chinery interacts specifically with a factor or factors bound to the transcriptional elements. All of these possibilities are compatible with our findings, but we believe that some aspect of point (ii) or (iii) is more likely. For SV40, polyomavirus, and adenovirus priming at the initiation step does not require RNA polymerase II, but is achieved by either a special virus encoded or cellular primase-polymerase. It is more difficult to distinguish between the functions (ii) and (iii), since both specific and nonspecific functions for DNA replication could be carried out by factors that have specificity in terms of DNA binding.

Association of transcriptional elements with origins of replication is a common observation. It is difficult to conclude whether this association is a general feature of origins, or if it reflects specific regulatory requirements for the respective replication systems, or a combination of both. An example that indicates that this association is not only a feature of viral regulation is the chorion genes of *Drosophila*. It has been shown that the chorion gene promoter resides within the origin of amplification for the chorion genes (*30*) and, even though this may represent a special case, it is possible that transcriptional elements are a general feature of cellular origins of replication as well. The link between enhancers, promoters, and replication origins in viruses may point to a method to regulate replication more directly than simply through transcriptional regulation of levels of trans-acting factors. If an intrinsic part of replication origins is a transcription factor binding site, the activity or state of this factor could directly influence the rate of replication initiation at the origin. In this way, a cycle of modifications (or other events) could serve to coordinately regulate both replication and transcription.

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46. We thank Kathy Jones and Bob Tjian for providing fractionated HeLa cell extracts and for help with the footprint assays, L. Berg for discussions, and M. Carey for advice concerning primer extension. Supported by PHS grant CA 42414 and American Cancer Society grant MV-91 (M.R.B.), by a fellowship from the Knut and Alice Wallenberg Foundation (A.S.), and by a predoctoral fellowship from the Office of Naval Research (G.L.B.).

23 January 1987; accepted 12 May 1987

Retroviruses and Mouse Embryos: A Rapid Model for Neurovirulence and Transplacental Antiviral Therapy

ARLENE H. SHARPE, RUDOLF JAENISCH, RUTH M. RUPRECHT

A murine model in which neurotropic retroviral infection can be studied over short periods of time was developed. Microinjection of Cas-Br-E virus into midgestation mouse embryos caused paralysis and death within 25 days after birth, in contrast to virus-infected neonates which develop disease only after 4 months. To evaluate whether antiviral drugs could cross the placental barrier and influence the course of the disease, the drug 3′-azido-3′-deoxythymidine (AZT) was administered to infected embryos through the drinking water of pregnant females. AZT treatment markedly retarded the onset and course of virus-induced central nervous system disease, permitting animals to survive beyond 4 months of age. These results are evidence for effective antiviral treatment during gestation and in the perinatal period and are of potential significance for the management of maternal transmission of the acquired immune deficiency syndrome (AIDS) virus.

BOTH THE PROGRESSIVE NEUROLOGical syndromes caused by human immunodeficiency virus [HTLV-III/LAV/HIV (*1*)] and the increasing frequency of acquired immune deficiency syndrome (AIDS) in the perinatal period (*2*) are serious complications of AIDS virus infection. No short-term animal model systems exist in which to study retroviral neurovirulence and treatment strategies for neonatal and in utero infections. Here we establish a murine model system that allows the rapid study of both the interaction of retroviruses with the developing embryo and virus-induced diseases of the central nervous system (CNS). We demonstrate that transplacental antiviral therapy with 3′-azido-3′-deoxythymidine (AZT) (*3*) is an effective treatment for rapidly fatal CNS disease following virus infection of embryos in utero.

The murine neurotropic type C retroviruses Cas-Br-E (isolated from a wild mouse population) and temperature-sensitive variants of Moloney murine leukemia virus (Mo-MuLV) induce progressive lower mo-

A. H. Sharpe, Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, and Departments of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115.
R. Jaenisch, Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142.
R. M. Ruprecht, Department of Medicine, Harvard Medical School, and Division of Cancer Pharmacology, Dana-Farber Cancer Institute, Boston, MA 02115.

tor neuron disease (*4*). Mice infected with Cas-Br-E develop hind-limb paralysis associated with spongiform changes in the anterior horns of the spinal cord, dentate nucleus of the cerebellum, and brainstem. Cas-Br-E infection exhibits some biological similarities to HIV infection: (i) Cas-Br-E may be transmitted via semen or milk, (ii) the viruses replicate in lymphocytes in early stages after exposure to virus (*4*), and (iii) both viruses can produce neuropathologic changes in the absence of an inflammatory infiltrate (*5*), in contrast to the neurotropic lentivirus visna (*6*).

To better understand the pathogenesis of Cas-Br-E paralytic disease, we investigated the interaction of this virus with midgestation mouse embryos. In previous experiments, infection of preimplantation and postimplantation mouse embryos with Mo-MuLV has served not only as a novel approach for the study of gene regulation during development, but also as a probe of molecular events occurring during preleukemic and leukemic phases of Mo-MuLV–induced disease (*7–10*). Postimplantation infection allows expression of virus in a wide variety of cell types, in contrast to postnatal infection, which restricts virus infection to cells primarily of the hematopoietic system (*8*). In particular, infection of postimplantation embryos with Mo-MuLV leads to infection of a wide variety of cell types in the nervous system, including astrocytes, oligodendrocytes, and spinal ganglia (*11*). Mo-MuLV, however, is not a neurovirulent virus and animals expressing even large amounts of virus in the CNS will not develop neurologic disease. We, therefore, introduced Cas-Br-E into embryos during midgestation at the stage of neural tube formation. Our aim was to investigate how infection of a wide variety of tissues with a neurovirulent virus would affect survival, onset, and spectrum of disease.

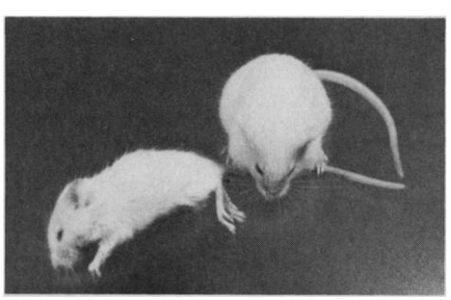

Fig. 1. Hind-limb paralysis in 3-week-old SWR/J mouse infected as a midgestation embryo. This mouse died 2 days after it was photographed. A noninfected littermate is shown for comparison. Cas-Br-E was microinjected into midgestation embryos as described (*8*). Pregnant mice were anesthetized and laparotomy was performed by a long ventral incision. The uterus was held with sharp forceps and cells producing molecularly cloned Cas-Br-E (clone NE-8) (*19*) were microinjected into individual embryos by introducing the micropipette into the ventral third of the decidual swelling. Approximately 0.1 to 0.5 μl (3×10^6 cells per milliliter) was injected per embryo.

Cas-Br-E virus was microinjected into postimplantation SWR/J mouse embryos during gestation between embryonic day (E) 8.75 and 9.0 (*8*). Approximately 63% (44 to 74%) of microinjected embryos survived to birth (Table 1), which is similar to that observed in studies with Mo-MuLV (*8*), and were born without obvious signs of disease or developmental anomaly. Animals were bled at 2 to 3 weeks of age and serum was assayed for viremia by radioimmunoassay (RIA). Viremia was used to assess the success of the microinjection technique because it is a highly sensitive indicator of viral replication (*10*). The RIA results indicated that 59 to 66% of microinjected embryos surviving after birth were successfully infected, which is comparable to previous results with Mo-MuLV (*8*).

Viremic animals derived from mouse embryos infected at midgestation began to show tremor between 8 and 14 days of age. These animals often were smaller and weighed less than uninfected age-matched controls. Disease progressed over the course of 1 to 2 weeks to complete hind-limb paralysis associated with prominent tremor. Infected animals also exhibited atrophy of hind-limb musculature, ruffled hair, and bladder incontinence (Fig. 1). Disease never involved the upper limbs. Animals died between postnatal day 19 and 25. The Fv-1 N host-mediated restriction of Cas-Br-E virus replication was maintained during infection at midgestation, as no animals derived from BALB/c or C57BL/6J embyros (Fv-1 B) infected with Cas-Br-E virus became viremic (Table 1). Histopathologic analysis of infected animals revealed spongiform changes in the CNS similar in location to that found in animals after postnatal infection (*12*). Thus, infection of midgestation embryos results in neurologic disease clinically and histopathologically similar to disease occurring in mice infected after birth. The course of disease, however, is telescoped in time. While animals infected postnatally develop tremor at 3 or 4 months followed by paralysis and death by 6 to 8 months, mice derived from infected midgestation embryos develop tremor and paralysis by 2 weeks and die of neurologic disease at 3.5 to 4 weeks (Fig. 2).

The murine retrovirus model described above appears ideally suited to test the biological efficacy of candidate antiviral agents targeted against neurovirulent retroviruses because the effects of an agent on symptoms and survival can be evaluated as early as 1 month after birth. Thus, noneffective agents can be quickly eliminated and more promising ones studied in further detail. To test the feasibility of this approach, we chose to examine the effects of therapy with AZT, a thymidine analog currently used to treat AIDS patients (*13*), on Cas-Br-E infection of SWR/J mice. AZT penetrates the blood-brain barrier, leading to CNS levels reaching 50% of serum levels (*13, 14*). Furthermore, AZT inhibits virus replication of Cas-Br-E, as tested by plaque formation on XC cells, with a median inhibitory concentration (IC_{50}) of 3 n*M* (0.8 ng/ml) and in the absence of cytotoxicity (*15*). In addition, oral AZT therapy effectively suppressed viremia and retroviral disease in BALB/c mice infected as adults with Rauscher murine leukemia virus complex (RLV) (*16*).

Oral AZT therapy, begun several hours after viral inoculation, dramatically altered the onset and course of neurologic disease in mice infected prenatally or postnatally. AZT was dissolved in the drinking water and administered to mothers before and after delivery and to weaned animals at two concentrations. In mice infected at midgestation, the onset of tremor was delayed from a median of 14 days in untreated viremic control animals to a median of 27 days in animals given AZT at 0.1 mg/ml ($P = 0.01$ by log rank) and a median of 50 days in mice given AZT at 0.2 mg/ml ($P < 0.0001$ by log rank) (Fig. 3A). Median survival for AZT-treated mice has not yet been reached

Table 1. Microinjection of mouse embryos in utero with Cas-Br-E. Embryos were microinjected in utero between E 8.75 and 9.0 as described (Fig. 1) with Cas-Br-E–producing NIH/3T3 cells or uninfected NIH/3T3 cells (mock infection). Viremic animals were identified by testing serum for p30 by RIA (*10*). AZT does not appear to affect embryo survival to birth ($P >> 0.05$ by Fisher Exact Test for all comparisons). Numbers of animals surviving to birth and developing viremia may be underestimated because of cannibalism of neonates by SWR/J mothers.

Strain	Injected with	Born/injected (%)		Viremic/alive at 2 weeks (%)	
		−AZT	+AZT	−AZT	+AZT
SWR/J	Cas-Br-E	46/73 (63)	89/120 (74)	21/32 (66)	32/54 (59)
SWR/J	Mock	8/15 (53)	40/63 (63)	0/8	0/40 (0)
BALB/c	Cas-Br-E	13/22 (59)		0/11 (0)	
C57BL	Cas-Br-E	12/27 (44)		0/10 (0)	

at either concentration, and is longer than 4 to 5 months, in contrast to median survival of 20 days in viremic, untreated controls (Fig. 3B). These results represent a statistically significant dose-response effect (P = 0.00008 for onset of tremor, $P < 0.00001$ for survival, as analyzed by dose rank in a Cox regression analysis). Most AZT-treated mice progressed from mild to more pronounced tremor over several months. Only at 6 months after birth did some of the AZT-treated mice begin to develop hind-limb paralysis. Mock-infected control mice treated with AZT or receiving no treatment have remained clinically well. After administration of AZT at 0.2 mg/ml in the drinking water beginning at E 9, an AZT concentration of 0.23 μg per gram of tissue in embryos was reached on E 12, as determined by high-performance liquid chromatography (*17*). This confirms that AZT effectively crosses the placental barrier.

Mice inoculated with virus postnatally also showed a remarkable alteration in disease course when their mothers were treated orally with AZT (0.1 mg/ml) followed by direct oral treatment of weaned mice. These animals developed complete hind-limb paralysis with a medium onset of 181 days in contrast to viremic, untreated controls, which developed paralysis at a median of 112 days ($P < 0.0001$ by log rank) (Fig. 3C). This is likely due to a reduction in virus titer as was shown previously for RLV-infected mice treated with AZT (*16*). Similarly, passive immunization against neurotropic MuLV was shown to reduce virus titers and to prevent paralysis (*18*).

To address the effect of transplacental therapy only, animals infected in utero received AZT from their mothers transplacentally only until birth. This regimen also significantly delayed the onset and progression of disease. In contrast to infected untreated mice that had a median time to tremor of 14 days, these mice began to develop tremor on postnatal day 32 with a median of 38 days (P = 0.01 by log rank, seven mice) and survived until postnatal day 99 to 127. Untreated mice had a median survival of only 21 days (P = 0.005 by log rank, seven mice). This clearly shows that transplacental treatment with AZT resulted in improved clinical outcome for offspring infected at midgestation. Because of small numbers of experimental animals, the effects of continuous AZT treatment as compared to transplacental treatment alone yielded no significant differences. In contrast, AZT therapy started only after birth did not appear to be as effective as treatment initiated transplacentally within 12 hours after virus infection. These results suggest that for antiviral therapy to be most effective, the drug has to be administered at the beginning of viral infection.

Mice infected as neonates (five mice) had a delayed onset of hind-limb paralysis as compared to 17 infected controls (median, 148 days versus 112 days, P = 0.04 by log rank), when AZT treatment was given only until weaning. In this case, continuous AZT therapy led to significantly longer paralysis-free survival (P = 0.003 by log rank) as compared to AZT therapy only during lactation.

In summary, using in utero infection of midgestation mouse embryos, we have developed a rapid model for studying neurotropic retrovirus disease. It is particularly significant that the disease developing in mice infected as midgestation embryos is similar to disease in mice infected as newborns. Because exposure of midgestation embryos to Mo-MuLV, in contrast to postnatal exposure, results in efficient infection of virtually all tissues (including all parts of the CNS) (*8, 9*), our results suggest that paralytic disease may be a consequence of a specific intracellular interaction of Cas-Br-E virus with cells in the lower spinal cord and may not be due to selective infection of these cells by neurotropic virus.

This model is well suited not only to the study of neurovirulence, but also to the evaluation of therapy directed to the CNS

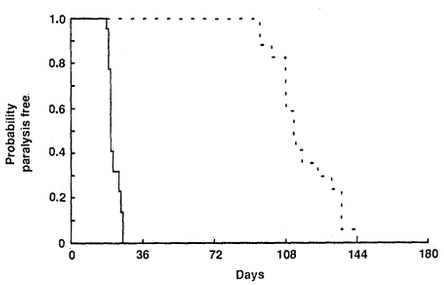

Fig. 2. Kaplan-Meier plot showing the probability of not developing full hind-limb paralysis versus time. SWR/J mice were infected either as midgestation embryos as described in the legend to Fig. 1. (———, 22 mice) or during the first 2 days after birth (- - - -, 17 mice). Neonates were infected intraperitoneally by injection of 2×10^4 plaque-forming units of Cas-Br-E virus in 0.1 ml. The virus was prepared by passing tissue culture supernatant, obtained from NIH/3T3 cells producing molecularly cloned Cas-Br-E (clone NE-8) (*19*), through a 0.22-μm Nalgene filter and quickly freezing and storing it in liquid nitrogen. Mice lost in the neonatal period due to maternal cannibalism were excluded from the analysis, and for animals infected as midgestation embryos, only viremic mice were scored due to the 66% success rate of infection. All mice were examined regularly and were scored as fully paralyzed when no motion was observed in the hind limbs or when mice with known neurologic dysfunction of the hind limbs were found dead. Moribund or fully paralyzed mice were sacrificed for humane reasons. The median time to full hind-limb paralysis was 20 days for midgestation infection and 112 days for neonatal infection ($P < 0.0001$ by log rank).

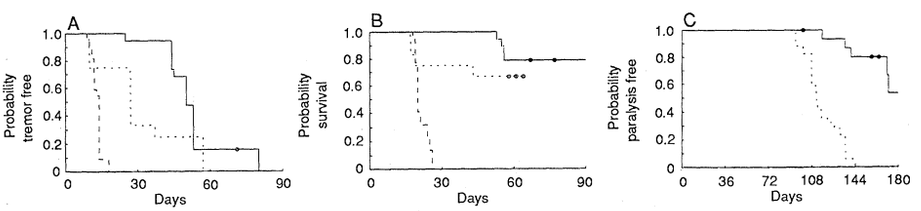

Fig. 3. Effect of AZT on onset of tremor, paralysis and survival of mice infected either as midgestation embryos or neonates, as shown by Kaplan-Meier analysis. (**A**) Probability to remain tremor-free for SWR/J mice infected in utero as described (Fig. 1). AZT was started 12 hours postoperatively by giving the drug in drinking water at 0.1 mg/ml (. . . .) or 0.2 mg/ml (———) to the pregnant females. Approximate doses based on weight and fluid intake ranged from 32 to 63 mg/kg/day for an AZT concentration of 0.2 mg/ml. At these doses, no maternal morbidity or mortality was observed. AZT treatment was continued throughout the life of the infected animals by first giving the drug at the same concentration to the lactating mothers after birth, followed by direct oral treatment of the infected offspring after weaning. Only viremic mice were scored and mice cannibalized during the perinatal period were excluded. Data are shown from 22 untreated mice (- - -), 12 animals whose mothers received AZT at 0.1 mg/ml (· · · ·), and 19 animals whose mothers received AZT at 0.2 mg/ml (——). One RIA-positive animal in the 0.2 mg/ml cohort was symptom free (-○-). (**B**) Same as (A), but showing the probability of survival. Animals alive at the time of analysis are shown by -○- (15 mice on 0.2 mg/ml; 8 mice on 0.1 mg/ml). To analyze dose effect, the doses were ranked (control = 1, 0.1 mg/ml = 2, 0.2 mg/ml = 3), and dose rank was entered into a Cox regression program as the covariate. (**C**) Probability to remain free of complete hind-limb paralysis versus time is shown for SWR/J mice infected as neonates during the first 2 days of life with Cas-Br-E virus as described (Fig. 2). AZT therapy was started 4 hours postinoculation by administering the drug in drinking water to lactating females at 0.1 mg/ml. This dose was well tolerated throughout the lactating period by both mothers and pups. After weaning, AZT was continued at 0.1 mg/ml in drinking water. -○-: 1 animal censored at 108 days due to accidental death; 8 animals without complete paralysis at time of analysis. Untreated control animals (. . ., 17 mice); AZT-treated mice (———, 16 mice). There were no differences in time to development of full hind-limb paralysis by sex nor any influence of sex on AZT therapy. Because onset of tremor and survival are not totally independent events (onset of tremor must precede death), a Bonferroni adjustment to the α-level for significance was performed for statistical tests on (A) and (B). P values <0.025 can be considered to be statistically significant for these comparisons.

sanctuary. Furthermore, therapeutic maneuvers designed to block the initial viremic phase of infection also may result in prevention of neurologic disease and could be tested in this model. We have shown that AZT dramatically alters the onset and course of retrovirus-induced neurologic disease in a dose-dependent manner. AZT effectively crosses the placental barrier and ameliorates disease in offspring infected in utero when treatment is given during gestation to the pregnant female. AZT therapy also leads to significant improvement in animals infected during the neonatal period. Thus, the murine neurotropic disease model permits rapid, cost-effective, quantitative assessments of treatment strategies that may be relevant to the therapy of neurologic manifestations of human retrovirus infections. Because AZT alters the onset and course of disease due to transplacental and perinatal retrovirus infection, we also can evaluate the efficacy of treatment during gestation and in the perinatal period, an important issue because of the increasing prevalence of pediatric AIDS (*2*).

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20. We thank P. Jolicoeur for Cas-Br-E–producing cells; S. Nusinoff-Lehrman (Burroughs Wellcome, Inc.) for AZT; P. Chassler, R. Curry, D. Grottkopp, and L. O'Brien for their excellent technical assistance; and J. Andersen for help with statistical analy-

A.H.S. is a Lucille P. Markey Scholar and this work was supported in part by a grant from the Lucille P. Markey Charitable Trust. R.M.R. is the recipient of a faculty development award from the Pharmaceutical Manufacturers Association Foundation. Supported in part by a contract from Commonwealth of Massachusetts Department of Public Health and NIH grant U01-AI24845-01 to R.M.R. and by NIH grants HD19015 and CA38497 to R.J.

17 April 1987; accepted 2 June 1987

Divalent Cations Directly Affect the Conductance of Excised Patches of Rod Photoreceptor Membrane

J. H. STERN, HANS KNUTSSON,* P. R. MACLEISH

Phototransduction in rod cells is likely to involve an intracellular messenger system that links the absorption of light by rhodopsin to a change in membrane conductance. The direct effect of guanosine 3′,5′-monophosphate (cGMP) on excised patches of rod outer segment membrane strongly supports a role for cGMP as an intracellular messenger in phototransduction. It is reported here that magnesium and calcium directly affect the conductance of excised patches of rod membrane in the absence of cGMP and that magnesium, applied to intact rod cells, blocks a component of the cellular light response. The divalent cation–suppressed conductance in excised patches showed outward rectification and cation-selective permeability resembling those of the light-suppressed conductance measured from the intact rod cell. The divalent cation–suppressed conductance was partly blocked by a concentration of the pharmacological agent L-*cis*-diltiazem that blocked all of the cGMP-activated conductance. Divalent cations may act, together with cGMP, as an intracellular messenger system that mediates the light response of the rod photoreceptor cell.

FESENKO *et al.* (*1*) REPORTED THAT guanosine 3′,5′-monophosphate (cGMP) directly activates the conductance of excised patches of rod cell membrane (*1–5*). We report here that, in the absence of cGMP, the divalent cations magnesium and calcium directly suppress the conductance of excised patches of rod membrane.

The effect of divalent cations on the rod membrane conductance was studied by exposing the intracellular side of inside-out, excised patches (*6*) to various bath concentrations of Ca^{2+} and Mg^{2+} (*7*). Patches were obtained from solitary rod photoreceptor cells dissociated from the tiger salamander retina (*4, 8*). In most experiments, the bath and pipette contained a simple salt solution of 120 m*M* NaCl, 3 m*M* KCl, 1 m*M* Hepes, and 0.02 m*M* phenol red at *p*H 7.3. In other experiments we used a stock solution containing 108 m*M* NaCl, 16 m*M* glucose, 3 m*M* KCl, 1 m*M* Hepes, 1 m*M* $NaHCO_3$, 1 m*M* sodium pyruvate, 0.5 m*M* NaH_2PO_4, and 0.02 m*M* phenol red at *p*H 7.3. The pipette solution always contained an additional 1 or 2 m*M* $CaCl_2$ and 1 or 2 m*M* $MgCl_2$, and the bath solution contained the Ca^{2+} and Mg^{2+} concentrations indicated. The bath solution was varied by moving the patch pipette to appropriate positions in front of a linear array of superfusion pipettes (diameter, 100 μm). Suction pipettes were made with a BB-CH pipette puller and Drummond 100-μl microcaps or Corning type 7740 capillary tubing; those having a final tip diameter of 1 to 2 μm were used immediately from the puller.

A current-clamp experiment in which 15-pA current pulses were applied to an excised patch of rod outer segment membrane is shown in Fig. 1A. Initially, the intracellular side of the membrane was exposed to a solution lacking divalent cations (*9*) and the conductance was high, as indicated by the small voltage deflection. The bathing solution was then changed to one containing 1 m*M* Ca^{2+}, and the conductance decreased two- to threefold. Changing the intracellular solution from 1 m*M* Ca^{2+} to 1 m*M* Mg^{2+} caused little further change (*10*). Figure 1A shows that both Ca^{2+} and Mg^{2+} caused rapid and reversible decreases in the patch membrane conductance in the absence of added cGMP. The effects of divalent cations were observed in this patch for 12 cycles of

Laboratory of Neurobiology, Rockefeller University, New York, NY 10021.

*Permanent address: Department of Electrical Engineering, University of Linkoeping, Linkoeping, Sweden.