resultant mutant proviruses transcribe very low levels of viral RNA compared to the wild-type HTLV-II provirus (*26*). Therefore, it appears that a primary effect of *rex* is exerted upon *tax* gene–mediated transactivation. Apparent differences between our results and theirs on transactivation may relate to the different constructs used to express the *rex* and *tax* nonstructural proteins. In addition, while we and other investigators have observed that the *tax* gene product is sufficient for low levels of transactivation (*11, 20*), optimal levels of transactivation are not achieved in the absence of the *rex* gene product (Fig. 3A). Identical results have recently been obtained in our laboratory with nonspliced constructs expressing *tax* and *rex*, respectively, in a variety of cell types (*26*).

The dose-dependent regulatory effect of *rex* on transactivation suggests a physiologic role in HTLV replication. HTLV-I and HTLV-II persist as proviruses in immortalized T cells. Uncontrolled positive feedback in transactivation could deplete host cell transcription factors, with detrimental effects on cellular homeostasis. Both the *rex* and *tax* gene products are encoded on the same mRNA. However, the sequences surrounding the *tax* and *rex* initiator Met codons favor translation of the *tax* gene product. Immediately after infection, only low levels of the *tax* and *rex* gene products would be present, enhancing transactivation. This would lead to further accumulation of viral mRNA, including spliced *tax/rex* mRNA. However, as *tax/rex* mRNA continues to accumulate, the intracellular levels of *rex* might increase to a degree sufficient to inhibit transactivation. In this fashion, the *rex* gene product may act to maintain viral transcription at appropriate levels, in effect, "buffering" the effect of the *tax* gene on viral transcription. Under some circumstances, accumulation of *rex* might result in profound inhibition of transactivation and conversion of productive viral infection to latent infection.

Many investigators have postulated a role for the *tax* gene product in cellular transformation by causing the inappropriate transactivation of cellular genes. Our results indicate that the *rex* gene of HTLV also plays a role in regulating viral gene expression, and could potentially affect cellular gene expression as well. If so, negative transcriptional regulation might also be involved in cellular transformation, similar to what has been demonstrated for transformation of rodent cells by the adenovirus transcriptional regulatory gene, E1A (*27*).

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18. To construct 91023-pX-b, a Nco I (nt 5179)–Sph I (nt 5123) HTLV-II fragment containing the *rex* initiator Met codon was ligated to the 5′ end of a Nco I (nt 5179)–Bam HI (nt 8550) fragment encoding p37^{xII} (derived from the 91023*x*II construct (*20*) and subcloned into pUC18). The resulting subclone was digested with Eco RI (in pUC18 polylinker) and Sph I, and ligated to a 15-nt linker, 5′-AATTCCAACACCAGG-3′, in which the 5′ terminal four and 3′ terminal three nucleotides were not base-paired. This provided consensus Kozak sequences directly about the *rex* initiator Met. The final fragment was subcloned into the Eco RI site of the p91023-B expression vector, downstream from the adenovirus major late promoter. The p91023-B vector contains an SV40 origin of replication [G. G. Wong *et al.*, *Science* **228**, 810 (1985)].
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29. We thank L. Souza at Amgen for preparation of oligonucleotide linkers; K. Shimotohno at the National Cancer Center Research Institute, Tokyo, for antibody to the *rex* protein; M. Peterlin for the HIV constructs; N. Nameri, D. Keith, H. Koga, D. Aboulafia, E. Chin, and S. Quan for technical assistance; W. Aft for preparation of the manuscript; and J. Gasson, J. Lugo, and J. Zack for critical review of the manuscript. Supported by a grant from the California Institute for Cancer Research; National Institutes of Health grants 1-K11 CA 01314, CA 32737, and 1 R01 CA 43370; and the University-wide Task Force on AIDS award R87LA057. A.J.C. is a Leukemia Society of America Fellow.

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Reduction of Naturally Occurring Motoneuron Death in Vivo by a Target-Derived Neurotrophic Factor

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Treatment of chick embryos in ovo with crude and partially purified extracts from embryonic hindlimbs (days 8 to 9) during the normal cell death period (days 5 to 10) rescues a significant number of motoneurons from degeneration. The survival activity of partially purified extract was dose-dependent and developmentally regulated. The survival of sensory, sympathetic, parasympathetic, and a population of cholinergic sympathetic preganglionic neurons was unaffected by treatment with hindlimb extract. The massive motoneuron death that occurs after early target (hindlimb) removal was partially ameliorated by daily treatment with the hindlimb extract. These results indicate that a target-derived neurotrophic factor is involved in the regulation of motoneuron survival in vivo.

EXPERIMENTS PERFORMED ALMOST 40 years ago by Hamburger and Levi-Montalcini (*1*) led to the idea that neurons within a population (for example, motoneurons or sensory neurons) are overproduced during development relative to the numbers present in the adult. Consequently, competition for a target-derived entity that is in limited supply is thought to result in the survival of only a portion of the original population. The experiments of Hamburger and Levi-Montalcini also paved the way for the discovery and characterization of nerve growth factor (NGF), the only known molecule that meets virtually all of the criteria for a target-derived neurotrophic factor that promotes the survival of developing neurons in vivo. Thus, NGF acts as a mechanism for regulating the population size of specific groups of neurons during periods of naturally occurring cell death (*2*). Although several other putative neurotrophic or survival agents (including motoneuron factors) have been isolated, characterized in vitro, and partially or completely purified (*3–5*), only one of these, brain-derived neurotrophic factor (BDNF), has been shown to regulate neuronal survival in vivo (*6*). Because putative neurotrophic factors characterized in vitro may promote neuronal survival by providing essential components that are missing or perturbed in the tissue culture environment, or by acting in a manner normally inoperative in vivo (*7*), such factors must be shown to be capable of affecting neuronal survival in vivo. In the chick embryo, 50% or more of the somatic motoneurons innervating skeletal muscle, at limb as well as nonlimb regions, degenerate between embryonic day (E) 5.5 and E12 (*8*). We found that hindlimb target tissues contain a putative neurotrophic factor that prevents the death of substantial numbers of motoneurons in vivo, in a manner consistent with its suspected role as a normal motoneuron survival factor.

Crude hindlimb or control tissue extracts were prepared from embryos at E8 to E9 when the hindlimb musculature is composed primarily, if not entirely, of primary myotubes and when normal motoneuron death is still occurring (*9*). Extracts were administered daily through a small window in the shell onto the vascularized chorioallantoic membrane beginning either on E5 or on E6. Control embryos received equal volumes of a physiological saline solution. Because motoneuron survival depends on neuromuscular activity (*10*), we also monitored embryonic motility to determine whether these extracts alter neuromuscular activity. In all cases, significantly more motoneurons survived in the lumbar spinal cord of embryos treated with crude hindlimb extracts than in controls (Fig. 1). Kidney, lung, and liver extracts were ineffective in rescuing motoneurons. Heat inactivation (60°C for 45 minutes) of crude hindlimb extracts eliminated the motoneuron survival activity. Although the amount of motoneuron survival varied between experiments, treatment with hindlimb extracts always resulted in a significant increase in motoneuron numbers. Before cell death, approxi-

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