performed in 20-Hz trains on 10 neurones in which APV was found to antagonize the physiologically evoked response. For nine of these neurones, APV was found to antagonize the response to the stimulus train. In particular, the later components of the response appeared more susceptible to APV (Fig. 3). Note that the APV-sensitive component of this response does not appear to be larger than the APV-insensitive component; this suggests that the APV-insensitive component becomes less effective during the stimulus train, possibly because of activation of inhibitory processes in the thalamus[13]. Clearly; experiments using intracellular recording would provide further information on this matter.

It is conceivable that, under physiological conditions, the NMDA receptor component potentiates an already present, but possibly sub-threshold, excitatory input mediated by non-NMDA excitatory amino-acid receptors (presumably of the kainate or quisqualate type). This hypothesis is attractive in view of recent work which shows that responses of neurones to NMDA are subject to voltage-dependent blockade by Mg^{2+} ions in the physiological concentration range for Mg^{2+} (refs 6, 14, 15), and it is noteworthy that a postsynaptic potential involving a short-latency non-NMDA component and an NMDA component of longer latency have been found in the spinal cord of *Xenopus*[16]. The recruitment of NMDA receptors could be a purely synaptic phenomenon similar to the APV-sensitive long-term potentiation observed in the hippocampus[7], and might involve one type of excitatory amino acid being released onto both NMDA and non-NMDA receptors. Alternatively, the two types of response could be mediated by two afferent pathways to the thalamus which terminate on the same neurone. Evidence for such multiple pathways already exists[17,18].

The NMDA receptor-mediated response of VB neurones to physiological stimulation of afferents provides a possible site of action for the dissociative anaesthetic ketamine, which has been shown to possess NMDA antagonist properties[6,19]. This contrasts with previous findings in the dorsal horns of the spinal cord[20] and medulla[3], where NMDA antagonists were found to be ineffective in blocking responses to air jet deflection of hairs and vibrissae. As there are no major procedural differences between the present experiments and those performed on the medullary dorsal horn[3], it seems likely that the observed difference reflects a regional difference between the dorsal horn and thalamus. Thus, it is possible that the thalamus is a major site for the anaesthetic action of ketamine.

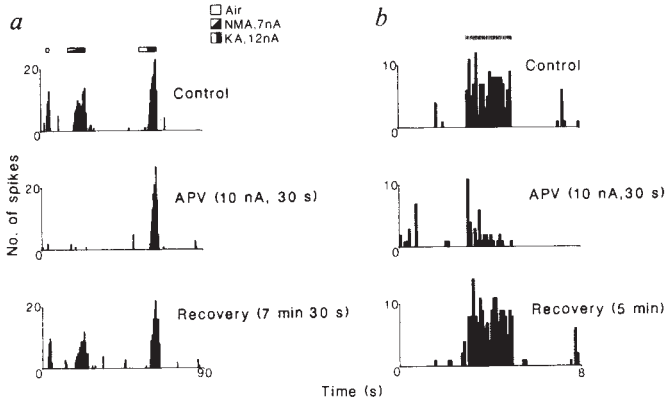

Fig. 3 *a*, Similar records to those in Fig. 1 (500 ms epochs), but from a neurone responding to air jet stimulation and iontophoretically applied NMA and kainate (KA). APV selectively and reversibly antagonized the responses to the air jet and NMA. *b*, Records from the same neurone as in *a*, showing the responses to a train of electrical stimuli (20 Hz, 0.1-ms pulse, 2 s duration, 100 ms epochs, 5 trials). The time of stimulation is indicated by the vertical lines above the top record. The middle record shows the response of the neurone during the ejection of APV, and the bottom record shows the recovery after termination of the APV ejection.

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Frequency-dependent involvement of NMDA receptors in the hippocampus: a novel synaptic mechanism

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Acidic amino acids, such as L-glutamate, are believed to be excitatory neurotransmitters in the mammalian brain[1,2] and exert effects on several different receptors named after the selective agonists kainate, quisqualate and *N*-methyl-D-aspartate (NMDA)[1]. The first two receptors, collectively termed non-NMDA receptors, have been implicated in the mediation of synaptic transmission in many excitatory pathways in the central nervous system (CNS), whereas NMDA receptors, with few exceptions, do not appear to be involved[2]; this is typified in the hippocampus where there is a high density of NMDA receptors[3] yet selective NMDA receptor antagonists, such as D-2-amino-5-phosphonovalerate (APV), do not affect synaptic potentials[4–11]. NMDA receptors have, however, been shown to be involved in long-term potentiation (LTP) in the hippocampus[6–11], a form of synaptic plasticity[12] which may be involved in learning and memory[11]. NMDA receptors have also been found to contribute to epileptiform activity in this region[13,14]. We now describe how NMDA receptors can participate during high-frequency synaptic transmission in the hippocampus, their involvement during low-frequency transmission being greatly suppressed by Mg^{2+}. A frequency-dependent alleviation of this blockade provides a novel synaptic mechanism whereby a single neurotransmitter can transmit very different information depending on the temporal nature of the input. This mechanism could account for the involvement of NMDA receptors in the initiation of LTP and their contribution, in part, to epileptic activity.

Experiments were performed on transverse hippocampal slices prepared from adult female rats as described previously[15]. The standard perfusion medium comprised (in mM): NaCl 124, $NaHCO_3$ 26, NaH_2PO_4 1.2, KCl 3 or 5, $MgSO_4$ 1 or 2, $CaCl_2$ 2, D-glucose 10. Extracellular and intracellular recordings were obtained from the CA1 cell body region using electrodes containing NaCl (1-4 M) and CH_3COOK (3 M), respectively. The Schaffer collateral-commissural pathway was stimulated with single shocks delivered at 10-30-s intervals to elicit 'low-

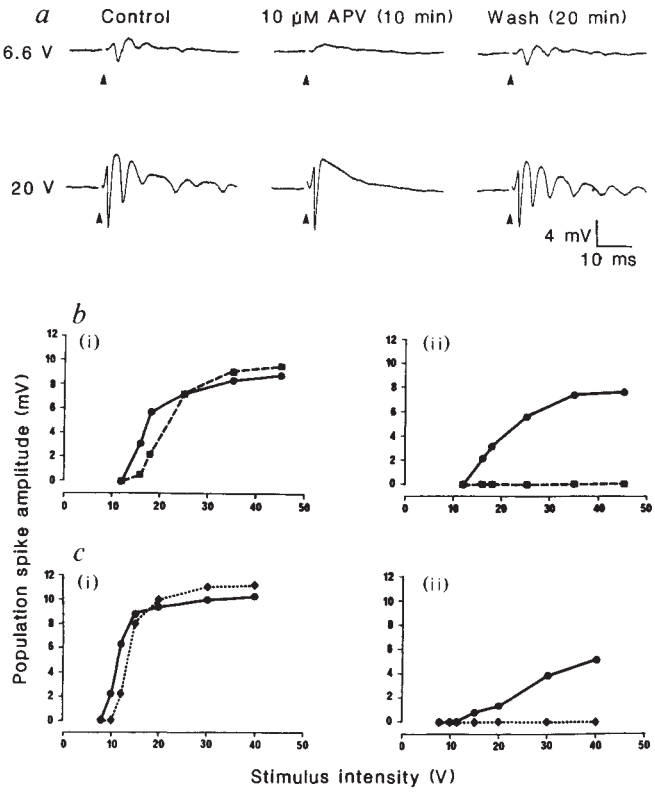

Fig. 1 Effects of NMDA antagonists on field potentials recorded extracellularly from the CA1 cell body region in response to low-frequency stimulation of the Schaffer collateral-commissural pathway. *a*, Effect of 10 μM APV on responses at two stimulus intensities (6.6 V and 20 V). *b*, Amplitude of the primary (i) and secondary (ii) population spikes as a function of stimulus intensity in control medium (continuous line) and in the presence of 20 μM APV (broken line) in another hippocampal slice. *c*, Data presented in the same manner as *b* for a different slice, showing the effects of 100 μM Mg^{2+}.

frequency' synaptic responses and for periods of 200 ms at 10-ms intervals to evoke 'high-frequency' responses. The selective NMDA antagonist APV was administered via the perfusion medium (flow rate 1–2 ml min^{-1}). In the absence of APV, high-frequency stimulation often produced LTP of the excitatory postsynaptic potential (e.p.s.p), therefore periods of high-frequency stimulation were delivered first in the presence and subsequently following washout of APV. The data were tested for significance ($P < 0.05$) using paired Student's *t*-tests.

Field potentials were recorded from the CA1 cell body region in response to low-frequency stimulation of the Schaffer collateral-commissural pathway with no Mg^{2+} in the bathing medium[16]. At low stimulus intensities the synaptic response was greatly suppressed (Fig. 1*a*) or abolished by 10 or 20 μM APV. In contrast, at higher stimulus intensities, the secondary population spikes were abolished while the primary population spike was little affected by APV (Fig. 1*a*, *b*), presumably because non-NMDA excitatory amino-acid receptors could sustain this component of the response[6]. These effects were fully reversible and seen in all 10 hippocampal slices examined. An identical pattern of antagonism was also observed with Mg^{2+} (100 μM) in all four slices tested (Fig. 1*c*).

These results demonstrate for the first time that NMDA receptors mediate a synaptic response in hippocampal neurones that has as low a threshold as that of non-NMDA receptors, provided Mg^{2+} is absent from the extracellular medium; this could be accounted for by the neurotransmitter having a high affinity for NMDA receptors, which would only be seen functionally when the Mg^{2+} block of NMDA channels was removed. The concentrations of Mg^{2+} (dose-dependent over the range 20–500 μM) that affect this component are the same as those that directly block postsynaptic responses to NMDA[1] and are much lower than those expected to affect significantly transmitter release[17] or membrane stabilization[18]; they are also much lower than the generally accepted levels of Mg^{2+} in extracellular fluid. It is likely, therefore, that a major action of Mg^{2+} in the hippocampus is to exert a powerful controlling influence on synaptic transmission by a direct interaction with the NMDA receptor system.

A second series of experiments was performed to determine the extent of the involvement of the NMDA receptor system in synaptic transmission in the hippocampus in the presence of physiological concentrations of Mg^{2+} (1–2 mM). Passive membrane properties, determined for 27 CA1 neurones (resting membrane potential −69 ± 1 mV; input resistance, calculated near the resting membrane potential, 27 ± 1 MΩ), were not altered significantly by APV (20 or 50 μM). Stimulation of the Schaffer collateral-commissural pathway at low frequencies evoked a fast e.p.s.p. which was unaffected by APV (Fig. 2*a*). Measurements (mean ± s.e.m.) of peak amplitude and duration at 50% of peak amplitude in control solutions (8.3 ± 0.6 mV; 9.6 ± 0.8 ms) and in the presence of APV (8.4 ± 0.6 mV; 9.3 ± 0.6 ms) were not significantly different ($n = 32$). The inhibitory postsynaptic potential (i.p.s.p.) which followed the fast e.p.s.p. was generally unaffected by APV (Fig. 2*a*).

In contrast to the lack of effect on low-frequency synaptic responses, APV had a marked effect on the synaptic response generated by high-frequency stimulation. During high-frequency stimulation, fast e.p.s.ps were associated with a slow depolarizing potential. APV (20 or 50 μM) had no effect on fast e.p.s.ps evoked during high-frequency stimulation (Fig. 2*c*); it did, however, reduce the size of the associated slow depolarization, measured from the pre-stimulus membrane potential to the base of the fast e.p.s.ps, in 17 of 22 neurones examined (Fig. 2*b*, *c*). The mean (±s.e.m.) amplitude of the depolarization, measured at 140–150 ms into the high-frequency train, for these 17 cells in the presence and after washout of APV, was 5.3 ± 0.9 mV and 8.3 ± 1.0 mV, respectively. The size of the NMDA receptor-mediated potential calculated individually for each cell varied between 0.9 and 5.1 mV (mean ± s.e.m., 3.0 ± 0.2 mV). This NMDA receptor-mediated e.p.s.p. became apparent in some cells as early as the second fast e.p.s.p. in the high-frequency train (Fig. 2*b*, *c*).

The lack of effect of APV on low-frequency synaptic transmission in the Schaffer collateral-commissural pathway is in agreement with previous reports using extracellular recording[5–9], but contrasts with a recent intracellular study which reported that APV depressed the fast e.p.s.p.[19]. The latter effect can be attributed to the use of high concentrations of the racemic mixture of APV, which has previously been shown to depress synaptic transmission in this pathway by an action unrelated to NMDA-receptor antagonism[7]. As low-frequency synaptic responses in this pathway can be reduced by broad-spectrum excitatory amino-acid antagonists, such as γ-D-glutamylglycine[6], it is believed that receptors of the kainate or quisqualate (that is, non-NMDA) type are responsible for fast e.p.s.ps. The present study has shown that an NMDA receptor-mediated e.p.s.p. can be recorded in this pathway during high-frequency stimulation, but the slow nature of this synaptic response as well as its activation characteristics clearly distinguished it from the fast e.p.s.ps. The observation that the NMDA receptor-mediated synaptic potential could often be detected as early as the second fast e.p.s.p. during high-frequency stimulation (see Fig. 2*b*, *c*) demonstrates that as few as two appropriately timed synaptic inputs may activate the NMDA receptor system in the hippocampus.

The finding that in the absence of Mg^{2+}, low-frequency stimulation activates a low-threshold NMDA receptor-mediated synaptic response, implies that NMDA receptors are normally acted on by the neurotransmitter released by low-frequency

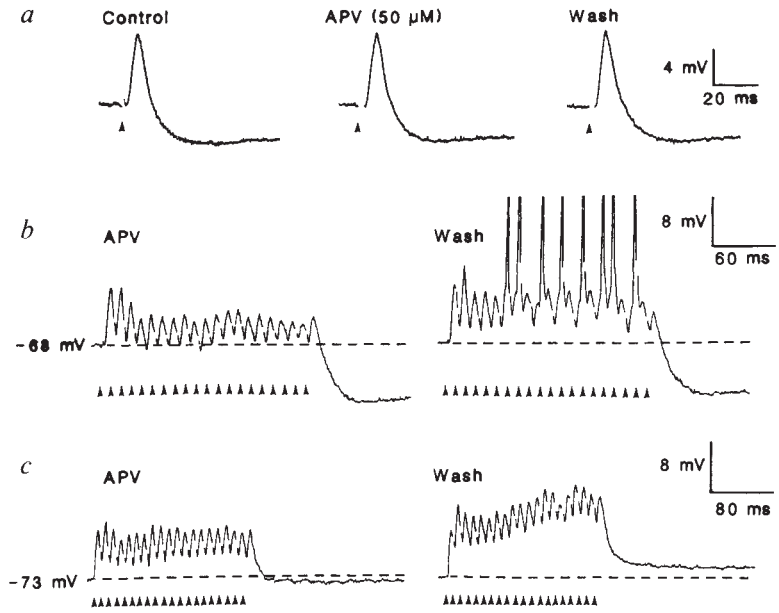

Fig. 2 Effect of 50 μM APV on synaptic responses recorded intracellularly in CA1 neurones in response to stimulation of the Schaffer collateral-commissural pathway. *a*, Averages of five successive records of synaptic potentials evoked at 30-s intervals before (control), during and after washout of APV. *b*, Single records of responses of the same cell to high-frequency stimulation (20 shocks at 10-ms intervals) in the presence and following washout of APV. The e.p.s.p.s shown in *a* (centre and right-hand records) were obtained immediately before the two respective periods of high-frequency stimulation. *c*, Single records of responses to high-frequency stimulation in another cell. The times of stimulation are indicated by arrowheads and stimulus artefacts have been blanked out for clarity. Stimulus intensity and membrane potentials were constant throughout. Action potentials in *b* are truncated.

stimulation. It is not necessary, therefore, to postulate that high-frequency stimulation is required either to release an additional agonist for NMDA receptors or to increase neurotransmitter release sufficient to activate the NMDA receptors due to their localization or activation characteristics. A simpler hypothesis[20,21] which agrees fully with the present observations is that a single neurotransmitter acts simultaneously on both non-NMDA and NMDA receptors but that the latter are prevented from participating appreciably in low-frequency synaptic transmission by Mg^{2+}. During high-frequency stimulation this Mg^{2+} blockade of the NMDA receptor system may be temporarily alleviated, allowing Ca^{2+} and Na^{+} to enter via the NMDA channels[6,22–25], and lead to the induction of LTP[26]. This inward movement of cations through the NMDA channels could account for the slow e.p.s.p. recorded in the present study. It is likely that depolarization of the synaptic membrane, caused in part by temporal summation of fast e.p.s.ps, could be the mechanism by which the blockade is reduced. In this respect, it has been shown using cultured neurones that Mg^{2+} directly blocks NMDA-gated channels and that this block is reduced by membrane depolarization[27]. In addition, it has been reported recently that pairing single afferent synaptic inputs with postsynaptic depolarization can activate the NMDA receptor system and elicit LTP[28].

It has been shown that NMDA receptors are involved in epilepsy[29]. In the presence of convulsant drugs, an NMDA receptor-mediated potential has been recorded extracellularly in the hippocampus during brief periods of high-frequency stimulation[8], and a small NMDA receptor-mediated component of the fast e.p.s.p. recorded extracellularly[21] or intracellularly[13,14] has been detected during low-frequency stimulation. The latter effect has been explained on the basis of removal of the Mg^{2+} block of NMDA channels, the necessary depolarization being provided by the fast e.p.s.ps prolonged by the action of the convulsant drug[14]. Such a mechanism could operate at the centre of an epileptic focus where synaptic inhibition may be impaired. The present results suggest a second way in which the NMDA receptor system may contribute to epileptic activity—by amplifying the synaptic output in response to a high-frequency input in the presence of fully functional synaptic inhibition (that is, absence of convulsant drugs). The synchronized, high-frequency discharge of neurones in epileptic tissue would provide ideal conditions for the activation of the NMDA receptor system such that this system could contribute to the propagation of epileptic activity through normal brain tissue.

It is now widely believed that NMDA receptors are important for synaptic plasticity in the hippocampus, but their function elsewhere in the brain is generally less clear. Although it is probable that NMDA receptors will be found to be involved in plastic processes in other regions of the CNS (for example, the cerebral cortex), their involvement in the discharge pattern of spinal interneurones[1], transmission of afferent sensory input[30] and generation of locomotor patterns[31] is also indicated. As Mg^{2+} ions are a potent NMDA antagonist in all regions of the brain so far examined, it is likely that a synaptic mechanism similar to that proposed here for hippocampal neuronal transmission will apply throughout the CNS.

Since submission of this manuscript, it has been reported[32] that postsynaptic hyperpolarization during conditioning reversibly prevents the initiation of LTP. These authors proposed that this was caused by the hyperpolarization preventing activation of voltage-gated calcium channels. On the basis of our present findings, we suggest a refinement of this hypothesis: that hyperpolarization prevented LTP by opposing the depolarization necessary to alleviate the Mg^{2+} block of NMDA channels.

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The inducible cytotoxic T-lymphocyte-associated gene transcript CTLA-1 sequence and gene localization to mouse chromosome 14

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Classical phenomenological approaches to the study of the mechanism of T-cell-mediated cytotoxicity[1–3] have now given way to a search for molecules involved in this function; this is attempted either by subcellular and biochemical fractionation of material from cytotoxic cells[4,5], or through the characterization of molecules recognized by cytotoxicity-inhibiting monoclonal antibodies (see ref. 6 for a review). Molecules having a role in cytotoxicity may also be identified by detecting the corresponding messenger RNA transcripts. Such an approach may include, as a first step, the search for transcripts as specific as possible to cytotoxic T cells; only secondarily can their actual relevance to cytotoxicity be investigated. We report here the preparation and systematic screening of a differential complementary DNA bank, in which we detected three distinct messenger RNA transcripts (CTLA-1, CTLA-2 and CTLA-3) present in various cytotoxic T cells but not (or less so) in a range of non-cytotoxic lymphoid cells. We describe the co-inducibility of these transcripts and of cytoxicity in thymocytes and hybridoma cells, the sequence of CTLA-1 cDNA, its protein homology with serine esterases and the localization of the corresponding gene to mouse chromosome 14.

A library was derived from cDNA obtained by subtractive hybridization[7–11] between cDNA from the B10.BR anti-H-2K^b cytotoxic T-cell clone KB5C20 (ref. 12; hereafter designated T_c) and mRNA from the B-lymphoma M12.4.1 (ref. 13; hereafter designated B). The 8,300 recombinant cDNA clones obtained were subjected to three successive rounds of screening with radioactive cDNA probes prepared from a range of cytotoxic and non-cytotoxic cells. In the first round of screening, 230 of the 8,300 cDNA clones gave a signal with a T_c probe but not with a B probe. These 230 T_c^+ B^- clones were subjected to a second round of screening using radioactive cDNA probes from T_c, B, T_h (T-helper cells, that is, the T-lymphoma EL4), thymocytes or brain. The results are summarized in Table 1. The 230 clones fell into a number of specificity patterns, with only 73 of them remaining 'T_c-specific'.

These 73 clones were hybridized with cDNA probes made from (1) RDM4, another non-cytotoxic T-cell lymphoma, detecting no clones, (2) EL4 cells, now activated with phorbol myristate acetate, which detected five 'activation-associated' clones, (3) lipopolysaccharide (LPS)-stimulated blasts, detecting two clones, and (4) a mixture of 2-day-old and 3-day-old cytotoxic concanavalin A (Con A) blasts, which failed to detect nine cDNA clones that previously were found to be positive with a T_c probe. The latter clones might, therefore, be associated with long-term *in vitro* growth of T cells rather than with cytotoxicity. Also, 8 of the 73 T_c^+ clones proved to be negative with a subtracted ($T_c - B$) cDNA probe, which was taken as an indication of non-T_c-specificity. Altogether, after this third round of screening, only 50 T_c-specific cDNA clones were retained for further study.

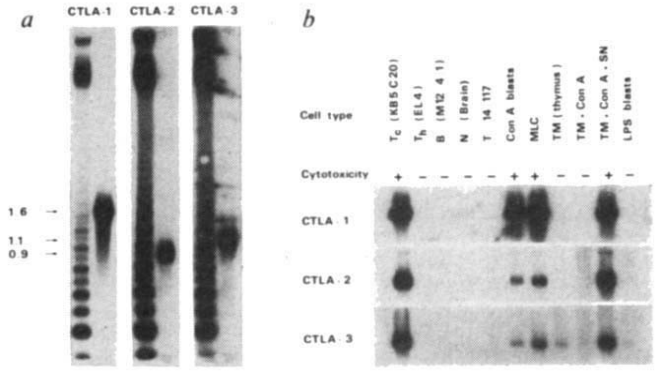

Fig. 1 Size and specificity patterns of CTLA-1, CTLA-2 and CTLA-3 RNA transcripts. Northern blots of RNA from the T_c clone KB5C20 (*a*) and from 11 cytotoxic and non-cytotoxic cell types (*b*) were probed with the inserts of plasmid M41D12, M41G12 or M11E6, representive of CTLA-1, CTLA-2 and CTLA-3, respectively. Cell types were as in Table 1, plus: T14.117, a hybridoma between a T-helper cell clone and the BW5147 thymoma[24]; MLC cells, A/J spleen cells stimulated for 5 days *in vitro* with C57BL/6 irradiated spleen cells; TM + Con A, C57BL/6 thymocytes incubated for 24 h in the presence of 5 μg ml^{-1} of Con A; TM + Con A + SN, TM + Con A further incubated for 5 days in the presence of IL-2-containing supernatant from PMA-stimulated EL4 C116 cells. At the time of RNA extraction, the cells were tested for cytotoxicity and classified as cytotoxic (+) or non-cytotoxic (−) according to the criteria given in Table 1. In *a*, an end-labelled 123-bp ladder (BRL) was used as a size marker. In *b*, a control actin probe gave a distinct band in all lanes (not shown). **Methods.** Total RNA (10 μg per lane) was run for 3–6 h at 2.5 V cm^{-1} on a 1.1% formaldehyde–agarose gel, in MOPS buffer, then transferred to nitrocellulose filters. Inserts, obtained either from CsCl-purified plasmids or from minipreparations of plasmids, digested with *Pst*I and separated on 2% LMP (low melting point) agarose (BRL) gels, were labelled by random priming[25] and used as probes on Northern blots. Blots were prehybridized in 5 × SSC, 50% formamide, 5 × Denhardt's, 5 mM EDTA, 0.1% SDS, 50 mM phosphate buffer *p*H 7 and 7% dextran sulphate, at 42 °C for 4–12 h. Hybridization was in the same buffer, to which the probe was added at 10^6 d.p.m. ml^{-1}, for 12 h at 42 °C. Washes were for 10 min in 2 × SSC, 0.1% SDS and for 30 min in 0.2 × SSC, 0.1% SDS at 65 °C.

In order to identify clones corresponding to the same genes, inserts (of 200 to 1,400 base pairs, bp) from individual clones were purified and hybridized to all of the 50 clones. This approach identified five groups of cDNA clones. Of these, on the basis of transcript size as determined by Northern blotting (Fig. 1*a*), three groups were clearly distinct. These were designated CTLA-1, CTLA-2 and CTLA-3, and included at least 3, 3 and 39 cDNA clones, respectively. Transcripts were detected as single bands of 1.6 kilobases (kb) for CTLA-1, 0.9 kb for CTLA-2 and 1.1 kb for CTLA-3 (Fig. 1*a*). CTLA-1, CTLA-2 and CTLA-3 transcripts were present in all the cytotoxic cell populations tested (T-cell clones, mixed leukocyte culture cells, Con A blasts and thymocytes activated with Con A plus interleukin-2 (IL-2)-containing EL4 supernatant). The CTLA-1 transcript was not detected in brain cells or in a range of non-

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