

## OLIGOBODIES: BENCH MADE SYNTHETIC ANTIBODIES

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**Abstract** Using synthetic peptides and a combinatorial library of 56 mer random oligonucleotides, we have developed reagents that behave as "synthetic antibodies". The results obtained with the protein phosphatase 2A as a model system are shown here. The specificity of these reagents, named "oligobodies", has been demonstrated by Western blot analysis and immunohistochemistry. The oligobodies have enormous advantages compared to antibodies: their production is independent of the immune system, they can be prepared in a few days and there is no need for a purified target protein. These reagents can be produced even if the corresponding protein was never isolated or purified, since only a partial DNA sequence from a database provides enough information to make them.

**Resumen** *Oligoanticuerpos: anticuerpos sintéticos de fácil preparación.* Usando péptidos sintéticos y una biblioteca combinatoria de oligonucleótidos de 56 bases de secuencia al azar, hemos desarrollado reactivos que se comportan como "anticuerpos sintéticos". Se muestran los resultados obtenidos utilizando a la proteína fosfatasa 2A como modelo. La especificidad de estos reactivos, que hemos llamado "oligobodies", ha sido demostrada por análisis de Western blot e inmunohistoquímica. Los "oligobodies" tienen ventajas enormes en comparación con los anticuerpos: su producción es independiente del sistema inmune, pueden ser preparados en unos pocos días y no se necesita una proteína pura. Estos reactivos pueden ser producidos aunque la proteína correspondiente no haya sido nunca aislada o purificada, dado que sólo una secuencia parcial de DNA obtenida de una base de datos provee la información necesaria para construirlos.

**Key words:** oligobodies, synthetic antibodies, aptamers, random oligonucleotides, selex

In order to produce polyclonal antibodies, it is generally necessary to have a purified protein, a good adjuvant, and inject the protein into rabbits or other animals. After approximately two months, it is possible to have some good titer, depending on the antigenicity of the injected protein. Then, to have good specificity, the antibody has to be affinity-purified from serum. The total procedure takes several months, and once the antibody is obtained, there is only limited amounts. One way to obtain industrial amounts of antibodies is to develop monoclonal antibodies. However, the procedure for making monoclonal antibodies is cumbersome, costly and complex screening procedures are needed to select adequate clones. An alternative approach, in case the target protein is not available, is to make synthetic peptides that correspond to possible antigenic sites of the protein. These peptides are then coupled to some other protein to increase their antigenicity (KLH, albumin,

etc.) and used to inject animals to produce polyclonal antibodies (monoclonal ones are difficult to obtain in this way). All these procedures are very time consuming and costly at industrial scale. Therefore, it would be of great interest to be able to develop "synthetic antibodies", without the need for an immune response, a purified protein, animals or cultured cells.

In the search for these "synthetic antibodies", one way is to explore libraries of combinatorial synthetic compounds. The idea of using a library of random oligonucleotides to select reagents that recognize proteins was proposed originally by Tuerk and Gold<sup>1</sup>, with the method called SELEX, and simultaneously by Ellington and Szostak<sup>2</sup>, who named aptamers to these reagents. Although about 150 reagents have been made against different proteins since the original SELEX method was described<sup>3</sup> and aptamers have been proposed recently as possible rivals for antibodies<sup>4</sup>, the aptamers do not have yet a niche in the market as replacement for antibodies. Probably different difficulties (mainly specificity and versatility) have precluded their competition in the market with antibodies.

We have also explored the possibility of obtaining synthetic antibodies using a library of synthetic oligo-

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nucleotides, although applying several strategies that differ from the method proposed originally by Tuerk, Ellington and others<sup>1,3,5</sup>. Using these strategies, we were able to produce oligonucleotides that behave as "synthetic antibodies", and we have probed their specificity using Western blot analysis and immunohistochemistry. The reagents were called "oligobodies" after their oligonucleotide origin and usefulness as antibodies.

## Materials and Methods

**Animals:** Cerebellar tissue was obtained from mice (inbred strain C57BL/6J Jackson Laboratories, Barr Harbor Maine, USA), raised in the Centro Nacional de Genética Médica, Instituto Malbrán, Buenos Aires, Argentina.

**Antibodies and synthetic peptides:** A polyclonal antibody obtained in rabbits, that recognize the 36 kDa catalytic subunit of mammalian PP2A, and the corresponding blocking synthetic peptide (amino acids 298-309: Pro-His-Val-Thr-Arg-Arg-Thr-Pro-Asp-Tyr-Phe-Leu) were acquired from Calbiochem (Calbiochem-Novabiochem Corp., La Jolla, CA, USA). The working dilution of this primary antibody was 1:500 for both Western blots and immunohistochemistry. The secondary antibody used to develop Western blots was a goat anti-rabbit, affinity-purified antibody, coupled to alkaline phosphatase (dilution: 1:10 000) (obtained from Sigma Co., St. Louis, MA, USA). For immunohistochemistry, a goat anti-rabbit antibody coupled to peroxidase (Promega, Madison, WI, USA) was used as secondary antibody (dilution 1:1 000). Staining was carried out with peroxidase substrate following the instructions from the manufacturer.

**Oligonucleotide library:** A library of 100 mer oligonucleotides (56 random) was custom synthesized at DNAgency (DNAgency, Malvern, PA, USA), having the following sequence: 5'-CTGCAGCCGCGGGGATCCT(N)<sub>56</sub>TCTAGTCAATTCAGCTTAGTGGC-3'.

**PCR amplification of the original synthetic oligonucleotide library:** The original synthetic oligonucleotide library was amplified by PCR using the primers 5'-CTGCAGCCGCGGGGATCCT-3' and 5'-GCCACTAAGCTTGAATTCGACTAGA-3' (custom made at DNAgency, Malvern, PA, USA). This initial amplification is necessary, since very low yield is obtained in the synthesis of long oligonucleotides (around 15% for a 100 mer oligonucleotide) and many truncated forms remain after the synthesis. Therefore, this first PCR amplification minimizes the presence of such truncated oligonucleotides and makes the library more stable upon storage (since it becomes double-stranded). The amplification reaction was done at a final volume of 100 µl containing: 5' and 3' primers (1.2 µM), Taq polymerase (5 U), template (5 µM), buffer 10X (KCl 500 mM, Tris-HCl 100 mM, pH 9.0 a 25°C, Triton X-100 1%), MgCl<sub>2</sub> (1.5 mM), dNTPs (0.25 mM each nucleotide), radiolabeled dCTP (1.5 x 10<sup>6</sup> cpm of (α-<sup>32</sup>P)dCTP, 3 000 Ci/mmol, NEG 513H, NEN) and RNase-free water. For PCR-amplification, an initial step of 5' at 94°C was followed by 35 cycles of: 94°C, 30"; 50°C, 30"; 72°C, 4" and a final step at 4°C.

**Double strand separation:** In order to separate both strands before incubation with the target, the library was re-amplified using a biotinylated down-stream primer (5'Biot-GCCACTAAGCTTGAATTCGACTAGA-3'). After amplification, 35 µl of the PCR reaction were incubated for 10 min with 30 µl of streptavidine coupled to paramagnetic particles (Streptavidin Magnasphere Z5482, Promega, Madison, WI, USA), washed with 90 µl of SSC (0.1 X) and eluted with 30 µl of NaOH (0.15N). The single strands eluted were immediately neutralized with 19 µl of sodium acetate (0.2 M) and then diluted to 350 µl using cold PBS containing 5% BSA.

We have observed that a permanent separation of both strands is not necessary to obtain species capable of binding to the target. Actually, a temporary separation of both strands obtained by heating the sample at 94°C for 5 min., followed by a rapid cooling with equal volume of PBS (0-4°C) is enough to obtain a good binding (as is done normally with Northern blots). However, the dilution of both strands has to be adequately tested, otherwise, the antisense strand would compete to form again the double strand, making the binding to the target almost impossible. For this reason, we used a permanent separation of both strands only in the first cycle of selection, when the template is highly concentrated and the binding species diluted among non-binding species. Therefore, after the first binding cycle a separation by heat is enough to obtain a good binding.

**Binding, PCR-amplification and selection:** For selection of binding species, a solid-phase method was used. A small piece of nitrocellulose membrane (0.12 cm<sup>2</sup>) (Schleicher and Schuell, Keene, NH, USA) was incubated with the target peptide at saturating concentration (10 mg/ml in water). The membrane with the adsorbed target peptide was then incubated in a 1.5 ml plastic tube containing 350 µl of PBS-BSA buffer (PBS, pH 7.4, BSA 5%) and 35 µl of the PCR amplified/radiolabeled library (final concentration of oligonucleotides is 1:10 from the original PCR reaction). The incubation was carried-out overnight at room temperature, with gentle agitation. The unbound oligonucleotides were washed 3 times (45 minutes each), using 1 ml of PBS with agitation (vortex at 300 rpm). Finally, the bound oligonucleotides were eluted by heating the membrane 10 minutes at 95°C, in 99 µl of PCR reaction without Taq and counted in a scintillation counter (Cherenkov counting) to determine the relative amount bound compared to controls (nitrocellulose blocked with 5% BSA). The membrane was then removed and the PCR reaction initiated after adding the enzyme to the remaining solution.

**Target switching:** After the first selection a strategy called "target switching" was used, which involves a change from the temporary target (synthetic peptide) to the final target, the protein. For this reason, a second selection of the oligobodies is done, which eliminates the remaining non-reacting oligonucleotides and those that cross-react with other proteins. To do this, we incubated the oligonucleotides of the first selection with the entire denatured protein in a Western-blot previously developed for PP2A/C using polyclonal antibodies and peroxidase (see below). Both strands obtained from the PCR in the first selection were separated by heat, diluted of 1/10 in PBS-5% BSA and incubated over night at room temperature with the Western blot membrane. The membrane was then washed with PBS (3 times, 15 min), the area corresponding to the PP2A protein band selected, and the bound oligonucleotides were eluted and re-amplified by PCR. This final product was stored as a double strand form at -20°C.

**Western blots:** Freshly isolated cerebelli were homogenized in PBS containing a cocktail of proteinase inhibitors (final concentration: EDTA 10 mM, phenantrolin 10 mM, E-64 10 µM, leupeptine 100 µM, aprotinine 10 mg/ml, pepstatin A 10 µM) (Sigma Co., St. Louis, MA, USA). Protein concentration was determined using the method of Lowry OH, et al.<sup>6</sup>. One hundred micrograms of protein was run in a 10% SDS-PAGE according to Laemmli<sup>7</sup>, transferred to nitrocellulose membrane<sup>8</sup> and blocked with PBS-BSA 5%. Incubation with the rabbit primary antibody (1/500) was performed overnight at 4°C, and with the secondary antibody was for 1 hour at room temperature (goat anti-rabbit phosphatase alkaline conjugated 1:10 000 in PBS-5%BSA and developed with NBT/BCIP or nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) (Gibco/BRL, Gaithersburg, MD, USA) for Western blot. Oligobodies-<sup>32</sup>P-labelled were incubated overnight at room temperature in PBS-5%BSA, washed 3 times with PBS and exposed for 1 hour with autoradiographic film.

Immunohistochemistry: Mouse cerebelli were dissected at different postnatal ages, fixed in alcohol/acid (95% ethanol: 5% acetic acid, 4 hours at -20°C), dehydrated, and embedded in paraffin. Five  $\mu\text{m}$  tissue slices were mounted in silanized (Silane, Sigma Co., St. Louis, MA, USA) coverglass, deparaffinized and rehydrated before use. Nonspecific binding sites were blocked using PBS-5%BSA during 1 hour and the slices were exposed to primary antibodies overnight at 4°C. Then, the slices were rinsed with PBS, incubated with the secondary antibody (goat anti-rabbit peroxidase 1:1 000) for one hour and developed with DAB (3-3'di-amino-benzidine tetrahydrochloride dihydrate) (Gibco BRL, Gaithersburg, MD, USA). The immunohistochemistry staining with oligobodies was performed using biotinylated-oligobodies prepared adding a 5'-biotinylated primer to the PCR reaction (5'-Biot-CTGCAGCCGC-GGGGATCCT-3') (custom made at DNAgency, Malvern, PA, USA). After PCR amplification, the cDNA strands were separated by heat (94°C, 5') and diluted ten times with PBS-5%BSA. The presence of biotin does not affect the PCR amplification or the binding to the target, since the bands obtained in a Western blot stained with biotinylated-oligobodies were identical to those developed using  $^{32}\text{P}$ -labelled-oligobodies (results not shown). Control samples were made preincubating the oligobodies with an excess of blocking peptide (overnight at 4°C). After incubation of slides with PBS-5% BSA to block nonspecific sites, oligobodies were incubated with the tissue for 1 hour at RT and then developed with streptavidin-peroxidase (Sigma Co., St. Louis, MA, USA).

## Results and Discussion

During the last 9 years, different oligonucleotide libraries have been used to produce reagents having certain capacity to recognize proteins. However, it is evident that the procedures are not yet satisfactory to produce synthetic antibodies, since only few aptamers that bind to proteins have been developed so far and their specificity has not been proved. Perhaps for this reason Jayasena et al.<sup>4</sup> believe that the value of aptamers in most cases will be in applications of formats where the performance of antibodies is inadequate rather than in replacement of assays that work well with antibodies. On the contrary, our results suggest that indeed the "oligobodies" will replace antibodies in most cases, since they have even better performance.

An ideal "synthetic antibody" should have three important characteristics: high specificity, high affinity, and versatility (the method should be able to generate synthetic antibodies against any desirable target). Although the reagents prepared so far have high affinity, the specificity in a mixture of proteins has not been really proven yet (i.e. by using Western blots). In addition, in order to obtain the reagents, it was necessary to start with a pure protein and many cycles of amplification/selection were required (10-25 cycles), a process that involves many intrinsic problems. More importantly, as shown by Morris et al.<sup>9</sup>, the oligonucleotides selected from a complex mixture of proteins, such as those found in cell membranes, seem to recognize only few proteins among several thousand of them (lack of versatility).

Using an interesting variation of SELEX, Xu and Ellington utilized synthetic peptides to generate aptamers against the Rev-binding element<sup>10</sup>. Since the protein was in a native form during the selection procedure, the specificity was studied using gel shift. Unfortunately, to prove specificity, only pure proteins were used in this assay (instead of using a mixture of proteins). Therefore, they were able to generate reagents that indeed recognized the protein Rev with high affinity, but the specificity of these reagents was not convincingly proved.

The present work is also based on the idea originally proposed by Tuerk et al.<sup>1</sup> and Ellington et al.<sup>2</sup>, that a combinatorial oligonucleotide library should contain species able to recognize any target. However, in order to produce highly specific synthetic antibodies ("oligobodies"), we had to use different strategies that differ substantially from the SELEX approach.

First, the initial library of 56 random nucleotides (plus 44 corresponding to flanking primers) was immediately amplified after synthesis. This was done due to the low yield of the synthesizers with long oligonucleotides (about 15% for 100 mer oligos) that produce original single strand libraries with many truncated species. This initial amplification minimizes the presence of such truncated forms and increases the concentration and stability of each full-length member. After this procedure, the library was re-amplified in the presence of  $^{32}\text{P}$  labeling and the strands separated for initial binding (see Materials and Methods for details).

The second strategy was to use synthetic peptides as "temporary targets", following a method similar to the one described by Xu and Ellington<sup>10</sup>, which was discussed above. Generally, a native target protein was used to select binding aptamers. Such procedure limits the conformational space to those reagents that recognize only the native protein, precluding their use as reagents in classical methods such as Western blots, immunoprecipitations or even immunohistochemistry. More important, such strategy limits the target to those epitopes accessible at the protein surface. Also, when using synthetic peptides there is no need for a pure protein, and any portion of the protein can be used to generate oligobodies directed against both the native or the denatured protein. In addition, by using the corresponding synthetic peptides, a partial cDNA or RNA coding sequence obtained from a database provides enough information to generate the oligobodies. Therefore, the advantages of using synthetic peptides are many.

The third strategy was to perform only one or at most two cycles of amplification/selection with the peptides as temporary targets. This strategy is critical and is clearly opposed to the SELEX strategy, which involves as many amplification/selection cycles as possible. In this way, using only one or two cycles, there is no bias in the library toward high affinity "epitopes" (which obviously are not

very abundant) before selecting for specificity. What is more important, the library remains rich in many different species that should contain those highly specific, regardless of their affinity. Indeed, if these highly specific species possess medium or high affinity is not important (monoclonal antibodies have generally low affinity compared to polyclonal antibodies, although they are very specific). Another problem that appears using many cycles of amplification/selection is the formation of high molecular weight polymers, which actually impair the selection process.

The fourth strategy was to switch from the temporary target (the peptide) to the final target (the protein either native or denatured) immediately after detection of some binding to the temporary target, a process that we have called "target switching". This step is also crucial to obtain oligobodies and it is preferably to use a very crude preparation of the final target (the protein), because the main objective is to select for those oligonucleotides of high specificity rather than those of high affinity. This is also a very important difference compared to the strategy followed by SELEX, in which the objective is to select first for high affinity species using many amplification/selection cycles, assuming that specificity will be a direct consequence of the high affinity obtained<sup>11</sup>. This "target switching" strategy with a crude preparation is decisive: if one first select for high affinity, then it is too late to select for specificity. Therefore, instead of performing again many cycles with the final target, we used immediately a mixture of proteins containing the target. In this way, regardless of their affinity, the specificity of the selected oligobodies is granted from the beginning. In addition, the recognition is done in a very specific way,

since the target has to be recognized among thousand of different proteins present in the mixture.

In consequence, the objective is first to select members of the library in a very specific way, and then the selection should be done for those of better affinity, among the specific ones. This is similar to what nature does: to obtain antibodies, the immune system first selects clones that produce specific antibodies, although they might be of low affinity (monoclonal antibodies are a good example), then nature selects for high affinity, selecting the appropriate clones or modifying them to obtain better affinity. We followed the same strategy, first the selection was done to obtain specificity and then we selected for high affinity (by extensive washing or by cloning). Even more so the affinity could be potentially improved in a later step, by introduction of random mutations in the selected species (*in vitro* evolution).

As an example of application of the above-mentioned strategies, we show here the results obtained using a synthetic peptide corresponding to the protein phosphatase PP2A. After amplification of the original library in the presence of <sup>32</sup>P labeled nucleotides, and separation of double strands by using avidine-sepharose, the peptides were bound to nitrocellulose (at saturating concentration) and incubated with the library. It is very important to note that we do not pre-adsorb the library with nitrocellulose, since that procedure might reduce the variety of the oligonucleotides present (the objective has always been to keep the library diversity at maximum). After binding to the peptides, the oligonucleotides were eluted and amplified for a further selection with the final target. We consider that one cycle of PCR amplification (two at most) after binding provides enough enrichment

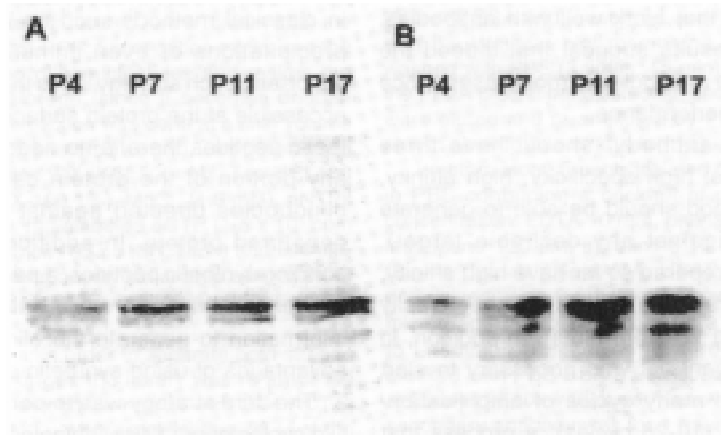


Fig. 1.— Western blot analysis of PP2A protein extracted from cerebellum. Detection of PP2A was carried out using A: primary polyclonal antibodies and secondary antibodies coupled to alkaline phosphatase. B: oligobodies radiolabeled with <sup>32</sup>P (autoradiography). Similar pattern at different postnatal ages (P#, in days) was obtained using both reagents.

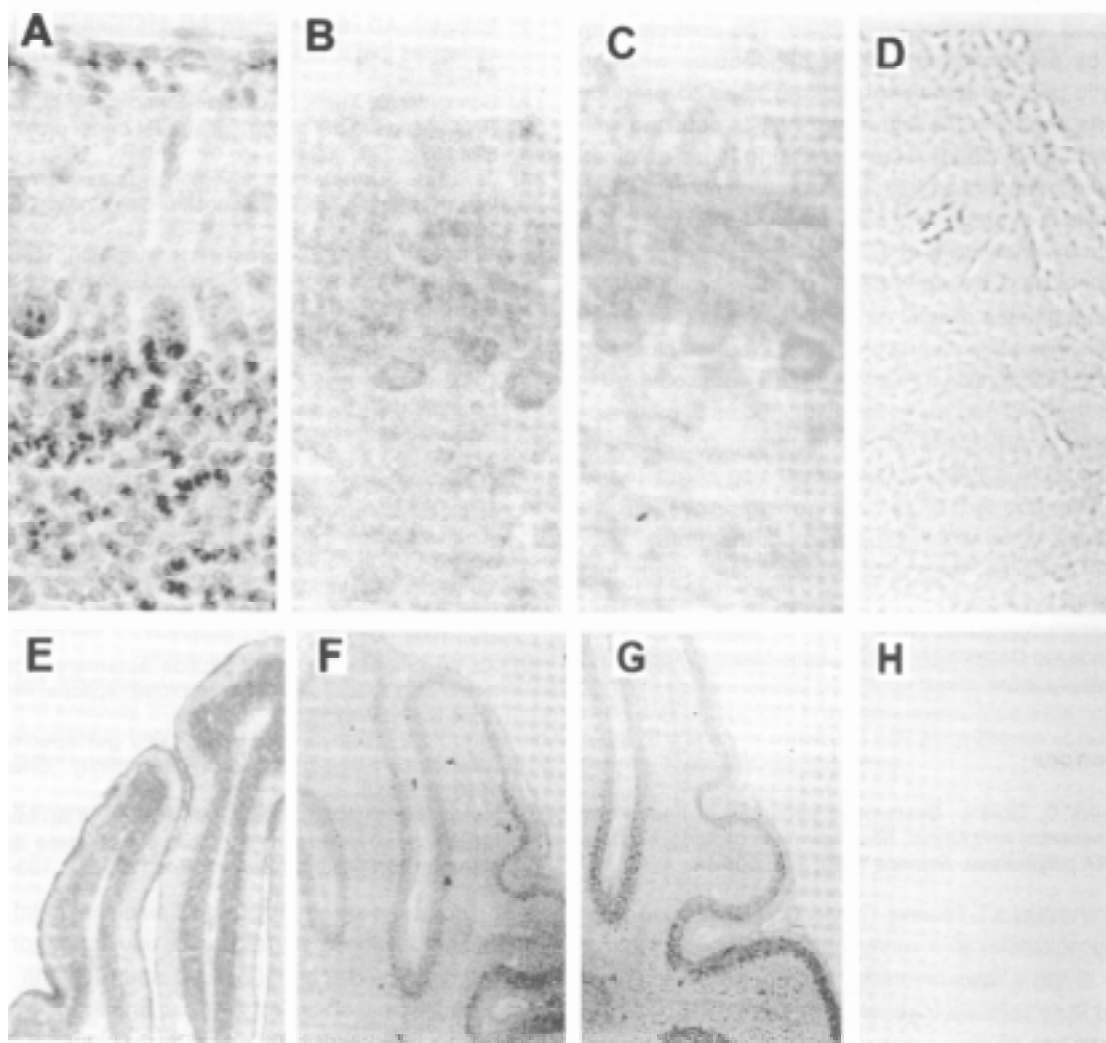


Fig. 2.— Immunohistochemistry of cerebellar slices at postnatal day 12. A, E: Hematoxylin-Eosin staining. B, F: Polyclonal antibodies (primary/secondary-peroxidase). C, G: oligobodies (oligobody-biotin/avidine-peroxidase). D, H: controls made with oligobodies preincubated with PP2A blocking peptide. Magnification was 400X for A, B, C (bright field) and D (phase contrast), 45X for E, F, G and H (bright field). Similar pattern of staining was obtained using either polyclonal antibodies or oligobodies.

to obtain oligobodies. As mentioned before, to use many cycles is adverse, since polymers are formed, the selection is biased toward those few members of high affinity, and what is worse, "diversity", which is the most important feature of a library to obtain specificity, is irremediable lost.

Then, the selected oligobodies were incubated with the target protein ("target switching", using PP2A in this case) contained in a mixture of proteins extracted with RIPA buffer from mouse cerebellum, and blotted on nitrocellulose. The spot corresponding to PP2A (developed in parallel with polyclonal antibodies) was cut, eluted and PCR amplified. In this way, we obtained for the first time "polyclonal obligobodies" that behave exactly as their polyclonal counterpart. As shown in Figure

1, the spots recognized using the oligobodies to develop the Western blots were identical to those recognized by polyclonal antibodies. These results indicate that very specific reagents that behave exactly as polyclonal antibodies were generated. These oligobodies can be cloned into vector T and screened to obtain very easily "monoclonal oligobodies" which are also suitable for immunoprecipitations (results not shown, manuscript in preparation).

We further compare both the polyclonal antibodies and the oligobodies studying their behavior as reagents for immunohistochemistry. Figure 2 shows the results obtained staining cerebellum slices with polyclonal antibodies against PP2A or by using oligobodies (labeled now by using a biotinylated primer). Both antibodies were

developed using avidine-peroxidase. The controls were made by pre-incubation of the oligobodies with the synthetic peptide (blocking peptide) used to produce them. As shown in the figure, the results obtained with the polyclonal antibody were identical to those obtained with the oligobodies and the pattern obtained using both antibodies is in agreement with the localization of PP2A reported by Hashikawa et al.<sup>12</sup> in rat cerebellum

In conclusion, we were able to develop synthetic reagents that behave exactly as synthetic antibodies. Their specificity has been clearly proven by Western blots and immunohistochemistry. These reagents will probably replace polyclonal and monoclonal antibodies in many applications. In this regard, a very important characteristic of oligobodies is their relative small size compared to antibodies (about 1/10 excluding the primers), which make them much more diffusible and, therefore, better putative "missiles" than antibodies to carry drugs into tumors.

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*We are free only to make the best of what we have, in the nowtime at our disposal. We send men to the moon and believe we have discovered tomorrow; but tomorrow is still growing out of all our yesterdays, and we decipher it in scraps and fragments like the arithmetic of the Incas... When you get to my age, you will find that there are few left with whom to share your past. The old go away. The young have no interest. You are there, a broken pillar in a wheatfield, the triumphs you celebrate long forgotten, the hands that raised you crumbled into dust and blown away.*

Sólo somos libres para hacer lo mejor con lo que tenemos en el instante a nuestra disposición. Mandamos hombres a la luna y creemos que hemos descubierto el mañana, pero el mañana está apenas surgiendo de nuestros ayeres, y lo desciframos en pedazos y fragmentos como la matemática de los Incas... Cuando se llega a mi edad, quedan pocos con quienes compartir el pasado. Los viejos se van. Los jóvenes no tienen interés. Uno está aquí, tal un pilar roto en medio de un campo, los triunfos largamente olvidados, las manos que nos criaron desechas en polvo y evaporadas.

Morris West (1916-1999)

*The Salamander*. New York: Pocket Book, 1974, p 131