Cloning of a Lymphoid-Specific cDNA Encoding a Protein Binding the Regulatory Octamer DNA Motif

LOUIS M. STAUDT,* ROGER G. CLERC, HARINDER SINGH, JONATHAN H. LEBOWITZ, PHILLIP A. SHARP, DAVID BALTIMORE

An octamer DNA sequence plays a critical role in directing transcription of immunoglobulin genes in B lymphocytes. A new technique of direct binding of radioactive DNA was used to screen a complementary DNA expression library from the BJAB cell line in λgt11 phage to derive molecular cDNA clones representing a putative B lymphocyte–specific octamer binding protein. The plaques were screened with DNA containing four copies of the octamer sequence and positive phage recombinants were identified. The fusion protein produced on inducing a lysogen of one phage bound to a monomeric octamer probe. The cDNA insert from this phage hybridized to messenger RNA found in B lymphocytes, but not in most other cells. Thus, this cDNA derives from a gene (*oct*-2) that specifies an octamer binding protein expressed preferentially in B lymphocytes, proving that, for at least one gene, a cell-specific transcription factor exists and its amount is controlled through messenger RNA availability.

AN "OCTAMER" SEQUENCE MOTIF has been implicated in the lymphoid-specific transcriptional activity of immunoglobulin promoters (*1*) and in the function of the immunoglobulin heavy chain and simian virus 40 (SV40) enhancers in lymphoid cells (*2, 3*). Paradoxically, the same motif is also a functional component of non–tissue-specific promoter and enhancer sequences (*4*). A DNA binding assay based on a shift of electrophoretic mobility has been used to define a widely occurring octamer binding protein (NF-A1) (*5, 6*) and a lymphoid-specific octamer binding protein (NF-A2) (*3, 7, 8*), as well as a testis-specific octamer binding protein (*9*). The various octamer binding proteins could either represent different modification states of a single protein or different proteins encoded by distinct genes. By screening the phage plaques of a λgt11 expression library with a DNA binding site probe (*10*) containing the octamer motif, we have cloned a partial cDNA that encodes an octamer binding protein, which is expressed predominantly in lymphoid cells. This suggests that the distinct regulatory functions of the octamer motif are mediated by octamer binding proteins encoded by distinct genes.

In an effort to clone the gene encoding the lymphoid-specific octamer binding protein, NF-A2, we constructed a randomly primed cDNA library in λgt11 using cytoplasmic poly(A)-containing mRNA from a human B cell lymphoma cell line, BJAB (*11*). We had previously observed that this cell line contained a particularly large amount of NF-A2 (*12*). By randomly priming cDNA synthesis, we expected to obtain recombinant phage encoding the octamer motif binding domain even if that domain was encoded by the 5′ region of a long mRNA. This library was screened by a modification of the method of Singh *et al.* (*10, 11*), with a ^{32}P-labeled DNA probe consisting of four copies of a 26-bp oligonucleotide containing the octamer motif derived from the variable region of a $V_\kappa 41$ immunoglobulin light chain promoter. To minimize detection of phage recombinants expressing proteins that bind nonspecifically to either double-stranded or single-stranded DNA, we included denatured, unlabeled calf thymus DNA in the binding buffer. From a primary screen of 450,000 phage plaques, three recombinants that bound this tetramer probe were isolated. Two of these phage, numbered 3 and 5, were detected specifically with the tetramer probe, but not with probes that lacked the octamer motif. The binding properties of these two phage were examined by the in situ assay with monomer and multimer probes. With a constant input of radioactive probe, these phage bound probes containing a single copy of the kappa (κ) promoter octamer motif less well than they bound the tetramer probe (Fig. 1). Even when four times as much monomer probe was used as tetramer probe, the monomer bound less well but the effect was greater with phage 5 than with phage 3. This suggests that multisite probes can simultaneously bind to several proteins on the membrane, thereby enhancing detection of recombinants encoding low-affinity DNA binding proteins (*10*). The stronger signal produced by the multimer probe with phage 5 (Fig. 1) was a consequence of its higher level of fusion protein production (as shown later), whereas its lower signal with the monomer probe reflected an apparently lower DNA binding affinity in vitro than that of the protein produced by phage 3.

The specificity of the DNA binding proteins encoded by the recombinant phages was investigated with extracts of induced phage lysogens. Lysogen extracts from both phages bound to the tetramer probe in a gel electrophoresis DNA binding assay, whereas lysogen extracts from nonrecombinant λgt11 showed no binding to this probe. Only the phage 3 extract bound strongly to the monomeric κ promoter probe. Because the inserts of phage 3 and phage 5 (1.2 and 0.45 kb, respectively) cross-hybridized, we chose phage 3 for further analysis.

Phage 3 encoded an octamer binding protein, as demonstrated by a competition DNA binding assay in which the lysogen extract was bound to the κ promoter probe in the presence of competing unlabeled DNA fragments containing either the wild-type or mutant octamer motifs (Fig. 2A). DNA containing the wild-type octamer sequence competed efficiently for binding, but DNA in which the octamer motif contained point mutations either did not compete or competed less well than the wild-type se-

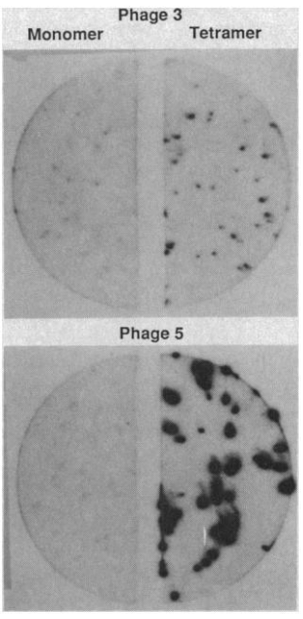

Fig. 1. Binding of octamer-containing monomer and tetramer DNA probes to phage 3 and phage 5 protein replica filters. Phage 3 and phage 5 were plated and protein replica filters were screened with either a ^{32}P-labeled monomer probe derived from the $V_\kappa 41$ promoter [Pvu II–Eco RI octamer-containing fragment from pSPlgVk (*5*)] or a tetramer probe containing four copies, in direct orientation, of a 26-bp octamer-containing oligonucleotide (*11*) derived from the $V_\kappa 41$ promoter (each at 1×10^6 cpm/ml).

L. M. Staudt and D. Baltimore, Whitehead Institute for Biomedical Research, Cambridge, MA 02142, and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.
R. G. Clerc, H. Singh, J. H. LeBowitz, P. A. Sharp, Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

*Present address: Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

quence. In fact, the two mutants that showed partial competition for the binding of the lysogen protein, TCATTTG*A*AT and *A*GATTTGCAT (Fig. 2A), were the only mutants that somewhat competed for the binding of NF-A1 and NF-A2 in a WEHI231 nuclear extract (*7*).

The phage-encoded octamer binding protein was further compared to NF-A1 and NF-A2 by a methylation interference assay (Fig. 2B). The interference pattern obtained with the lysogen extract was centered over the octamer motif and was similar to the patterns of NF-A1 and NF-A2 from a BJAB or WEHI 231 nuclear extract (*7*). The minor differences between the patterns of the phage-encoded and the mammalian proteins probably reflect slight differences in affinity or specificity of the recombinant protein either because of its incomplete structure or its fusion to β-galactosidase.

To examine whether the phage-encoded β-galactosidase fusion protein in lysogen extracts was the octamer binding protein, we subjected these extracts to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred the separated proteins to nitrocellulose filters. After a denaturation-renaturation procedure (*10*), the filters were treated with either the ^{32}P-labeled octamer-containing tetramer probe (OCTA) or a nonspecific DNA probe (pUC) (Fig. 3A). The same filters were subsequently treated with an antiserum specific for β-galactosidase (Fig. 3B). The OCTA probe specifically bound to the β-galactosidase fusion proteins of phage 3 and phage 5 to a much greater extent than the pUC probe. The apparent molecular size of the largest fusion proteins of phage 3 and phage 5 (a pair at 150 kD and one at 135 kD, respectively) suggests that the cDNA inserts were completely translated. Proteolysis probably accounted for the multiple fusion proteins detected in each lysogen extract.

The phage 3 cDNA insert, which defines what we refer to as the *oct*-2 gene, was used to probe a DNA blot of human and mouse genomic DNAs that had been digested with various restriction enzymes. Each digest showed a simple hybridization pattern, consistent with *oct*-2 being a single copy gene that is highly conserved. Rearrangement or amplification of the gene was not detected in a survey of eight lymphoid and nonlymphoid cell lines including BJAB.

Expression of the *oct*-2 gene was assessed by RNA blot analysis of mRNA from 13 lymphoid and nonlymphoid cell lines. This gene was almost exclusively expressed in lymphoid cells (Fig. 4, A and C). All five B lymphoma cell lines (Fig. 4A, lanes 2, 3, 8, 9, and 10, and Fig. 4C, lane 1), including pre-B and mature B cells, and one of three T lymphoma cell lines (Fig. 4A, lanes 7, 11, and 13) expressed a family of six mRNAs. Of the five nonlymphoid cell lines tested, only a glioma cell line, U1242, showed detectable expression of this gene (Fig. 4A, lane 5). Even at low stringency we could not detect any mRNA in nonlymphoid cell lines that might encode a more widely occurring protein such as NF-A1. The various mRNAs, estimated to be 7.2, 5.8, 5.4, 3.7, 3.1, and 1.3 kb in length, were expressed in varying relative amounts in the positive cell lines. Whether these mRNAs represent alternative mRNA splicing or specific mRNA degradation products remains to be determined. Highly purified preparations of NF-A2 consist of three or more major polypeptides clustered around 60 kD (*13, 14*), possibly due to the heterogeneity of *oct*-2 mRNAs.

Previously, we and others (*3, 7, 8*) showed that the octamer binding protein NF-A2 varied considerably in abundance among lymphoid cell lines. The relation between mRNA complementary to the *oct*-2 gene and NF-A2 was assayed by the gel-electrophoresis DNA binding assay (Fig. 4B). The cell line, BJAB, which expressed the largest amount of mRNA (Fig. 4A, lanes 9 and 10) showed the largest amount of NF-A2 [Fig. 4B, lane 9; the upper intense bands represent larger, undefined

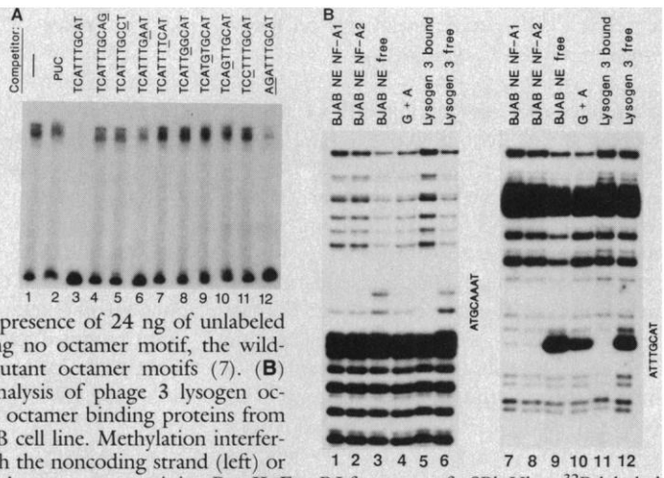

Fig. 2. Specificity of the octamer binding activity in extracts of lysogens of phage 3. (**A**) Phage 3 lysogen extracts (*10*) were assayed in a mobility-shift DNA binding assay (*7*) with the octamer-containing Pvu II–Eco RI fragment from pSPlgVk (*7*) as the ^{32}P-labeled probe. Binding reactions were carried out in the absence or presence of 24 ng of unlabeled competitor DNA containing no octamer motif, the wild-type octamer motif, or mutant octamer motifs (*7*). (**B**) Methylation interference analysis of phage 3 lysogen octamer binding proteins and octamer binding proteins from nuclear extracts of the BJAB cell line. Methylation interference was performed (*7*) with the noncoding strand (left) or the coding strand (right) of the octamer-containing Pvu II–Eco RI fragment of pSPlgVk as ^{32}P-labeled probes. The probes were partially methylated and used in preparative mobility-shift DNA binding assays. DNA present in the bound bands representing complexes with NF-A1 and NF-A2 from a nuclear extract from the BJAB cell line (lanes 1, 2, 7, and 8) or the band formed by the phage 3 lysogen extract (lanes 5 and 11) or unbound DNA was isolated, cleaved at the modified purine residues, and subjected to denaturing polyacrylamide gel electrophoresis (PAGE).

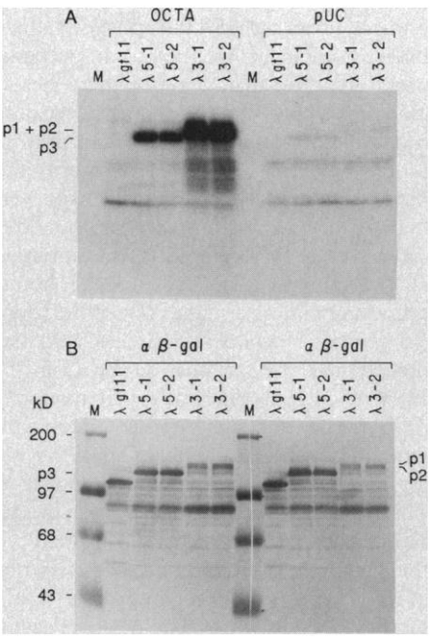

Fig. 3. Southwestern blot analysis of the DNA binding activity encoded by phages 5 and 3. (**A**) Bacterial lysogens of phage 5 (called 5-1 and 5-2) of phage 3 (called 3-1 and 3-2) and of phage λgt11 were isolated. Total protein from induced cultures of the lysogens was separated in duplicate on a 10% SDS-PAGE and transferred by electroblotting to nitrocellulose (Schleicher & Schuell BA85). The immobilized proteins were denatured in 50 m*M* tris-HCl, *p*H 8, 7*M* guanidine-HCl, 50 m*M* dithiothreitol (DTT), 2 m*M* EDTA, and 0.25% BLOTTO (*10*) for 60 min at room temperature and allowed to renature in 50 m*M* tris-HCl, *p*H 8, 100 m*M* NaCl, 2 m*M* DTT, 2 m*M* EDTA, 0.1% NP-40, and 0.25% BLOTTO (*10*) for 24 hours at 4°C. The duplicate filters were probed, with the [α^{32}P]dATP-labeled tetramer probe (OCTA) (Fig. 1) and a control pUC 19 DNA probe (Eco RI–Hind III 50-bp fragment), each filter at 10^6 cpm/ml in 10 m*M* tris-HCl, *p*H 8, 1 m*M* EDTA, 50 m*M* NaCl (TNE 50) for 60 min at room temperature. The filters were washed three times for 10 min each in TNE 50 at room temperature. (**B**) The same filters analyzed in (A) were reacted with rabbit antibodies to β-galactosidase and subsequently with mouse antibody to rabbit immunoglobulin G horseradish peroxidase conjugate (*10*).

complexes as described in (7)]. Nuclear extracts from the pre-B cell lines, 70Z and 38B9, showed very little NF-A2 (Fig. 4B, lanes 5 and 6) and, correspondingly, expressed very little mRNA [more poly(A)-containing mRNA from these two cell lines was analyzed] (Fig. 4A, lane 2, and Fig. 4C). Of the three T lymphoma cell lines tested, EL4 was the only line that expressed mRNA (Fig. 4A, lanes 7, 11, and 13) and was also the only line that showed a clearly identifiable NF-A2 band (Fig. 4B, lanes 1 to 3). Although NF-A2 was believed to be exclusively expressed in lymphoid cells (*3, 7, 8*), the glioma cell line U1242 expressed both the *oct*-2 gene and an octamer binding protein that comigrated with NF-A2 in the gel electrophoresis assay (Fig. 4B, lane 11). Nuclear extracts from two other glioma cell lines, U1240 and U563, which were negative for *oct*-2 expression, did not generate a complex that comigrated with NF-A2 (Fig. 4B, lanes 12 and 13). The significance of this apparent nonlymphoid coexpression of NF-A2 and *oct*-2 transcripts is not clear.

We had already shown that NF-A2, but not NF-A1, was inducible in pre-B cells by treatment of the cells with bacterial lipopolysaccharide (LPS) and that this induction required new protein synthesis (7) (Fig. 4B, lanes 4 and 5). Therefore, we prepared poly(A)-containing mRNA from the pre-B cell line 70Z/3 before and after LPS treatment and observed that LPS increased the expression of the *oct*-2 gene (Fig. 4C, lanes 1 and 2). Thus, in every instance, expression of the *oct*-2 gene correlated with the presence of NF-A2, suggesting that *oct*-2 encodes the NF-A2 DNA binding protein.

The molecular cloning of a lymphoid-restricted cDNA that encodes an octamer binding protein demonstrates that higher eukaryotes utilize different genes to encode proteins that bind a common regulatory motif. The widely occurring (NF-A1) and lymphoid-specific (NF-A2) proteins make indistinguishable contacts with the octamer sequence (7) yet appear to have distinct functional properties. Structure-function studies of other transcription factors, notably those belonging to the steroid-receptor gene family (*15*), suggest that the DNA binding activity and the transcriptional regulatory activity often reside in discrete protein domains. Our data suggest that similar diversification of function among proteins that bind the octamer motif has occurred during evolution. Thus, NF-A1 could mediate the functions of the octamer sequence that are not tissue-specific (*16*). The cloning of a cDNA representing the *oct*-2 gene that is lymphoid-restricted in its expression suggests that the product of this gene, and not that of the NF-A1 gene, is probably responsible for the lymphoid-specific functions of the octamer sequence. Indeed, NF-A2, the putative protein product of the *oct*-2 gene, has been shown to function as a transcription factor for immunoglobulin promoters in vitro (*14, 17*). A further understanding of B cell development should come from analysis of the mechanisms underlying the lymphoid-specific expression of the *oct*-2 gene.

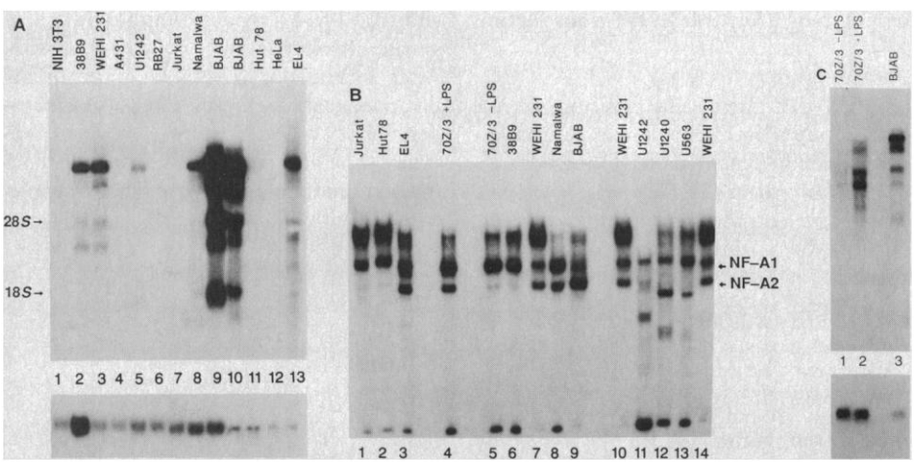

Fig. 4. Expression of *oct*-2 and NF-A2 in cell lines. (**A**) RNA blot analysis of mRNA from lymphoid and nonlymphoid cells hybridized with the *oct*-2 gene (upper panel) or a rat α-tubulin gene probe (lower panel). Poly(A)-containing mRNA (3 μg, lanes 1 and 3 to 9; 20 μg, lane 2) or total mRNA (30 μg, lanes 10 to 13) was analyzed (*18–20*) from the following cell lines: (lane 1) NIH 3T3, mouse fibroblast; (lane 2) 38B9, mouse pre-B cell line; (lane 3) WEHI 231, mouse mature B cell line; (lane 4) A431, human epidermal cell line; (lane 5) U1242, human glioma cell line; (lane 6) RB27, human retinoblastoma cell line; (lane 7) Jurkat, human T cell line; (lane 8) Namalwa, human mature B cell line; (lane 9) BJAB, human mature B cell line [poly(A)-containing mRNA]; (lane 10) BJAB (total mRNA); (lane 11) Hut78, human T cell line; (lane 12) HeLa, human cervical carcinoma cell line; (lane 13) EL4, mouse T cell line. (**B**) Mobility-shift DNA binding assay of octamer binding proteins in lymphoid and nonlymphoid cell lines. Nuclear extracts (8 μg of protein) were assayed (7) with the octamer-containing $V_κ41$ ^{32}P-labeled probe (legend to Fig. 2). The bands corresponding to NF-A1 and NF-A2 are indicated. The cell lines tested, in addition to those listed in the legend to (A), were 70Z/3, mouse pre-B cell line untreated (lane 5) or treated (lane 4) with bacterial lipopolysaccharide (LPS) for 24 hours (7), U1240 and U563, and human glioma cell lines (lanes 12 and 13). (**C**) RNA blot analysis of mRNA from 70Z/3 cells with and without treatment with LPS hybridized with the 1.2-kb cDNA segment from phage 3 (upper panel) or a rat α-tubulin probe (lower panel). Poly(A)-containing mRNA was prepared from 70Z/3 cells untreated (lane 1, 12 μg) or treated with LPS for 24 hours (7) (lane 2, 12 μg). Lane 3 contains BJAB poly(A)-containing mRNA (1 μg) (legend to A).

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11. A randomly primed cDNA library in λgt11 was generated [U. Gubler and B. J. Hoffman, *Gene* **25**, 263 (1983)] with the use of cytoplasmic poly(A)-containing mRNA from the BJAB cell line and random hexamers (Pharmacia) to prime the first strand cDNA synthesis. The unamplified library contained 500,000 recombinants. The probe for the library screen was constructed by cloning four copies of the oligonucleotide

GATCCTTAATAATTTGCATACCCTCA

GAATTATTAAACGTATGGGAGTCTAG

in direct orientation into the Bam HI site of the pUC polylinker and ^{32}P-labeling the 112-bp Sma I–Xba I fragment. The library was screened with the tetramer probe (*10*), except for the following modification. Previous screenings with poly(dI-dC) · poly(dI-dC) as the nonspecific competitor DNA yielded recombinant phage encoding single-stranded DNA binding proteins. The signal from these phages, but not from phages encoding sequence-specific DNA binding proteins (*10*), could be effi-

ciently competed with sonicated and denatured calf thymus DNA (5 μg/ml) and therefore this nonspecific competitor was substituted for poly(dI-dC) · poly(dI-dC) in all the screens described.
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18. The mRNA was subjected to electrophoresis through a formaldehyde-containing 1.3% agarose gel and transferred to a nitrocellulose filter (*19*). After prehybridization, the filter was hybridized at high stringency with a labeled 1.2-kb cDNA segment from phage 3 (*19*). The filter was washed in 0.2× saline sodium citrate and 0.1% SDS at 68°C and autoradiographed with an intensifying screen at −70°C for 24 hours. The filter was stripped by washing in 50% formamide, 10 m*M* tris (*p*H 7.4), and 1 m*M* EDTA at 68°C for 1 hour, and then rehybridized with a ^{32}P-labeled rat α-tubulin cDNA probe (*20*) to control for the amount of mRNA loaded.
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Chemotransduction in the Carotid Body: K^+ Current Modulated by P_{O_2} in Type I Chemoreceptor Cells

José López-Barneo, José R. López-López, Juán Ureña, Constancio González

The ionic currents of carotid body type I cells and their possible involvement in the detection of oxygen tension (P_{O_2}) in arterial blood are unknown. The electrical properties of these cells were studied with the whole-cell patch clamp technique, and the hypothesis that ionic conductances can be altered by changes in P_{O_2} was tested. The results show that type I cells have voltage-dependent sodium, calcium, and potassium channels. Sodium and calcium currents were unaffected by a decrease in P_{O_2} from 150 to 10 millimeters of mercury, whereas, with the same experimental protocol, potassium currents were reversibly reduced by 25 to 50 percent. The effect of hypoxia was independent of internal adenosine triphosphate and calcium. Thus, ionic conductances, and particularly the O_2-sensitive potassium current, play a key role in the transduction mechanism of arterial chemoreceptors.

The mammalian carotid bodies are arterial chemoreceptors responsible for the hyperventilation observed in physiological and pathological conditions that produce a decrease in blood P_{O_2} (*1*). The chemoreceptor, or type I, cells transduce the decrease in P_{O_2} and produce a neurosecretory response. The neurotransmitters released by these cells set the level of electrical activity in the afferent fibers of the carotid sinus nerve (*2*).

The mechanism involved in the transduction of the hypoxic stimulus has been elusive (*3*). Either low P_{O_2} or high external K^+ can induce release of dopamine from type I cells, which is dependent on external Ca^{2+} and is inhibited by Ca^{2+} channel blockers such as nitrendipine and nisoldipine. On the basis of these data, it has been suggested that membrane depolarization may play a part in the response of type I cells to hypoxia (*4*).

The electrophysiological characteristics of type I cells are not well known. It has been reported that these cells are nonexcitable because, after impalement with microelectrodes, action potentials have not been recorded from them (*5*). However, these data cannot be considered definitive because, possibly as a result of cell damage, the average resting potential (−19.8 mV) was low enough to produce a complete inactivation of the ionic conductances that produce depolarization of the membrane. We have now found that type I cells have voltage-dependent Na^+, Ca^{2+}, and K^+ channels and that the K^+ channel activity is inhibited by low P_{O_2}.

Our experiments were performed on cultured type I cells isolated from rabbit carotid bodies. These cells were easily recognized by their small diameter (10 to 14 μm) and their birefringent appearance under the light microscope. The dispersed cells released dopamine in response to hypoxia. Clean carotid bodies were incubated for 30 min in a Ca^{2+}- and Mg^{2+}-free Tyrode solution with collagenase (3 mg/ml) and trypsin (1 mg/ml). After incubation, cells were mechanically dispersed with a fire-polished Pasteur pipette and washed twice to remove the enzymes. The final pellet was resuspended in 5 ml of minimum essential medium supplemented with glutamine (1%) and bovine fetal serum (5%). Cells were plated on sliv-

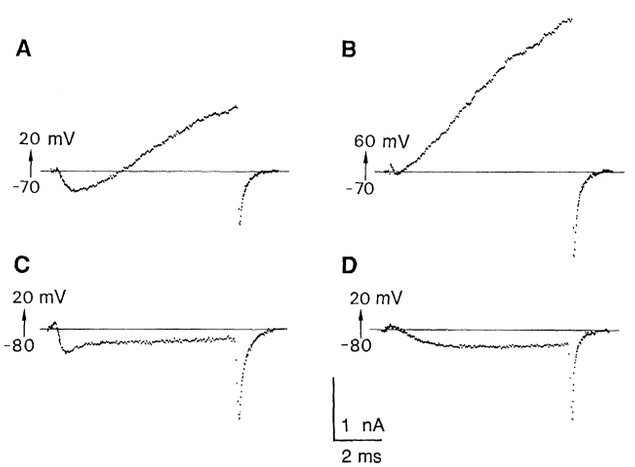

Fig. 1. Voltage-dependent ionic currents recorded in type I chemoreceptor cells of the carotid body. (**A** and **B**) Inward and outward currents recorded by voltage steps to 20 mV (A) and 60 mV (B) from a holding potential of −70 mV. External solution: 140 m*M* NaCl, 10 m*M* $CaCl_2$, 2.7 m*M* KCl, and 10 m*M* Hepes; internal solution: 80 m*M* potassium glutamate, 30 m*M* KCl, 20 m*M* KF, 2 m*M* $MgCl_2$, 10 m*M* Hepes, and 1 m*M* EGTA. (**C** and **D**) Na^+ and Ca^{2+} currents recorded after depolarization to 20 mV from a holding potential of −80 mV; K^+ currents were abolished by replacement of internal K^+ with Cs^+ (C), and Na^+ currents were blocked by bath application of TTX (1 μg/ml) (D). External solution as in (A) and (B); internal solution: 110 m*M* CsCl, 20 m*M* CsF, 2 m*M* $MgCl_2$, 10 m*M* Hepes, 5 m*M* EGTA, and 2 m*M* Mg^{2+}-ATP. The *p*H of all solutions was 7.3, and P_{O_2} was equilibrated to 150 mmHg. Linear ionic and capacitance currents were subtracted with a P/4 procedure (*10*). Temperature was 20° to 22°C.

J. López-Barneo and J. Ureña, Departamento de Fisiología, Facultad de Medicina, Universidad de Sevilla, Avenida Sánchez Pizjuán, 4, 41009 Sevilla, Spain.
J. R. López-López and C. González, Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, Calle Ramón y Cajal, 5, 47005 Valladolid, Spain.