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The use of the rat LAP LCR and promoter for the high-level constitutive expression of K^+ channel cDNAs in a rat liver cell line

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Abstract Locus control regions (LCRs) are *cis*-acting elements that confer position-independent and copy-number-dependent expression upon associated genes in transgenic mice. Here we show the second example of the use of an LCR (the rat LAP LCR) in a stable expression vector system, used here in conjunction with the rat liver (NRLM) cell line. Non-transfected NRLM cells are electrically silent and highly suitable for patch clamp electrophysiology. We report reliable constitutive expression from two different K^+ channel cDNAs; the voltage-gated rat clone Kv3.4 and the inward rectifier mouse clone Kir2.1. We further show that constitutive expression levels are stable for at least 8 weeks from initial recording.

Key words: Locus control region; Liver activating protein; K^+ channel, voltage-gated; Inward rectifier; Expression system

1. Introduction

Locus control regions (LCRs) are *cis*-acting elements that confer position-independent expression upon their associated genes and gene clusters in the genome. LCRs are further characterised by the presence of nuclease-sensitive domains. For example, the extensively characterised LCR of the human β-globin gene cluster is delineated by a group of four nuclease-sensitive domains located upstream of the cluster [1–3]. These domains are rich in transcription factor binding sites, and a possible role of the LCR is to keep associated promoters free of histones and allow transcription initiation to occur unimpeded [4].

Presently, only one eukaryotic expression vector system employing LCR sequences has been reported. This, the LCR/MEL system [5], uses sequences from the human β-globin LCR upstream of a β-globin gene backbone where heterologous cDNAs can be inserted in the expression vector. Following stable transfection into mouse erythroleukaemia (MEL) cells and subsequent induction to differentiation, high levels of heterologous protein can be produced. The system has been routinely used in our laboratory for the stable and functional expression of K^+ channel cDNAs and genes [6–8].

Recently, a novel LCR has been identified upstream of the rat gene coding for the liver-enriched transcriptional activator protein (LAP) [9], which binds to albumin D promoter elements [10]. This 2.8 kb region has been used to direct position-independent and copy number dependent expression in transgenic mouse livers [9]. LAP mRNA has also shown to accumulate at high levels in lung, less in spleen and kidney and at much lower levels in testis and brain [9].

To try to meet the need for a high-level, constitutive, stable expression system for ion channels to allow easier coordination of expression and analysis by electrophysiology, we have taken the 2.8 kb LAP LCR region along with the LAP promoter and employed them in a novel expression vector. In the following report we describe stable, high-level, constitutive expression of K^+ channels in a rat liver epithelial cell line under control of the LAP LCR and promoter.

2. Materials and methods

2.1. Production of the LAP expression vector pCLAPE6 and Kir2.1 and Kv3.4 expression constructs

Construction of the LAP LCR expression vector pCLAPE6 was based upon sequences from the vector pCDNA3 (Invitrogen) and LAP LCR and promoter sequences from the construct S1LAP (a gift from Prof. U. Schibler, Geneva, Switzerland). Fig. 1 shows the construction of pCLAPE6 (see also Table 1).

Two K^+ channel expression constructs were made. The inward rectifier mIRK1 (Kir2.1) coding sequence [11] was isolated on an *Eco*RI-*Xho*I fragment from a PCR derived clone [7] and cloned between the same sites of pCLAPE6 (Fig. 2). The rat Raw3 (Kv3.4) voltage-gated channel cDNA [12] (a kind gift from Prof. Olaf Pongs, Hamburg, Germany) was isolated on an *Asp*718-*Not*I fragment and cloned between the same sites of pCLAPE6.

Adherent normal rat liver epithelial (NRLM) cells were a gift from Dr. T. Gant (Leicester, UK). The NRLM cell line was established from a 10-day-old male Fischer (F344) rat [13].

2.2. Tissue culture and transfections

NRLM cells were grown in Williams E medium (Gibco BRL) supplemented with 10% fetal calf serum and GlutamaxI (Gibco BRL) and resuspended by the addition of 0.2 g/l EDTA in PBS prior to transfection. Expression constructs were linearised with *Sca*I and transfected by electroporation as described previously [6] with the following changes. Firstly, following electroporation, cells were diluted in fresh culture medium to 1×10^3–1×10^4/ml; secondly, G418 (0.8 mg/ml) was used for selection; and thirdly, adherent colonies were isolated using cloning cylinders (Sigma) and EDTA (0.2 g/l) in PBS. Individual colonies were grown on and finally resuspended with EDTA to detach and dissociate individual NRLM cells prior to their use for recording.

2.3. Electrophysiological recordings and data analysis

Whole-cell currents were recorded using an Axopatch 200 A amplifier. Current records in response to voltage steps were filtered at 5 kHz (−3 dB, 8-pole Bessel), digitized at 10 kHz using a TL-1 Labmaster interface (Axon Instruments), and analysed on a 486 computer. A suite of programs developed using the AxoBASIC library [14] was used both to apply voltage clamp command potentials and for analysis of whole-cell currents. Patch pipettes were pulled from thin-walled borosilicate tubing (o.d. 1.5 mm; Clarke Electromedical, UK) and fire polished. Their resistance when filled with pipette solution was in the range 5–10 MΩ. The pipette-filling (intracellular) solution contained (mM): KCl, 140; $MgCl_2$, 1; EGTA, 10; HEPES, 10; pH adjusted to 7.2 at 22°C. Extracellular solutions contained (mM):

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KCl, 70 or 5; NaCl, 70 or 135; $CaCl_2$, 2; $MgCl_2$, 2; HEPES 10; pH adjusted to 7.3 at 22°C.

Analogue means were used to cancel capacity transients as far as possible. Series resistance (R_s) was 11.2 ± 1.0 MΩ (n = 40). Up to 80% compensation for series resistance was used. Experiments were performed at room temperature 18–25°C.

3. Results

Owing to the high levels of LAP mRNA accumulation in liver, initial experiments were performed to characterise the suitability of a number of liver cell lines for patch clamp studies. The NRLM cell line was chosen for transfection experiments owing to the absence of any observed large native voltage-activated currents (Fig. 3A). The NRLM cells used in this study had a mean membrane capacitance of 21.4 ± 1.8 pF (n = 40). Fig. 3 shows the results obtained after transfecting NRLM cells with the pCLAPE6/Kir2.1 expression construct.

3.1. Functional expression of Kir2.1

Membrane currents were recorded from single NRLM cells

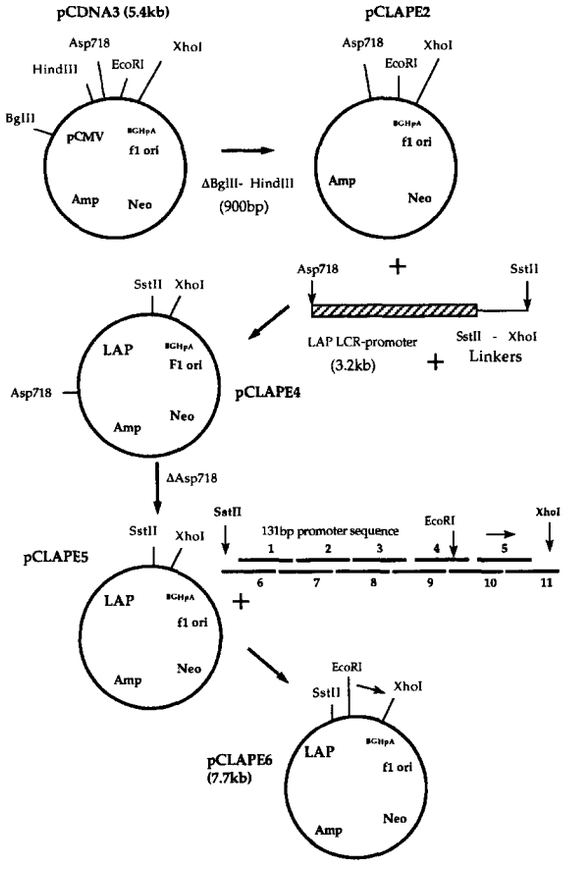

Fig. 1. Production of pCLAPE6. pCDNA3 was cut with *Bgl*II and *Hin*dIII to remove the CMV enhancer and promoter sequences and these two sites filled in and religated to give pCLAPE2. Next, an *Asp*718-*Sst*II fragment containing the full LAP LCR and main promoter sequences was ligated into the *Asp*718 and *Xho*I sites of pCLAPE2 to give pCLAPE4. The *Asp*718 site of pCLAPE4 was then removed by digestion with *Asp*718, filling in and religation to give pCLAPE5. Finally, 131 bp of missing 3′ promoter sequence (to position −40 to the ATG start codon) and a set of unique restriction sites were introduced on a set of 11 overlapping oligonucleotides (Table 1) of which the internal ones were 5′ phosphorylated; all were annealed and then ligated between the *Sst*II and *Xho*I sites of pCLAPE5 to give pCLAPE6 (Fig. 2).

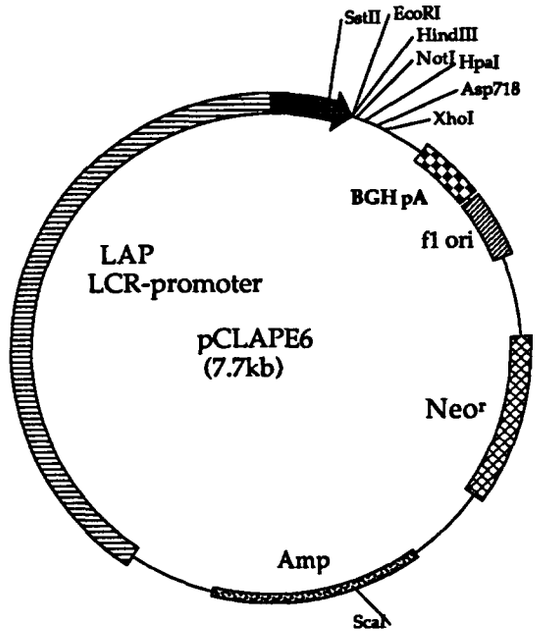

Fig. 2. pCLAPE6. The main features of pCLAPE6 are the LAP LCR and promoter sequences, ampicillin resistance gene (Amp.), neomycin resistance gene (Neo^r), f1 origin of replication (f1 ori), bovine growth hormone polyA signal (BGH pA) and unique restriction enzyme sites for inserting heterologous cDNAs (*Eco*RI, *Hin*dIII, *Not*I, *Hpa*I, *Asp*718 and *Xho*I) and the unique *Sca*I site for linearising expression constructs prior to transfection.

using the conventional whole cell clamp technique and in response to voltage steps from a holding potential of −17 mV (the K^+ equilibrium potential, E_K) to test potentials ranging between −120 and +60 mV, in 10 mV increments. The extracellular and intracellular K^+ concentrations were 70 and 140 mM respectively. Under these conditions, no significant whole cell currents were detected in non-transfected cells (Fig. 3A). In contrast, substantial inward currents were recorded from transfected NRLM cells at membrane potentials negative to E_K, while much smaller outward currents were recorded at potentials positive to E_K (Fig. 3B). This is consistent with the expression of the Kir2.1 gene to produce a homotetrameric Kir2.1 channel [7,15]. The current-voltage relation for this cell is illustrated in Fig. 3C, and shows pronounced inward rectification. The current amplitudes shown in Fig. 3C were divided by the K^+ driving force to obtain the chord conductance ($g_K = I/(V-E_K)$). The normalized conductance-voltage relation is shown in Fig. 3D and can be fit with a Boltzmann equation of the form:

$$g_K/g_{K(max)} = \{1 + \exp[-(V-V_{0.5})/k]\}^{-1}$$

where $V_{0.5}$ gives the voltage at which $g_K/g_{K(max)}$ is 0.5, and k (mV) is a steepness factor indicating the voltage change for an e-fold increase in conductance, at very low conductance. Mean values for $V_{0.5}$ and k were found to be −31.9 ± 0.8 mV and 10.1 ± 0.6 mV (n = 10) respectively. These values are similar with a $V_{0.5}$ value of −26 mV and a k of −10.4 mV found for wild-type Kir2.1 channels stably expressed in MEL cells [7].

3.2. High levels of expression

A large fraction of any given colony of transfected cells expressed inwardly rectifying whole cell currents. For exam-

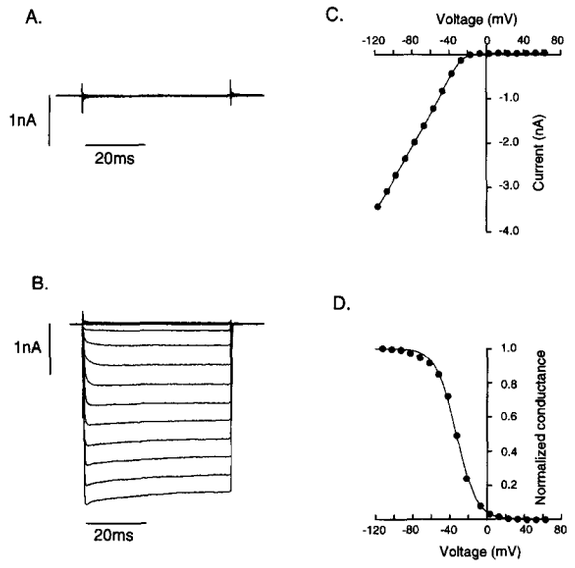

Fig. 3. Expression of Kir2.1 in NRLM cells. Whole-cell currents recorded from non-transfected NRLM cells (A) and NRLM cells transfected with the pCLAPE6/Kir 2.1 expression construct (B) in response to voltage steps from a holding potential of −17 mV (E_K) to test potentials from −120 to +60 mV in 10 mV increments. Extracellular and intracellular K^+ concentrations were 70 and 140 mM respectively. C: Current voltage relation for cell shown in B. D: Voltage dependence of the normalised conductance for cell shown in B, data points (filled circles) fitted with a Boltzmann equation (solid line). $V_{0.5}$ value = −33.1 mV, k value = 10.6 mV.

ple, in one colony, out of nine cells from which whole cell currents were recorded, eight gave inwardly rectifying currents. The amplitude of the inward currents, measured at −100 mV, ranged from 0.25 to 3.5 nA with a mean of 1.6 ± 0.4 nA (n = 8). This value corresponds to a current density of 76.3 ± 16.2 pA/pF. Furthermore, high fractions of colonies expressing Kir2.1 were seen in seven separate G418 resistant Kir2.1 expressing cell lines (Fig. 4).

3.3. *Functional expression of voltage-activated Kv3.4 K^+ channels*

To investigate further the viability of the LAP LCR expression system, NRLM cells were stably transfected with a pCLAPE6/Kv3.4 expression construct. Non-transfected NRLM cells again displayed no voltage-activated currents in response to a 100 ms voltage step from a holding potential of −80 mV to potentials between −60 and +60 mV (Fig. 5A). In transfected cells, transient outward currents were evoked when the membrane potential was stepped positive to −30 mV (Fig. 5B). The activated current decayed rapidly during the 100 ms depolarizing pulse as expected for the A-type Kv3.4 channel. The current-voltage relation for Kv3.4 is shown in Fig. 5C.

Fig. 5D (filled circles) shows the voltage dependence of the normalized peak conductance. The data points can be fit with a single Boltzmann equation to obtain the voltage of the half-maximal activation, $V_{0.5}$ and the steepness factor, k. For six cells, the mean value of $V_{0.5}$ was found to be 14.8 ± 1.5 mV, and the mean value for k, 10.9 ± 0.3 mV. Expression of Kv3.4 cRNA in *Xenopus laevis* oocytes produces A-type K^+ channels with a $V_{0.5}$ of 14 mV and a k value of 9.7 mV [12]. The voltage dependence of inactivation is also shown in Fig. 5D (open circles). This relationship was determined by stepping to +30 mV from different holding potentials, ranging from −100 to +20 mV in 10 mV increments. The results are plotted as the fraction of the current elicited by stepping from a holding potential of −100 mV. The results were fitted with a single Boltzmann from which the value for half-inactivation was found to be −26.7 ± 2.7 mV, with a steepness factor of −12.6 ± 1.2 mV (n=4). Kv3.4 channels expressed in *Xenopus laevis* oocytes have been shown to half inactivate at −29.7 mV, with a steepness factor of 12.2 mV [12].

The time constants of inactivation varied considerably from cell to cell. The fastest decay measured at +50 mV was 18 ms, and the slowest 227 ms. This wide scatter tends to be a characteristic feature of cloned A-type K^+ channels [12,16,17] and may occur as a direct consequence of the regulation of fast inactivation by cysteine oxidation [17]. Consistent with this, we found that the inclusion of reducing agents such as dithiothreitol (10 mM) or reduced glutathione (10 mM) in the patch pipette restored fast inactivation to the majority of cells (data not shown).

The potential for long-term expression was also investigated using single Kir2.1 and Kv3.4 G418-resistant expressing lines. We have observed continual high levels of whole-cell Kir2.1 and Kv3.4 currents from these clonal lines for 8 weeks (approximately 66 passages) following initial colony isolation.

4. Discussion

This paper reports the first example of the use of an LCR for constitutive expression of functional K^+ channel cDNAs. We have demonstrated the expression of members of two different classes of K^+ channel, the inward rectifier type Kir2.1 and the transient A-type Kv3.4, both displaying similar

Table 1
Sequences of the 11 overlapping oligonucleotides encoding 131 base pairs of the 3′ LAP promoter sequence and new unique restriction enzyme sites

Oligonucleotide	Sequence 5′-3′
1	GGCCGGGCAATGACGCGCACCGACCCGGCGGCG
2	GGGCGGCGGGAGGGGCCCCGGCGTGACGCAGCC
3	CGTTGCCAGGCGCCGCCTTATAAACCTCCGCTC
4	GGCCGCCGCCGAGCCGAGTCCGAGCCGCGCAG
5	AATTCCGCCCAAGCTTGGGCGGCCGCGTTAACGGGGTACCCCC
6	CGTCATTGCCCGGCCGC
7	CCCCTCCCGCCGCCCCGCCGCCGGGTCGGTGCG
8	CGGCGCCTGGCAACGGGCTGCGTCACGCCGGGG
9	GCTCGGCGGCGGCCGAGCGGAGGTTTATAAGG
10	AAGCTTGGGCGGAATTCTGCGCGGCTCGGACTCG
11	TCGAGGGGGTACCCCGTTAACGCGGCCGCC

biophysical properties to those reported following expression in *Xenopus* oocytes.

One of the main advantages of using LCRs in expression vectors is in overcoming the problems of integration site 'position effects' for stable expression. In the absence of an LCR, the chromatin integration site of an expression construct will have a dramatic effect on the level of transcription of the associated cDNA or gene [18]. LCRs overcome these position effects and provide a powerful tool for the guaranteed high level expression of stably integrated expression constructs. Analysis of whole cell currents from single cells of seven pCLAPE6-Kir2.1, G418-resistant, stably transfected colonies showed high numbers of functional channels in most of the analysed cells (Fig. 4). This observation supports the position independent function of the LCR.

Another strong feature of this expression strategy is the constitutive nature of the expression. We have shown that expression levels are high from initial G418 selection and have observed continual high levels of expression from a high fraction of cells from one pCLAPE6/Kv3.4 and one pCLAPE6/Kir2.1 cell line for at least 8 weeks with continual culture. For long-term analysis and expression protocols, cells can be easily harvested for liquid nitrogen storage at an early passage stage, and fresh low passage cell aliquots thawed at regular intervals. The constitutive nature of expression has also allowed us to propagate a large number of NRLM cell lines harbouring different K^+ channel constructs with much easier coordination for patch clamp analysis. This contrasts with the need for an induction step before analysis can be performed in the LCR/MEL system.

The β-globin LCR in the LCR/MEL system is already being used for the expression of a wide range of proteins, including ion-channels and such proteins as type I and II tumor necrosis factor receptors [19] and human calcitonin receptors [20]. Therefore, the use of the LAP LCR based expression system reported here opens up the possibility for the functional and high level expression of other proteins.

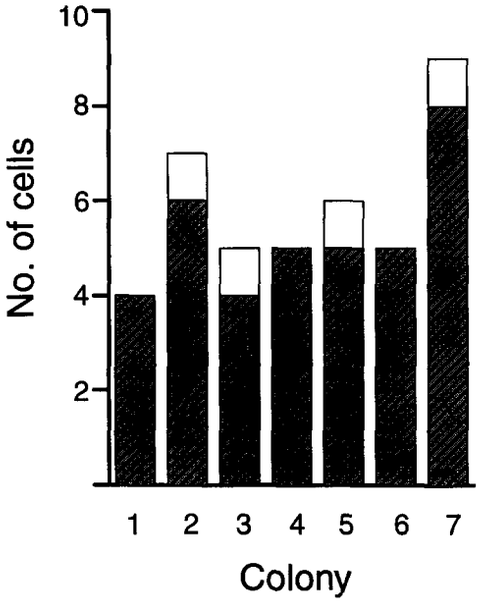

Fig. 4. High levels of expression. Bar chart showing the levels of expression of Kir2.1 across seven G418-resistant colonies. Number of cells from each colony for which whole cell recordings were made is shown by the total height of each bar, the number of cells from this total expressing Kir2.1 (>100pA inward current at −100 mV) is shown by the shaded area.

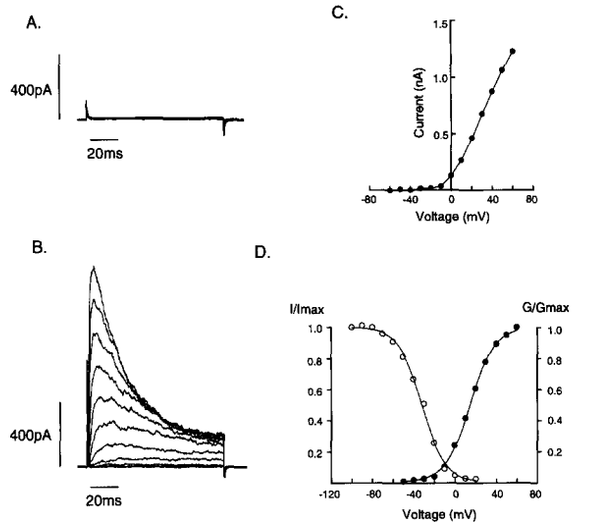

Fig. 5. Expression of Kv3.4 in NRLM cells. Whole-cell currents recorded from (A) non-transfected NRLM cells and (B) NRLM cells transfected with the pCLAPE6/Kv3.4 expression construct in response to 100 ms depolarising voltage steps from a holding potential of −80 mV to potentials between −60 and +60 mV. Extracellular and intracellular K^+ concentrations were 5 and 140 mM respectively. C: Current-voltage relation for cell shown in B. D: Voltage dependence of conductance (filled circles) and steady-state inactivation (open circles). The data points have been fitted with single Boltzmann equations to give values of $V_{0.5} = -30$ mV and $k = 11$ mV for the inactivation curve.

The LAP expression system may permit expression in cell lines derived from other tissues than liver due to the identification of LAP mRNA in lung, spleen, kidney, testis and brain, expression in cell lines derived from these tissues may be possible.

The use of the LAP LCR for the high level constitutive expression of K^+ channel cDNAs shows progress in heterologous expression technology. The use of a simple plasmid vector for inserting cDNAs and a simple transfection protocol into NRLM cells provides a route to reliable, high level constitutive expression.

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