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Promoter–cDNA-directed heterologous protein expression in *Xenopus laevis* oocytes

[chloramphenicol acetyltransferase/secreted alkaline phosphatase/422(aP2) protein/glucose transporter GLUT1/glucose uptake]

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ABSTRACT **Heterologous proteins can be expressed in *Xenopus laevis* oocytes by cytoplasmic microinjection of mRNA. To circumvent limitations inherent in this approach we investigate direct nuclear injection of strong viral expression vectors to drive transcription and subsequent translation of cDNAs encoding cytoplasmic, secreted, and plasma membrane proteins. After several viral promoters had been tested, the pMT2 vector was found to be a superior expression vector for *X. laevis* oocytes capable of directing expression of high levels of functional heterologous proteins. Typically the amount of protein derived from transcription–translation of the microinjected cDNA accounts for ≈1% of total non-yolk protein. Moreover, the inefficiency usually associated with nuclear injections was overcome by coinjection of pMT2 driving expression of a secreted alkaline phosphatase as an internal control to select positive-expressing oocytes. Using this method, we have successfully expressed high levels of chloramphenicol acetyltransferase, the adipocyte-specific cytosolic 422(aP2) protein, and the membrane-associated glucose transporter GLUT1. The system described should be applicable to a wide variety of proteins for which cDNAs are available. Hence, the cumbersome and often inefficient *in vitro* synthesis of mRNA for studying ion channels, receptors, and transporters as well as for expression cloning in *Xenopus* oocytes should no longer be necessary.**

Xenopus oocytes have been widely used to study protein function and regulation. A broad range of heterologous proteins has been successfully expressed in oocytes by cytoplasmic microinjection of mRNA (1, 2). mRNA encoding a specific protein can be synthesized *in vitro* from the corresponding cDNA and subsequently microinjected. If a cDNA is unavailable, total mRNA may be used. This approach has proven successful for studying ion channels, receptors, and transporters (3, 4); it has also been used for expression cloning of proteins the functions of which can be readily measured in oocytes (5, 6). Disadvantages of this method are that *in vitro* preparation of mRNA is time consuming, costly, and limited by the ability to transcribe the cDNA of interest. In addition, the structure and size of the cDNA may preclude use of this method.

To bypass *in vitro* mRNA synthesis cDNA constructs can be directly microinjected into nuclei of intact oocytes (1, 7). A limited number of successful reports using nuclear injection of promoter cDNA have been described (8, 9). Two reasons why this method has not gained widespread use have been the technical difficulty of nuclear injections and low levels of cDNA-derived protein expression. Recently, a system using coinjection of cDNA and vaccinia virus was developed (10). Although this technique proved successful for several cDNAs, the level of protein expression attained was not consistently as high as that achieved by mRNA injection. An additional drawback of the method is the requirement of viral infection.

Therefore, we sought to develop a simple, yet efficient, system for expressing proteins in oocytes. Our approach was to identify an expression vector that upon nuclear injection into oocytes would yield high levels of functional protein. After testing several viral promoters, we selected pMT2 (11, 12) as a superior oocyte expression vector. The inefficiency of nuclear injections, as well as the lack of expression in some oocytes, was overcome by coinjection of a pMT2 expression vector containing the test cDNA along with a pMT2 expression vector containing a secreted alkaline phosphatase cDNA. Thus, secretion of alkaline phosphatase activity into the surrounding medium was used to select positively expressing oocytes. In this paper, we describe a system for expressing high levels of functional proteins in *Xenopus laevis* oocytes by nuclear injection of promoter–cDNA constructs. This method eliminates the need to synthesize mRNA *in vitro* to express proteins in oocytes.

MATERIALS AND METHODS

Recombinant Plasmids. The eukaryotic expression vector pMT2 (Genetics Institute, Cambridge, MA) was linearized with *Eco*RI. After linearization the ends were filled in with Klenow polymerase in the presence of dNTPs. A chloramphenicol acetyltransferase (CAT) cDNA was blunt-end subcloned into this site in the sense orientation. A cDNA corresponding to a secreted form of alkaline phosphatase (SEAP; ref. 13) was subcloned in a similar fashion. pMT3 was created by subcloning a double-stranded oligonucleotide as follows:

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5'     GGCGGCCGCCCGGGTATCGATACCGTCGACCTCGAGGTACCG     3'
3' ACGTCCGCCGGCGGGCCCATAGCTATGGCAGCTGGAGCTCCATGGCTTAA 5'
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containing the restriction sites (*Not* I, *Sma* I, *Cla* I, *Sal* I, *Xho* I, and *Kpn* I) into the *Pst* I and *Eco*RI sites of the pMT2 vector. The *Pst* I and *Eco*RI sites were regenerated. The sequence of this oligonucleotide is based on the multiple cloning site of pBluescript (Stratagene). A full-length 422(aP2) cDNA (14) was subcloned into the *Cla* I and *Xho* I sites of pMT3. A full-length glucose transporter 1 (GLUT1) cDNA (15) was subcloned into the *Not* I and *Cla* I sites of pMT3. Purified plasmid DNA was resuspended in 10 mM Tris·HCl buffer, pH 7.4/1 mM EDTA (TE buffer). DNA was diluted in TE buffer before injection. The plasmid pGEM-4Z/PLAP489 was used for *in vitro* synthesis of SEAP mRNA (16).

Oocyte Isolation and Injection. Adult *X. laevis* females were obtained from Nasco (Fort Atkinson, WI) and maintained as described (17, 18). Lobes of ovary were surgically removed,

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Abbreviations: GLUT1, glucose transporter 1; SEAP, secreted form of alkaline phosphatase; CAT, chloramphenicol acetyltransferase; AdMLP, adenovirus major late promoter.

rinsed with Ca^{2+}-free modified Barth's solution (MBS), and freed of follicle cells with 0.2% collagenase (Sigma, type IA) in Ca^{2+}-free MBS. Oocytes were maintained in complete MBS/2.5 mM sodium pyruvate/penicillin at 100 units/ml/streptomycin at 1 mg/ml (MBS^+) at 18°C for 16–20 hr before injection. Healthy oocytes were visually selected, and nuclear injection of DNA was performed by impaling the animal hemisphere (≈1000 injections per hr). The injection volume (10–30 nl ± 15%) was controlled with an automatic pressure generator (Narishige USA, IM-200). Injected oocytes were incubated for 18 hr at 18°C in MBS^+, and damaged oocytes were discarded.

Alkaline Phosphatase Assay. Oocytes injected with either pMT2–SEAP (nuclear injection) or SEAP mRNA synthesized *in vitro* (cytoplasmic injection) and control oocytes were transferred individually to 96-well flat-bottom culture plates and maintained in 0.2 ml of MBS^+ at 18°C. At the indicated times after injection, alkaline phosphatase activity in the culture medium of each oocyte was assayed (16). The linear reaction rate was determined for each well at 10- to 20-min intervals by measuring absorbance at 405 nm in an automatic plate reader.

CAT Assay. Forty-eight hours after injection oocytes were homogenized in 0.25 M Tris·HCl buffer, pH 7.8, containing a mixture of protease inhibitors (19). The homogenate was subjected to repeated freeze-thawing and mixing and was then centrifuged for 2 min at 16,000 × *g* in a microcentrifuge. The supernatant was heated to 65°C for 15 min and after cooling was centrifuged for 5 min at 16,000 × *g*. The supernatant was assayed for CAT activity (20). Initial experiments were performed on pooled sets of 20 oocytes, whereas individual oocytes were assayed when pMT2–SEAP was coinjected with pMT2–CAT as described in the text.

Hexose Transport Assay. Hexose uptake rate was determined as described (18) with minor modifications. Briefly, individual oocytes were incubated for 30 min at 18°C in 5-ml glass vials containing 0.5 ml of MBS^+. 2-Deoxy-D-[U-^{14}C]glucose (500 nmol; 800,000 cpm) was then added, and incubation at 18°C was continued for an additional 30 min. Hexose uptake was terminated by two rapid washes with 3 ml of ice-cold phosphate-buffered saline (PBS), after which oocytes were lysed in 1 ml of 0.1 M NaOH/0.1% SDS, and cell-associated radioactivity was determined.

Immunoblotting of CAT, 422(aP2), and GLUT1 Proteins. Seventy-two hours after nuclear coinjection of pMT2–SEAP and either pMT3–422(aP2) or pMT3–GLUT1, control (noninjected) oocytes and oocytes positive for SEAP activity were homogenized in ice-cold 50 mM Hepes buffer, pH 7.5/1 mM EDTA/protease inhibitors as in ref. 19. For pMT3–422(aP2)-injected oocytes, homogenates were centrifuged at 4°C for 15 min at 16,000 × *g*. For pMT3–GLUT1-injected oocytes, homogenates were centrifuged at 1000 × *g* for 10 min at 4°C, after which resulting supernatants were centrifuged at 150,000 × *g* for 30 min. Samples of CAT and 422(aP2) cytosolic extracts prepared as described above were mixed with electrophoresis sample buffer containing 6% SDS and 60 mM dithiothreitol. After being boiled for 5 min, samples were submitted to SDS/12.5% PAGE. High-speed pellets from control and pMT3–GLUT1-injected oocytes were resuspended in the Hepes/EDTA buffer and in some cases were treated with peptide N-glycosidase F at 1 unit per 100 μg of protein (Boehringer Mannheim)/15 mM phosphate buffer, pH 7.5/8 mM EDTA/1 mM phenylmethylsulfonyl fluoride/1% Triton X-100 for 48 hr at 37°C. Samples were mixed with electrophoresis sample buffer containing 6% SDS, 7 M urea, and 300 mM dithiothreitol and submitted to SDS/10% PAGE. Proteins were transferred for 12 hr to nitrocellulose filters (Sartorius) after which filters were blocked for 12 hr at 4°C in TTBS (25mM Tris·HCl, pH 7.5/150 mM NaCl/0.05% Tween 20/0.001% Thimerosal)/1% nonfat dry milk. For immunological detection of CAT protein, a purified rabbit antibody (5 Prime → 3 Prime Inc.) was used. For immunological detection of 422(aP2) protein, an affinity-purified rabbit antibody against a 422(aP2) carboxyl-terminal synthetic peptide (21) was used. For immunological detection of GLUT1 protein, polyclonal rabbit anti-human erythrocyte glucose transporter antibodies (22) were used. Blots were developed by the Enhanced ChemiLuminescence method (Amersham) using horseradish peroxidase-conjugated goat anti-rabbit IgG.

RESULTS AND DISCUSSION

Heterologous protein expression in *Xenopus* oocytes is a powerful system for studying protein function and regulation; however, its application is limited by the ability to synthesize high-quality mRNA *in vitro*. The size and structure of the cDNA often limits *in vitro* mRNA synthesis. In addition, the quality of mRNA varies from preparation to preparation. Therefore, we sought to bypass *in vitro* mRNA synthesis by direct nuclear injection of promoter–cDNA constructs. First, we determined whether strong viral promoters could drive expression of a cDNA when injected into the nucleus of *X. laevis* oocytes. To this end we tested the capacities of three different viral promoters to direct expression of the reporter gene CAT. We selected this cDNA because *Xenopus* oocytes do not possess endogenous CAT activity and CAT protein is both readily assayed and relatively stable in animal cells. The Rous sarcoma virus, simian virus 40, and adenovirus major late (AdMLP) promoters were all active in *Xenopus* oocytes (Fig. 1*A*). The AdMLP was substantially more active than the other promoters. Activity depended upon the nuclear injection of vector DNA because neither cytoplasmic injection of the DNA nor nuclear injection of buffer alone resulted in detectable CAT activity (results not shown). The amount of CAT activity generated suggested that significant levels of transcription of the CAT gene and subsequent translation of CAT mRNA had occurred.

The fact that nuclear injection of AdMLP–CAT led to a significant level of CAT activity indicated that the AdMLP is active in *Xenopus* oocytes. Because the AdMLP had been previously subcloned as part of the expression vector pMT2 (11, 12), we subcloned the CAT gene into this vector. In addition to the AdMLP, pMT2 has sequence elements that

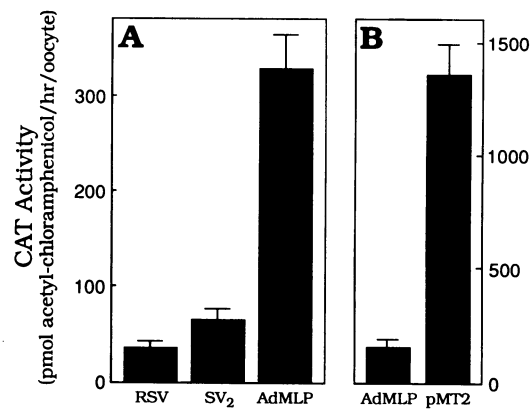

FIG. 1. Effectiveness of selected viral promoters for driving CAT expression in *X. laevis* oocytes. (*A*) Plasmids with the Rous sarcoma virus (RSV), simian virus 40 (SV_2) or AdMLP promoter driving CAT expression were injected (30 ng of DNA per oocyte) into the nuclei of *X. laevis* oocytes. (*B*) Plasmids with either the AdMLP or the viral-based expression vector pMT2 directing CAT expression were injected (0.3 ng of DNA per oocyte) into the oocyte nucleus. In both *A* and *B*, 48 hr after injection three sets of 20 oocytes for each construct were assayed for CAT activity. Results are expressed as means ± SD.

increase mRNA stability and translatability in mammalian cells, but their function in amphibian cells has not been reported. Injection of pMT2–CAT led to 5- to 10-fold greater CAT expression when compared with oocytes injected with AdMLP–CAT (Fig. 1*B*). The magnitude of activity attained suggested that expression from CAT cDNA could be detected in individual oocytes.

To identify oocytes that exhibited promoter–cDNA-directed protein expression, we constructed a pMT2-secreted alkaline phosphatase expression vector. A mutated form of human placental alkaline phosphatase (SEAP) that is secreted into the culture medium has been described (13), and SEAP mRNA synthesized *in vitro* has been used as a coinjected internal standard to identify oocytes that efficiently express injected mRNA (16). Our initial experiments focused on the ability to detect SEAP activity in the medium of individual oocytes. Twenty hours after nuclear injection of pMT2–SEAP, single oocytes were transferred to 96-well microtiter plates. After incubation at 18°C for an additional 24–120 hr, an aliquot of the medium from each oocyte was removed and assayed for alkaline phosphatase activity. Forty-eight hours after nuclear injection, individual oocytes exhibited high SEAP activity that continued to increase up to 96 hr, and by 144 hours had plateaued (Fig. 2). The activity generated from pMT2–SEAP-injected oocytes was ≈1000-fold higher than that achieved by cytoplasmic injection of *in vitro*-synthesized SEAP mRNA.

The level of alkaline phosphatase activity expressed depended upon the quantity of plasmid DNA injected. Activity was evident when 0.03–30 ng of DNA was injected per oocyte; however, the highest activity was obtained when 0.3 ng was injected (Fig. 2). We suggest that the decreased activity seen when higher levels of the expression vector were injected was a result of promoter competition for pMT2-specific transcription factors.

Our results using the SEAP expression vector to identify injected oocytes capable of transcription and translation suggested the possibility of coinjecting pMT2–SEAP with other test pMT2–cDNA vectors. To assess the feasibility of this approach pMT2–SEAP and pMT2–CAT were coinjected. After incubation, SEAP assays were conducted on the medium, and CAT assays were performed on the extract from each oocyte. Table 1 shows that oocytes positive for SEAP activity also exhibited CAT activity, whereas oocytes negative for SEAP activity did not exhibit CAT activity.

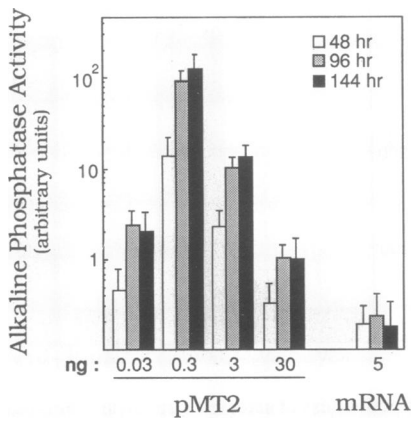

FIG. 2. pMT2-directed expression of secreted alkaline phosphatase from injected *X. laevis* oocytes. Either plasmid pMT2–SEAP was injected (0.03, 0.3, 3, or 30 ng of DNA per oocyte) into the oocyte nucleus, or *in vitro*-synthesized SEAP mRNA was injected (5 ng of RNA per oocyte) into the oocyte cytoplasm. Alkaline phosphatase enzymatic activity in the medium of individual oocytes was assayed at 48, 96, and 144 hr after injection. Activity is expressed as means + SD of at least 10 positive oocytes on a logarithmic scale.

Table 1. SEAP and cytoplasmic CAT activities of *X. laevis* oocytes coinjected with pMT2–SEAP and pMT2–CAT

Oocytes	No.	SEAP activity, arbitrary units	Cytoplasmic CAT activity, nmol of product per hr per oocyte
Negative	22	None	None
Positive	8		
A8		196	12
A10		200	19
B1		248	25
B6		19	13
B7		289	13
B9		416	29
C5		45	7
C9		525	33

pMT2-secreted alkaline phosphatase (SEAP) and pMT2-CAT plasmid DNAs were coinjected (0.3 ng of total DNA per oocyte) into the nuclei of 30 oocytes. Forty-eight hours after injection, activities of SEAP in the medium and cytoplasmic CAT were measured as described. In this table, positive oocytes are indicated by their original location within the 96-well microtiter plate. Correlation coefficient for expression of SEAP and CAT activities was 0.89.

Typically 30–65% of promoter–cDNA-injected oocytes expressed the encoded proteins. The correlation coefficient (r) between SEAP and CAT activities for oocytes scoring positive was 0.89 (Table 1). This high correlation suggested that SEAP activity would be useful not only to identify functional oocytes but also as a predictor of total promoter–cDNA-directed protein expression.

To directly measure CAT protein expression and to rule out the possibility of only a few CAT protein molecules exhibiting high enzymatic activity, immunoblots were performed on oocyte cytoplasmic extracts that tested positive for CAT activity. A very strong signal was elicited with as

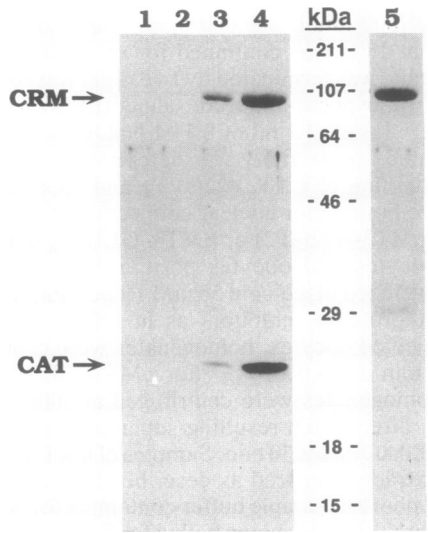

FIG. 3. Immunoblot analysis of CAT protein from nuclear injected *X. laevis* oocytes. Before SDS/12.5% PAGE, cytoplasmic extract from a single SEAP-expressing oocyte (lanes 1–4) prepared 48 hr after nuclear coinjection of pMT2–SEAP and pMT2–CAT expression constructs (0.3 ng of total DNA per oocyte) and an uninjected control oocyte (lane 5) were diluted in sample buffer. Proteins were transferred to nitrocellulose filters and probed with purified rabbit anti-CAT antibody and developed by the Enhanced ChemiLuminescence method. Amount of extract loaded per lane was 0.3, 1.0, 3.0, and 10% of a positive-expressing oocyte for lanes 1–4, respectively, whereas amount of extract loaded for control represented 10% of an uninjected oocyte. Positions of prestained molecular mass markers in kDa, CAT protein as well as nonspecific cross-reacting material (CRM) are indicated.

little as one-tenth of an oocyte (Fig. 3), although a weak but detectable signal was observed when one-hundreth of an oocyte was assayed. Similar results were obtained for SEAP protein secreted into the medium (data not shown). Therefore, the high amount of CAT and SEAP enzymatic activity seen was, indeed, from high levels of protein expression.

To quantitate the absolute level of promoter–cDNA-directed heterologous protein expression, pMT2-SEAP was coinjected with the full-length 422(aP2) cDNA subcloned into pMT3. The pMT3 expression vector is a pMT2 derivative containing the multiple cloning site described. The 422(aP2) cDNA had been cloned and sequenced in this laboratory (14) and shown to encode a cytosolic protein that is an endogenous substrate of the insulin receptor tyrosine kinase in 3T3-L1 adipocytes (23, 24). The availability in this laboratory of native 422(aP2) protein as well as purified anti-422(aP2) antibody made it possible to quantitate its level of expression. Fig. 4 shows that immunoblotting results indicated a signal was elicited with as little as one-tenth of an oocyte; a weaker, but detectable, signal was observed when one-thirtieth of an oocyte was analyzed. No endogenous signal was detected in control oocytes. Quantitation of band intensities by laser scanning densitometry and comparison to a 422(aP2) protein standard curve (1–30 ng) showed that 3 days after nuclear injection of pMT3-422(aP2) ≈50 ng of 422(aP2) protein can be detected per oocyte. This level of expression represents ≈1% of total non-yolk oocyte protein and an estimated 10% of total oocyte protein synthesized during this period.

To further verify the applicability of this system, we extended our studies by expressing a functional membrane protein, the erythrocyte glucose transporter GLUT1. To this end, we coinjected pMT2–SEAP along with pMT3 containing the murine GLUT1 cDNA (15). Seventy-two hours after nuclear coinjection, individual oocytes positive for SEAP activity were selected and assayed for hexose uptake. Injection of pMT3–GLUT1 resulted in a 20- to 50-fold increase of 2-deoxyglucose transport activity over basal activity (Fig. 5*A*). This result was specific for the GLUT1 cDNA because nuclear injection of other cDNAs did not significantly affect rate of sugar uptake. The observed increase in hexose transport activity after nuclear injection of pMT3–GLUT1 was the result of expression of fully processed glucose transporter molecules as confirmed by immunoblot analysis (Fig. 5*B*). Positive-expressing oocytes exhibited a diffuse band (centered at 50 kDa) reminiscent of the heavily glycosylated erythrocyte glucose transporter. Comparison with membranes prepared from 3T3-L1 preadipocytes indicated that the level of expression in one oocyte was equivalent to the amount of GLUT1 in ≈2 × 10^6 3T3-L1 preadipocytes (results not shown). As expected, pretreatment of oocyte membranes containing expressed GLUT1 with peptide N-glycosidase F resulted in a sharper band with a lower molecular mass of ≈35 kDa. These findings indicate that the *Xenopus* oocyte can express and posttranscriptionally modify high levels of a functional membrane protein.

The methodology described here provides a rapid, straightforward technique for expressing high levels of functional proteins in *X. laevis* oocytes. In contrast to the widely used method of cytoplasmic mRNA injection, direct nuclear injection of promoter–cDNA constructs eliminates the problems associated with *in vitro* mRNA synthesis. Proteins that have not been successfully expressed in oocytes because of difficulties in synthesizing mRNA *in vitro* may now be studied. We have been able to detect proteins encoded by cDNAs up to at least 5 kilobases in length (results not shown). Preliminary experiments indicate that this approach can also be used to study adipocyte-specific gene expression (unpublished results). Proteins can be directly expressed from the pMT2 (or pMT3) vector for studying ion channels, receptors, and transporters as well as for expression cloning in *Xenopus* oocytes. In addition, the selection of positively expressing oocytes by coinjection of pMT2–SEAP and subsequently assaying for SEAP activity permits the analysis of individual

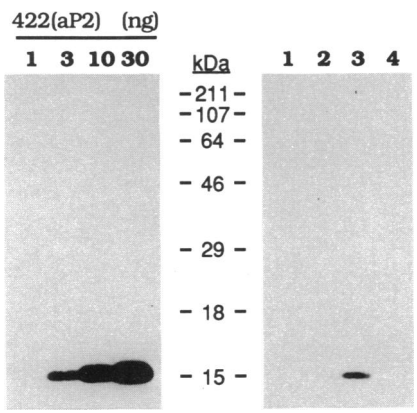

FIG. 4. Quantitative immunoblot analysis of 422(aP2) protein from nuclear-injected *X. laevis* oocytes. Before SDS/12.5% PAGE, cytoplasmic extract from a single SEAP-expressing oocyte (lanes 1–3) prepared 3 days after nuclear coinjection of pMT2-SEAP and pMT3-422(aP2) (0.3 ng of total DNA per oocyte) and an uninjected control oocyte (lane 4) were diluted in sample buffer. To compare levels of specific protein expression, purified 422(aP2) protein (1, 3, 10, and 30 ng) was loaded on the same gel. Proteins were transferred to nitrocellulose filters and processed for immunoblotting. Blots were probed with affinity-purified rabbit antibodies against a 422(aP2) carboxyl-terminal synthetic peptide (21) and developed as described in the legend for Fig. 3. Amount of extract loaded per lane was 1, 3, and 10% of an oocyte for lanes 1, 2, and 3, respectively, whereas amount of extract loaded for control represented 10% of an oocyte (lane 4). Positions of prestained molecular mass markers are indicated. 422(aP2) protein has an apparent molecular mass of 15 kDa.

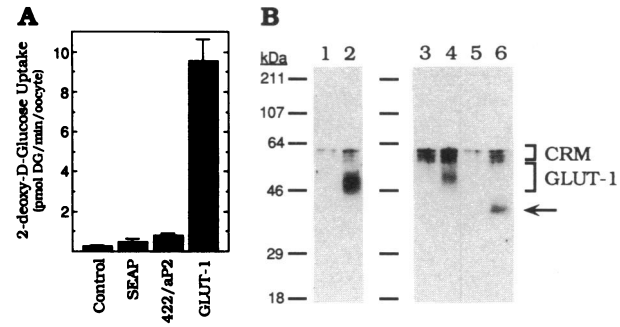

FIG. 5. Hexose transport activity and immunoblot analysis of GLUT1 protein from nuclear-injected *X. laevis* oocytes. (*A*) Three days after nuclear injection of pMT2–SEAP (0.3 ng of DNA per oocyte) or nuclear coinjection of pMT2–SEAP and either pMT3–422(aP2) or pMT3–GLUT1 (0.3 ng of total DNA per oocyte), individual SEAP-expressing oocytes were tested for 2-deoxy-D-glucose (DG) uptake. Results are compared with that obtained with uninjected (control) oocytes and are the means + SD of 5–10 oocytes. (*B*) Membranes were prepared from uninjected control oocyte (lanes 1, 3, and 5) and from SEAP-expressing oocytes (lanes 2, 4, and 6) 3 days after nuclear coinjection of pMT2–SEAP and pMT3–GLUT1 (0.3 ng of total DNA per oocyte). Samples were pretreated for 48 hr at 37°C in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of peptide N-glycosidase F. Before SDS/10% PAGE, all samples were diluted in sample buffer containing urea. Proteins were transferred to nitrocellulose filters and processed for immunoblotting. Blots were probed with polyclonal rabbit anti-human erythrocyte glucose transporter antibodies (22) and developed as described in the legend for Fig. 3. Amount of membranes loaded per lane was equivalent to one oocyte. Positions of prestained molecular mass markers, nonspecific cross-reacting material (CRM), GLUT1, and unglycosylated GLUT1 (arrow) are indicated.

oocytes, thereby eliminating the need for pooling oocytes for statistical purposes.

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