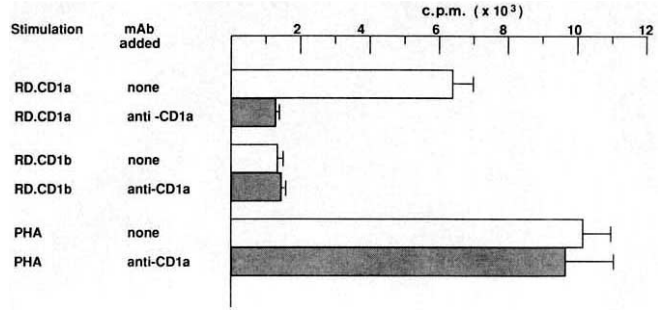

FIG. 4 Recognition of CD1a stimulates IL-2 production by CTL BK6. Culture supernatants of BK6 stimulated with CD1 transfectants of RD cells or with phytohaemagglutinin (PHA) in the presence or absence of anti-CD1a monoclonal antibody (mAb) were tested for IL-2 activity using IL-2-dependent cell line CTLL20. Results expressed as c.p.m. of [^{3}H]thymidine incorporation by CTTL20 cells.
METHODS. BK6 cells (2.5×10^4) were cultured for 24 h in 96-well flat bottom microtitre plates (Flow Laboratories) with 10^4 RD.CD1a (CD1a$^+$) or RD.CD1b (CD1b$^+$) or with PHA (Gibco, 1:1,000 dilution) in 0.2 ml culture medium (RPMI-1640, 10% human serum, 2 mM L-glutamine, 1 mM HEPES, 1% penicillin-streptomycin) containing 1 ng ml^{-1} phorbol myristate acetate (PMA) (Sigma). IIIE5 (anti-CD1a) ascites fluid was added to a final dilution of 10^{-3}. IL-2 was assayed by a modification of a published method[33], using 4,000 CTLL20 cells and a 1:2 dilution of supernatant. Results are means ±1 s.e.m. of triplicate cultures, and are representative of three separate experiments. [^{3}H]thymidine incorporation by CTLL20 in the absence of IL-2 for the experiment shown was 1,158 ± 115 c.p.m. Similar results were obtained in the absence of PMA, although the magnitude of response was lower (data not shown).

these molecules, give rise to the hypothesis that some T cells recognize antigens presented by CD1 molecules. □

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Voltage-dependent anion channels in the plasma membrane of guard cells

Bernhard U. Keller*†, Rainer Hedrich* & Klaus Raschke*

*Pflanzenphysiologisches Institut der Universität Göttingen, 34 Göttingen, FRG
†Max-Planck-Institut für biophysikalische Chemie, Am Fassberg 34 Göttingen, FRG.

WHEN plants assimilate CO_2 from the atmosphere, water vapour escapes. This exchange of gases can be moderated by the plant through volume changes of guard cells, which in pairs surround each stomatal pore in the epidermis and act as turgor-operated valves. When light intensity diminishes or water stress develops, guard cells shrink and stomata close. This process requires the release of ions from the guard cells. Voltage-dependent K^+ channels[1,2] provide a path for cations. The required corresponding passage for anions, particularly chloride and malate[3], is not known at the molecular level. We have applied the patch-clamp technique[4] to guard-cell protoplasts of *Vicia faba* and found strongly voltage-dependent activities of single anion channels in the plasma membrane (plasmalemma). These channels have a conductance of 39 pS in 100 mM KCl and are permeable for chloride and malate. They become active at membrane potentials positive to −80 mV. Their maximum activity occurs at −40 mV. The resulting outward Cl^- currents of individual guard cells are at least 25 pA. These voltage-dependent anion channels provide a mechanism for the control of salt efflux from guard cells.

Patch-clamp experiments were performed on guard-cell protoplasts isolated from *Vicia faba* by a gentle procedure (no agitation) at the temperature of plant cultivation[5]. High-resistance membrane seals[4] were formed between the patch pipette and the plasma membrane (Fig. 1). Fluxes of K^+, Cl^- and malate^{2-} were recorded at the cellular and single-channel level using the whole-cell configuration as well as isolated membrane patches[4].

Whole-cell currents were measured in the presence of 150 mM KCl in the pipette and 30 mM KCl in the bath (Fig. 2*a*, filled circles). The membrane conductance was studied as a function of the membrane voltage applied to the cytoplasmic side of the plasma membrane. The relationship between current and voltage depended on the voltage range considered. At membrane potentials below −100 mV, large inward currents occurred. They were identified as inward K^+ currents by reversal potential analysis[2]. Membrane potentials above −20 mV, particularly positive potentials, caused outward K^+ currents; at +50 mV they were as large as 100 pA. In the voltage range between −100 mV and −80 mV, which corresponds to the resting potentials of guard cells[6], the current was close to zero and the resistance of the plasma membrane was larger than 10 GΩ. Increasing the potential to values positive to −80 mV led to an inward current with a maximum conductance at −40 mV, the reversal potential for K^+.

To test whether this current was carried by anions, K^+ was replaced by Cs^+ or *N*-methylglucamine (NMG), which are known to permeate K^+ channels at negligible rates[7] (Fig. 2*a*, open circles). Below −100 mV and above −20 mV, currents were much smaller than they were in the presence of K^+, or even zero. Their fluctuations reached a maximum amplitude at −41 mV (Fig. 2*b*) and they disappeared at +39 mV (Fig. 2*b*), close to the Nernst potential for Cl^- (+40 mV). These results provide evidence that anions are the main charge carrier[5] of the whole-cell currents in the presence of CsCl solutions. At −81 mV, the opening and closing of single anion channels could be clearly discerned, even in whole-cell recordings.

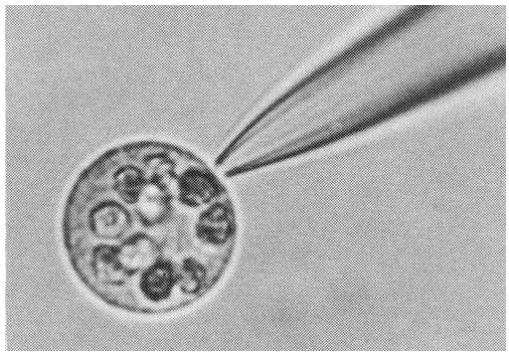


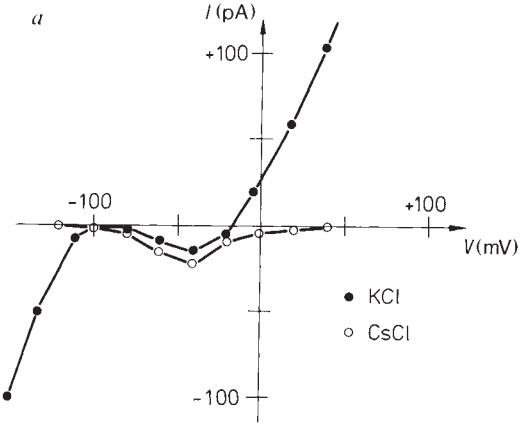
FIG. 1 Micrograph (Nomarski interference contrast) of a guard-cell protoplast from *Vicia faba*.
METHODS. Guard-cell protoplasts were isolated without agitation by overnight incubation at 20 °C in small Petri dishes containing 2.5 ml of 400 mM D-mannitol, 10 mM Na-ascorbate, 1 mM $CaCl_2$, 2% (w/v) cellulase (Onozuka RS), 1% pectinase (for example, Mazerozyme), 0.5% BSA, 0.05% kanamycin sulphate, pH 5.5, with osmolality adjusted to produce incipient plasmolysis of guard cells. Protoplasts were purified by filtration and centrifugation[5].

In inflated guard cells, malate is the major anion. Replacing chloride by malate left the voltage dependence of the anion current essentially unchanged. The maximum current still occurred close to −40 mV, but was reduced to values below 10 pA. After raising concentrations of cytosolic free Ca^{2+} to >5 μM, anion currents could be measured at voltages negative to −60 mV. At a Ca^{2+} concentration in the pipette of >2 mM, whole-cell anion currents did not exceed 30 pA in the presence of CsCl solutions.

After withdrawal of the pipette from the membrane surface, single anion channels and K^+ channels were recorded in the outside-out patch configuration in K^+ salt solutions (Fig. 3). To avoid interferences by the activity of K^+ channels, we limited the investigation to voltages positive to −90 mV, at which inward K^+ channels were closed. Between −90 mV and −40 mV, the membrane current was dominated by the opening of single anion channels. Downward current deflections represented single-channel currents of 3.9 pA at −90 mV. In excised patches, anion channels were most active when the concentration of free Ca^{2+} on the cytoplasmic side of the membrane was raised to 3–5 μM. Channel activity occurred in bursts separated by silent periods of 0.2 to 0.9 s duration. At voltages positive to −20 mV, the opening of outward K^+ channels appeared as upward current deflections. Multiple current levels indicated the simultaneous opening of several K^+ channels in the patch. At voltages above +20 mV, currents through the plasma membrane were dominated by the activity of K^+ channels, with a current amplitude of 2 pA at +70 mV (Fig. 3*a*).

For single anion channels, a main conductance level of 39 pS in 100 mM KCl was calculated from current-voltage measurements (Fig. 3*b*). Occasionally, channel openings at lower conductance levels could be observed, as shown in Fig. 3*c*. To assess the selectivity of the anion channels, Cl^- was replaced by several other anions. In reversal-potential measurements we found a permeability sequence of $NO_3^- > Cl^- > malate^{2-}$. Anion permeation was blocked by the addition of 10 μM Zn^{2+}, a well-known blocker of anion channels[8] (Fig. 4).

The anion currents we observed differ significantly from the anion currents recently described in the plasma membrane of plant cells. Anion currents in suspension-cultured cells of *Asclepias tuberosum* were elicited by hyperpolarizing instead of depolarizing voltages and had a magnitude of 300–400 pA in the whole-cell configuration[9]. The corresponding single-channel conductance was 100 pS compared with 39 pS for guard cells,



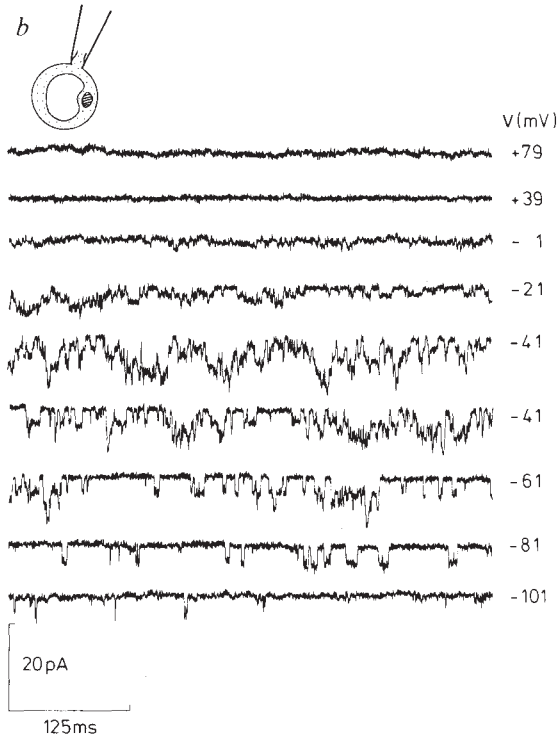
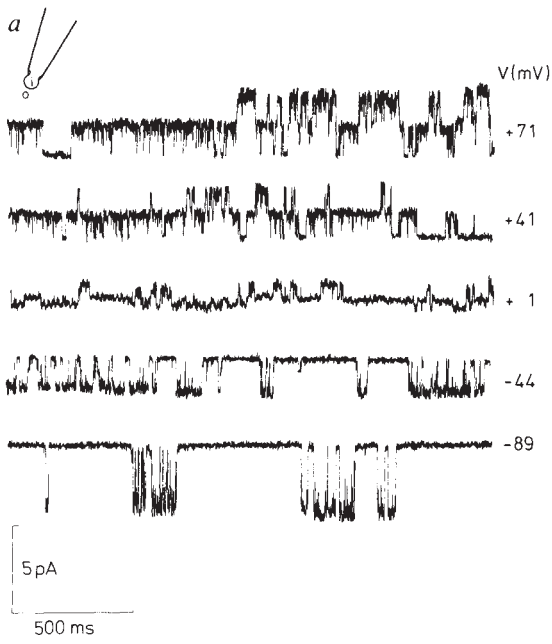
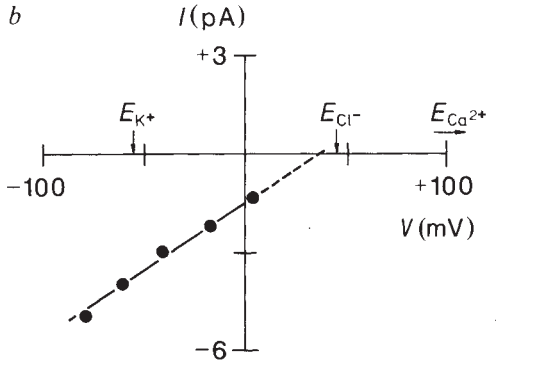


FIG. 2 Whole-cell currents of guard-cell protoplasts. *a*, ●, current–voltage (*I*–*V*) relationship of protoplasts bathed in 30 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES–Tris buffer, pH 5.5. Pipette solution, 150 mM KCl, 2 mM $MgCl_2$, 2 mM Mg ATP, 1 mM EGTA, 10 mM HEPES–Tris buffer, pH 7.2. Current–voltage relationships were determined by continuously increasing the voltage from −150 mV to +50 mV. Voltage and current were plotted on an *x*–*y* screen and corrected for the (ohmic) resistance of the membrane seal. Downward currents corresponded to cation influx into (or anion efflux from) the protoplast or both. ○, current–voltage relationship of protoplasts bathed in 30 mM CsCl, 1 mM $CaCl_2$, 2 mM Mg $MgCl_2$, 10 mM HEPES–Tris buffer, pH 5.6. Pipette solution was 150 mM CsCl, 2 mM $MgCl_2$, 2 mM Mg-ATP, 1 mM EGTA, 10 mM HEPES–Tris buffer, pH 7.2. *b*, Whole-cell currents recorded at various voltages *V* in asymmetrical CsCl solutions corresponding to those in Fig. 2*a* (○). Membrane potentials were controlled by an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt), and data were stored in digital form on a video-tape recorder. Off-line analysis was performed on a PDP-11/73 computer after filtering the data at 2 kHz (8-pole Bessel filter). All membrane potentials refer to the cytoplasmic side of the plasma membrane. Experiments were performed at room temperature (20 ± 2 °C).





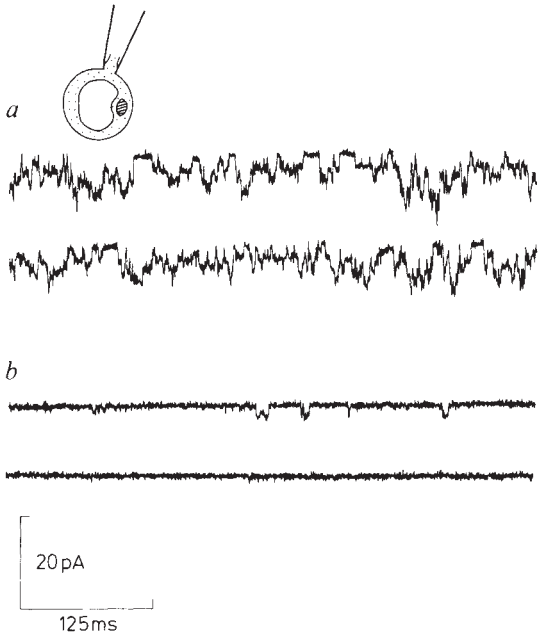


FIG. 4 Block of anion channels by extracellular Zn^{2+}. *a*, Currents through several anion channels were recorded in the whole-cell configuration. Protoplasts were bathed in 30 mM CsCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES–Tris buffer, pH 5.6. Pipette solution, 150 mM CsCl, 2 mM $MgCl_2$, 2 mM MgATP, 1 mM EGTA, 10 mM HEPES–Tris buffer, pH 7.2. The membrane potential was held at −41 mV. *b*, Addition of 10 μM $ZnCl_2$ to the bath solution blocked the activity of anion channels.

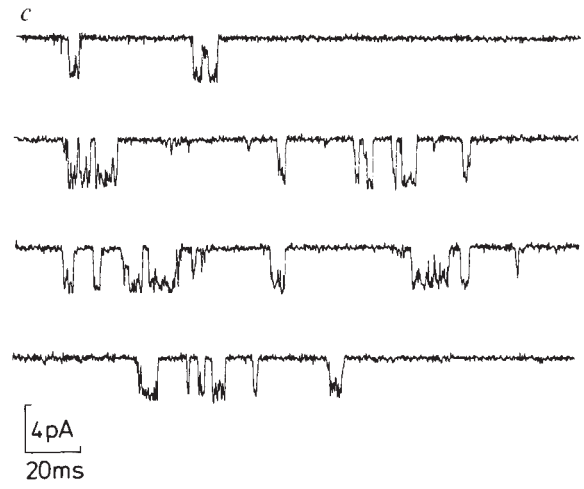


FIG. 3 Single-channel recordings from excised patches. *a*, Single K^+- and anion-selective channels measured in outside-out patches in an asymmetrical solution of KCl. Pipette solution, 100 mM KCl, 2 mM $MgCl_2$, 2 mM Mg-ATP, 1 mM EGTA, 10 mM HEPES–Tris buffer, pH 7.2; Bath solution, 10 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES–Tris buffer, pH 5.8. Upward current deflections at positive membrane voltages correspond to K^+ currents flowing through open K^+ channels. Current recordings at negative voltages are dominated by the opening and closing of single anion channels. Note the gating of both K^+- and anion channels at $V = +1$ mV. *b*, Single-channel current–voltage relationship. Single-channel current–voltage curve observed in a 10-fold gradient of KCl. Pipette solution, 100 mM KCl, 2 mM $MgCl_2$, 2 mM Mg-ATP, 0.1 mM $CaCl_2$, 1 mM EGTA, 10 mM HEPES–Tris buffer, pH 7.3; Bath solution, 10 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES–Tris buffer. pH 5.5. The Nernst potentials for Cl^-, K^+ and Ca^{2+} were $E_{Cl} = -44.1$ mV, $E_{K^+} = -55.0$ mV and $E_{Ca^{2+}} > +100$ mV, respectively (values corrected for ionic activities). Slope conductance, 39 pS. *c*, Currents through single anion-channels. Single-channel currents were recorded at −82 mV in the presence of the non-permeating cation NMG. Pipette solution, 100 mM NMG-Cl, 2 mM $MgCl_2$, 2 mM Mg-ATP, 1 mM EGTA, 10 mM HEPES–Tris buffer, pH 7.4. Bath solution, 10 mM NMG-Cl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES–Tris buffer, pH 5.5. Note the occurrence of channel substates in the first record from top.

indicating a striking discrepancy between anion transport in non-differentiated suspension-cultured cells and guard-cell protoplasts from developed leaves.

Our results also disagree with those of Schroeder and Hagiwara[10], who showed instantaneously activated, almost voltage-insensitive anion conductances in guard-cell protoplasts from *Vicia faba*. These anion currents were observed at high concentrations of cytoplasmic Ca^{2+} and exceeded outward K^+ currents by a factor of three (0.7 nA at −70 mV). Outward anion currents of that magnitude did not occur in guard cells embedded in the epidermis, where anion release during stomatal closure was equivalent to 30–40 pA[11]. Therefore, the anion channels shown here provide suitable molecular pathways to determine the magnitude of anion release from guard cells. Whether Ca^{2+}-activated anion conductances function in addition or alternatively to the voltage-regulated anion channels described here needs to be investigated further.

An early proposal was that the release of salts by guard cells is determined primarily by the anion permeability of the plasma membrane and that an increase in this permeability was sufficient to initiate stomatal closure[3,12]. The anion channels that we have described here would allow the depolarization required for salt release from guard cells. The ensemble conductance of the anion channels permeable to Cl^- and malate^{2-} can account for the observed rates of anion loss during stomatal closure[11]. The anion channels in guard cells indeed seem to provide a mechanism for controlled salt release. We propose that this

type of anion channel also functions in other types of plant cells, where it may be involved in the release of salts for the purpose of osmoregulation and for the control of turgor or cell volume. □

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Trans-dominant inactivation of HTLV-I and HIV-1 gene expression by mutation of the HTLV-I Rex transactivator

Laurence Rimsky, Madeleine Duc Dodon*, Eric P. Dixon & Warner C. Greene

Howard Hughes Medical Institute, Departments of Medicine and Microbiology-Immunology, Duke University Medical Center, Durham, North Carolina 27710, USA
* Immuno-Virologie Moléculaire et Cellulaire, UMR30-CNRS/UCBL, Faculté de Médecine A Carrel, 69372 Lyon Cedex 8, France

THE *rex* gene of the type I human T-cell leukaemia virus (HTLV-I) encodes a phosphorylated nuclear protein of relative molecular mass 27,000 which is required for viral replication[1–3]. The Rex protein acts by promoting the cytoplasmic expression of the incompletely spliced viral messenger RNAs that encode the virion structural proteins[4,5]. To identify the biologically important peptide domains within Rex, we introduced a series of mutations throughout its sequence. Two distinct classes of mutations lacking Rex biological activity were identified. One class corresponds to *trans*-dominant repressors as they inhibit the function of the wild-type Rex protein. The second class of mutants, in contrast, are recessive negative, rather than dominant negative, as they are not appropriately targeted to the cell nucleus. These results indicate the presence of at least two functionally distinct domains within the Rex protein, one involved in protein localization and a second involved in effector function. The *trans*-dominant Rex mutants may represent a promising new class of anti-viral agents.

Oligonucleotide-directed mutagenesis was employed to alter the primary sequence of the *rex* gene at 18 discrete sites (Fig. 1*a*). The boxed amino acids were replaced with a dipeptide, aspartic acid–leucine, by insertion of an in-frame oligonucleotide duplex that contains a diagnostic *Bgl*II restriction site. Each of these *rex* mutants were then inserted into the pBC12/CMV eucaryotic expression vector[6] and the mutations verified by DNA sequencing. For functional analysis, a Rex-responsive reporter plasmid, pgTAX-LTR, was prepared (Fig. 1*b*). The pgTAX-LTR vector contains the two protein-coding exons of the *tax* gene separated by the HTLV-I *env* gene and the complete HTLV-I 3′ long terminal repeat (LTR). Contained within this 3′ LTR is a *cis*-acting Rex response element (RexRE) which seems to correspond to a complex RNA stem–loop structure[5,7]. Either direct or indirect binding of Rex to this RexRE probably underlies its post-transcriptional mechanism of action. The pgTAX-LTR vector does not itself produce Rex, because of a mutation introduced at the Rex initiator codon. In the absence of Rex, pgTAX-LTR produces the Tax protein, reflecting the translation of the spliced HTLV-I mRNA species that lacks virtually all of the *env* sequences. When pgTAX-LTR is co-transfected with the *rex* expression plasmid, pREX, however, synthesis of the HTLV-I Env protein is activated. This protein (relative molecular mass 68,000 (68K)) is readily identified by immunoprecipitation with the anti-HTLV-I envelope monoclonal antibody, 0.5-alpha (ref. 8) (Fig. 2*a*; lane 1). In contrast, no HTLV-I Env protein is detected when pREX is replaced by either pREV (lane 2) or pCMV-IL-2 (lane 3), which encode the HIV-1 Rev protein and human interleukin-2 polypeptide respectively. Also, no Env protein is identified when cells are transfected with pREX in the absence of pgTAX-LTR (lane 4). HTLV-I Env expression by pgTAX-LTR, therefore, is specifically induced in the presence of the wild-type Rex protein.

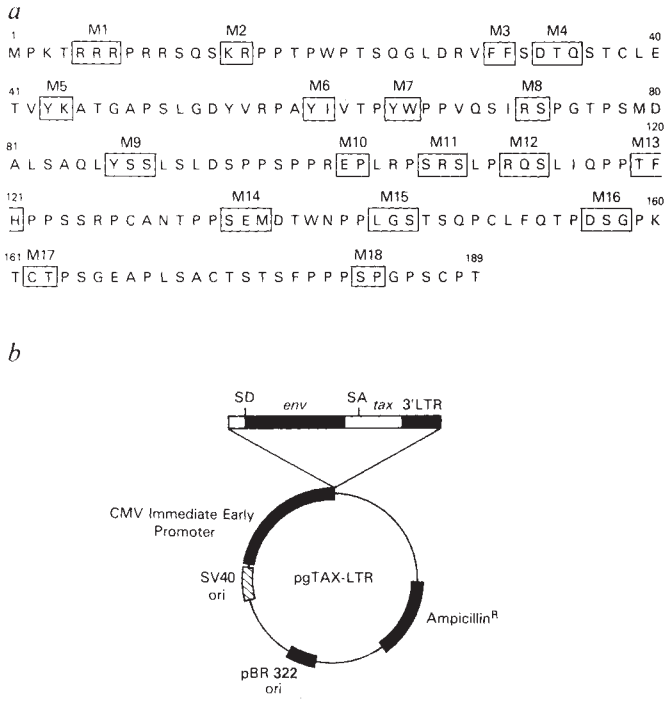

FIG. 1 *a*, Amino-acid sequence of the HTLV-I Rex protein and location of the 18 point mutations introduced. Using oligonucleotide-directed mutagenesis in the M13 bacteriophage[21], nucleotides encoding each of the boxed amino acids were removed and replaced in frame by an oligonucleotide encoding the aspartic acid–leucine dipeptide. Each mutant was subsequently inserted into the pBC12/CMV expression vector and the presence of the mutation confirmed by DNA sequencing of the double-stranded supercoiled templates. *b*, Structure of the Rex-responsive pgTAX-LTR expression vector. The 3′ end of the CR-1 HTLV-I provirus (a gift from Dr Flossie Wong-Staal) from the *Hin*dIII site at map position 5,013 (ref. 22) through to the 3′ LTR, was inserted into the pBC12/CMV expression vector. This fragment contains the two coding exons for Tax (white boxes), the complete *env* gene, and the entire 3′ LTR, including the Rex response element[5]. Although this vector contains the coding region for Rex, it does not synthesize this protein because of the introduction of a mutation at the *Sph*I site, which is coincident with the Rex translation initiation codon[14]. Expression of these HTLV-I sequences is promoted by the immediate early region of the human cytomegalovirus (CMV) and additional polyadenylation sequences provided by the 3′ region of the rat pre-proinsulin gene[6]. This vector produces Env only in the presence of Rex, but Tax is synthesized in the presence or absence of Rex.