Direct autoradiography after radio-ligand binding to identify mammalian cell clones expressing hormone receptor cDNA

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Screening and isolation of membrane bound hormone-receptor cDNA clones is elegantly accomplished by radiolabeled-hormone binding assay. In this communication, we describe a method of screening mammalian cells, transfected with eukaryotic cDNA expression libraries, that utilizes the interaction between ligand and receptor. The method simply involves the transfection of mammalian cells with cDNA expression library, incubation of radiolabeled-ligand with the cells on culture plate, followed by exposure to X-ray film and subsequent identification of cells expressing receptor and isolation of cDNA clones from the cells.

Cloning hormone receptors often requires partial amino acid sequences, antibodies, and/or elaborate assays. Generally, these are not available to research groups and cloning becomes difficult and expensive. Therefore it is desirable to implement simpler and less elaborate methods of cloning receptors and binding proteins such as radioligand binding assays. A ligand binding assay designed to screen for binding proteins expressed in *E. coli* transformed with λ gt11 cDNA libraries has been previously described (1). Although this method effectively identifies clones that express either prokaryotic binding proteins or proteins that are not glycosylated in the natural state, it is ineffective in the identification of eukaryotic glycoprotein because *E. coli* does not support glycosylation. Most mammalian membrane associated receptors are glycoproteins. Expression of these proteins in *E. coli* would yield a nonfunctional receptor that would not be detected by a ligand binding assay. It is therefore necessary to express and screen eukaryotic membrane receptors in mammalian cells which not only support glycosylation but also proper post-translational processing. Recently eukaryotic membrane receptor cDNAs have successfully been cloned using antibodies (2) and/or microscopic screening of radioligand bound to cell cultures on slide glass (3, 4). The slide glass culture plate has to be fixed with glutaraldehyde and coated with photographic emulsion, followed by autoradiographic development (3). The method described in this communication is simpler and less time consuming. Furthermore, more cells can be screened at a time. It has successfully been used to isolate LH/CG receptor clones as previously implicated (5).

Polyadenylated mRNA was isolated from superovulated rat Corpora lutea using Fast Track (Invitrogen). The manufacturer's suggested protocol was followed with one exception that, rather than using tissue cultured cells, superovulated ovaries were sliced

on ice with a razor blade and RNA was extracted. A cDNA library was constructed using Librarian I (Invitrogen). First and second strand cDNAs were synthesized and ligated to BstXI nonpalindromic linkers. Double strand cDNAs were size-fractionated on agarose gels to isolate cDNAs larger than 2 kb. The >2 kb fragments were ligated to BstXI site of pCDM8 (2), a gift from Drs Brian Seed and Alejandro Aruffo, and *E. coli* MC1061/p3 was transformed. Derivatives of pCDM8 are now commercially available, which carry a convenient polylinker site. The library size was approximately 7×10^5 . The eukaryotic expression vector is capable of extrachromosomal replication in cell lines, such as Cos 1, Cos 7, CV1 and WOP cells which express the SV40 large T antigen or are latently infected with polyoma virus. This vector and its derivatives are easily recovered from the mammalian cells and are capable of transiently over-expressing proteins.

pCDM8 carrying cDNA inserts were purified twice on CsCl density gradient and divided into 20 groups. Each group was used independently in the transfection of Cos cells by the DEAE Dextran Method (6). Transfected cells were cultured on 10 cm plates for 2-3 days. Culture plates were incubated with 125 I-hCG at 37°C for 60 min, washed several times to remove unbound hormone, and thoroughly drained. As shown in Figure 1, the plates were sequentially fitted with polyethylene film, inner ring, outer ring, X-ray film, intensifying screen, lead shield and cover.

It is important that the polyethylene film adheres to the surface of culture monolayer without air pockets. For this purpose thinner Handiwrap is better than thicker Saran Wrap. To press down the Handiwrap film, a plastic ring was inserted that tightly fit against the inside wall of the plates. Next, outer plastic ring that tightly fit the out side of the plate wall was placed over the excess part of the polyethylene film and around the culture plates. This secured the polyethylene film in place. The inner and outer rings were made out of used X-ray film. A circular piece of X-ray film was cut in the dark using a hardboard pattern and placed on the inner bottom of the plates. The X-ray film and the inner wall of the plates were marked. A circular intensifying screen and a circular polyethylene lead shield were placed on top of X-ray film. This lead shield was used to protect the X-ray film from outside radiation as well as to apply pressure to hold down the intensifying screen and X-ray film. The intensifying screen

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and lead shield are not absolutely necessary, however. Finally, the plates were wrapped with aluminum foil, covered with a black plastic bag, and kept at -75°C for 3–7 days. The resulting autoradiogram was used to identify the locations of bound radiolabeled hormones (Figure 2). The positions on the plates corresponding to radioactive spots identified by autoradiogram were marked on the outer bottom of the plates. Sections measuring 2×2 mm were cut by melting with a hot surgical blade. (Melting was found to be easier than mechanically cutting the plates.) The cut plate pieces, including polyethylene film, were immersed in $200\ \mu\text{l}$ of Hirt Solution (7) consisting of 0.6% sodium dodecylsulfate and 10 mM EDTA, for 20 min to recover the pCDM8 plasmids. After spinning at $14,000 \times g$ for 5 min, the supernatant was recovered, mixed with $50\ \mu\text{l}$ of 5 M NaCl, and allowed to incubate on ice for 5 h. The supernatant was collected, phenol-extracted and ethanol-precipitated repeatedly. One hundred μl of competent host strain, *E. coli* MC1061/p3, was transformed with $8\ \mu\text{l}$ of the precipitate for 30 min on ice. The cells were heat-shocked at 40°C for 1 min, mixed with $500\ \mu\text{l}$ of SOC medium (2), and incubated for 1 h at 37°C . The transformed cell mixture was incubated overnight in 50 ml of LB medium containing tetracyclin. Amplified DNA was purified on CsCl density gradient centrifugation for transfection of Cos cells. This procedure was repeated several times to isolate single clones.

Monolayer Cos cells were transfected with varying concentrations of the pCDM8 library, cultured for varying periods of time, and incubated with ^{125}I -hCG. The optimal condition was $20\ \mu\text{g}$ of DNA/ml for transfection and 62 h culture of transfected cells. After washing cells, they were exposed to X-ray film. Five plates out of approximately two hundred plates showed evidence suggesting the interactions between receptor and radiolabeled hormone. The positions corresponding to these positive interactions in each plate were cut, pooled, and treated with Hirt solution. Plasmids were recovered, amplified and used to transfect Cos cells in 10 plates. Since the spots of the first round screen were not so distinct enough to be seen on print, they are not presented in Figure 2. After four false chases, we were successful. Two plates in the second round screen exhibited ^{125}I -hCG bound spots as shown in Figure 2, which were more populous and conspicuous than the spots of the first round screen. Positions corresponding to the spots were cut. Each plate piece was separately extracted with Hirt solution and plasmids were recovered for the transfection of Cos cells for the third round screen. The result of the fourth round screen showed more spots of ^{125}I -hCG binding (Figure 2).

The plasmids recovered from the fourth round screen were amplified and isolated on CsCl centrifugation (8). To verify the specificity of ^{125}I -hCG binding, monolayer murine Leydig tumor cells, which express the LH/CG receptor, were incubated with ^{125}I -hCG and exposed to X-ray film. Radioactive spots were seen on the autoradiogram (Figure 2). In contrast, no spots were detected on the autoradiogram of Cos cells transfected with pCDM8 with no insert. In addition, when Cos cells were transfected with the LH/CG receptor cDNA vector and incubated with ^{125}I -hCG in the presence of cold hCG, no radioactive spots were found on the autoradiogram (data not shown). Taken together, these results demonstrate the specificity of ^{125}I -hCG binding to the transfected Cos cells. Finally, the sequence of the isolated clone confirmed the rat LH/CG receptor as reported previously (9). Binding of ^{125}I -hCG is dependent on successful transfection and expression and furthermore, the transfection

efficiencies are less than 100%. Therefore, we would not expect all of the transfected cells to bind ^{125}I -hCG even in the fourth round.

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Figure 1. The schematic presentation of the X-ray film exposure. Cos cells on culture plates were transfected with pCDM8 cDNA library and incubated with ^{125}I -hCG. The plates were sequentially fitted with polyethylene film, inner ring, outer ring, X-ray film, intensifying screen, lead shield and cover.

Figure 2. Autoradiograms of culture plates treated with ^{125}I -hCG. The autoradiograms of the second and fourth round screens of transfected Cos cells are shown. In a negative control, no spots are seen on the autoradiogram of Cos cells transfected with pCDM8 with no insert. As a positive control, monolayer murine Leydig tumor cells, which express the LH/CG receptor, were incubated with ^{125}I -hCG and exposed to X-ray film.