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Isotope Labeling in Mammalian Cells

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Abstract

Isotope labeling of proteins represents an important and often required tool for the application of nuclear magnetic resonance (NMR) spectroscopy to investigate the structure and dynamics of proteins. Mammalian expression systems have conventionally been considered to be too weak and inefficient for protein expression. However, recent advances have significantly improved the expression levels of these systems. Here, we provide an overview of some of the recent developments in expression strategies for mammalian expression systems in view of NMR investigations.

Keywords

Isotope labeling; Nuclear magnetic resonance; Recombinant protein expression; Human embryonic kidney cells

1. Introduction: Mammalian Cell Expression System

1.1. Principle of Mammalian Cell-Mediated Protein Expression

Mammalian cell expression systems are being increasingly used to express proteins for structural biology. This is evident from the increase in the number of structures available in the Protein Data Bank (PDB) (1) of proteins purified from such sources. The main reason for switching to these higher eukaryotic expression systems is the ability to produce biologically active cell surface receptors and secreted glycoproteins. The functionality of these proteins is linked to the requirement for posttranslational modifications, such as

glycosylation and disulfide bond formation, which can often only be satisfied by using mammalian systems and not other eukaryotic systems, such as yeast and insect cells.

Protein expression in mammalian cells involves transfection with a plasmid carrying the gene of interest under the control of a mammalian promoter. The mammalian gene can be introduced into cells either by way of transient transfection or by developing a stable cell line. The former can be achieved with both adherent and suspension cells using polyethylenimine (PEI). The only advantage of this method over stable cell line preparation is that milligram quantities of protein can be prepared in a few days time compared to the months required by the latter method (2–4). However, there are several disadvantages associated with the transient transfection approach (1) Not every protein, in particular membrane proteins, give high yields when transiently transfected; (2) Very large quantities of DNA need to be prepared; (3) The process needs to be repeated every time protein is needed, adding labor and cost. Stable cell line creation overcomes these limitations, although 2–6 months are required to establish a high-level expression system. The effort is often justified, however, by the advantages of a stable mammalian cell line over a transient transfect (1) The yield is typically very high; (2) The process becomes fast and robust once the cell line is created for a particular protein; (3) Stable cell lines are usually created using calcium phosphate transfection, an efficient and inexpensive method; (4) Cell lines can be established in cellular backgrounds that are specifically tailored to the needs of the protein being expressed. For example, heterogenous glycosylation is a problem in NMR studies which typically require highly homogenous material. To overcome this problem, cell lines can be used that are deficient in complex heterogenous glycosylation capabilities (5); (5) The ability to create inducible cell lines is an additional advantage. In cases where constitutive expression of a particular protein is toxic to cells, placing the gene under an inducible promoter can allow expression of otherwise toxic proteins; (6) Finally, stable cells can be easily scaled up by transferring them to suspension cultures in spinner flasks or bioreactors.

Episomal vectors developed from viruses, such as Epstein–Barr virus (EBV) (6), bovine papilloma virus (BPV) (7), BK virus (BKV) (8), and Simian virus 40 (SV40) (9) are mainly used for transfection. A list of commonly used vectors can be found in (10). The advantages of using episomal vectors over integrating genes into the DNA of the host cell are numerous (1) Episomal expression will result in gene expression independent of the regulatory mechanisms of the host cell and the position of integration in the host genome, leading to higher expression levels since these factors may unfavorably influence expression. Random integration may also disrupt the characteristics of the gene of interest (11); (2) There is no interruption in host cell gene expression, which is often the case with integrative vectors, the latter leading to transformation of the host cell and undesirable effects on protein production; (3) Episomal vectors can exist in multiple copies in a cell, thus leading to amplification of the gene of interest. The main criteria for selecting a suitable episomal vector are (1) high copy number in *E. coli* for large-scale DNA production; (2) a strong mammalian promoter for high expression levels of the gene of interest in mammalian cells; and (3) small size so that genes of different lengths can be easily cloned into the vector. Thus, the vector typically consists of a strong mammalian promoter to drive expression of the mammalian gene of interest, a viral origin of replication activated by viral early genes, which is required to carry out its replication thereby maintaining the vector in the host system, and a eukaryotic selection marker, usually an antibiotic resistance gene, such as neomycin, to select for transfected cells. The human cytomegalovirus promoter has been shown to be very powerful in both HEK293 and CHO cells and is also active in most mammalian cells (12).

1.2. Mammalian Cell Lines

Mammalian cell lines used for transfection with virus-based vectors carrying the gene of interest usually already carry vectors encoding viral early genes corresponding to the origin of replication used by the new vector. Some of these early genes are SV40 T antigen, EBNA-1, E1, and E2 which bind to SV40, EBV, and BPV ori, respectively. The early genes act in *trans* to initiate replication by binding to the ori and they also act as enhancers, thereby increasing the copy number of viral vector and the expression levels of the protein of interest (13). Since very high copy numbers of the viral genes can lead to host cell death, regulation of viral replication can be imposed by transfecting mammalian cells with a different vector carrying the early genes (13). Mammalian cell lines that are widely used for protein expression are Chinese Hamster Ovary (CHO) and Human Embryonic Kidney 293 (HEK293) cells. Both are suitable for use in adherent and suspension cultures. The advantage of using CHO cells is the availability of various auxotrophs that can be used as selection markers for transfection. An example of such an auxotroph is dihydrofolate reductase (DHFR) deficient cells which are triple auxotrophs for hypoxanthine, glycine, and thymidine (14). Transfection of foreign genes along with DHFR genes in these cells allows for the selection of clones in a medium devoid of the above nutrients. Another advantage of this system is that it helps amplify the foreign gene when DHFR deficient cells are grown in the presence of methotrexate, which blocks DHFR activity. This causes the transfected cells to deal with low DHFR activity by amplifying the copy number of DHFR, thus amplifying the copy number of the transfected gene of interest. The disadvantages of the CHO cell line is that these cells do not carry all of the sugar transferring enzymes (15) that are present in human cell lines. This may lead to the production of functionally nonrelevant proteins. Further, some of the posttranslational modifications of human proteins are not appropriately carried out in these cells (16). It has also been seen that expression levels are usually lower in CHO cells than in HEK293 cells (12, 17). Therefore, the human mammalian cell line, HEK293, is used for proteins that require functionally sensitive posttranslational modifications.

There are two mutant cell lines of HEK293, HEK293E, expressing EBNA-1, and HEK293T, expressing SV40 large T antigen, that increase the copy number of plasmids with EBV and SV40 origins of replication, respectively, thereby increasing expression levels of the protein of interest (13).

1.3. Expression and Labeling of Recombinant Proteins in Mammalian Cells

Uniform isotope labeling of proteins expressed in mammalian cells is still under development (18). This is because mammalian cells, like insect cells, are unable to grow in the type of minimal media that are used for bacteria where glucose or glycerol and ammonium chloride are the only sources of carbon and nitrogen, respectively. Higher eukaryotes, like the organisms they represent, require certain essential amino acids, without which they are unable to grow. Thus, the use of simple sources for ^{13}C and ^{15}N isotope labeling of proteins in bacteria is not possible for mammalian cells. A number of recent approaches, however, have been developed to address this complication. The first uniformly isotope-labeled protein purified from mammalian cells was obtained by Hansen et al. (19) followed by (20, 21). The method involved purification of a mixture of isotope labeled amino acids from an acid hydrolysate of algae or bacteria grown in $^{15}\text{NH}_4\text{Cl}$ and ^{13}C glucose or $^{13}\text{CO}_2$ to prepare uniformly labeled urokinase in a Sp2/0 mouse myeloma cell line. Different purification protocols of the labeled mixture were tested but only acid hydrolysis, which removes bacterial and algal products that are toxic to mammalian cells, proved to work. The mixture, however, needed to be supplemented with commercially available amino acids that would degrade during hydrolysis, particularly glutamine and cysteine. A commercially available form of ^{15}N -cysteine was used. Because of the high cost

involved in purchasing ^{15}N -glutamate, ^{15}N -, and $^{15}\text{N}/^{13}\text{C}$ -glutamine was synthesized from labeled glutamate (available commercially), $^{15}\text{NH}_4\text{Cl}$, and ATP. Dialyzed serum was used to prevent dilution of isotope labeled amino acids with unlabeled amino acids from the serum. Such purified amino acid mixtures are commercially available today. One such commercially available mixture from Martek Biosciences Corp. was tested to support expression in CHO cells (20). The growth medium was optimized in terms of its concentration, removal of amino acids, such as aspartic acid and asparagine, and addition of amino acids, such as arginine, cysteine, and glutamic acid after isolating and purifying from the mixture so that the highest level of protein expression was obtained. However, there are serious concerns regarding the toxic effects of feeding these mixtures to mammalian cells, leading to extensive methods of purification and optimization, and making it a time and cost inefficient method. HEK293 cells, the most commonly used system of expression for preparing isotope-labeled samples, have, to date, not been grown successfully in the presence of these mixtures (Klein-Seetharaman, unpublished results). An uniformly labeled ^{15}N TGF β 1 sample was prepared from CHO cells by growing them in Minimum Essential Medium (MEM, Gibco) with dialyzed serum, uniformly labeled ^{15}N labeled choline and uniformly ^{15}N -labeled amino acids (except tryptophan which was labeled only in the backbone nitrogen) (22), demonstrating the utility of CHO systems. The total cost involved was \$1,000/L. Therefore, this method could prove to be expensive if the protein to be labeled does not have high expression levels, as is the case with most membrane proteins.

Mammalian growth media containing a mixture of certain isotope-labeled amino acids are commercially available and have been recently used for labeling the G-protein coupled receptor, bovine rhodopsin, using ^{15}N labeled medium in which GKLQSTVW amino acids were ^{15}N labeled (23). If all the amino acids in the mixture do not cover the complete sequence of the protein to be labeled, then this method may not lead to complete labeling, as was the case with rhodopsin where 50% of amino acids were labeled (23). Apart from the high costs involved, an additional problem is the inability to perform perdeuteration because of the sensitivity of mammalian cells to deuterium oxide. Therefore, the most widely used method to obtain structural information on mammalian proteins by NMR has been by conducting amino acid type selective (AATS) isotope labeling. AATS labels a protein with specific isotope-labeled amino acids rather than uniform labels and yields structural information selectively for the amino acid(s) used in labeling. This has been successfully done with numerous proteins (24–26). Extensive AATS labeling has been carried out with rhodopsin, which was labeled by using ^{15}N -isotopes of tryptophan (26), lysine (25), histidine (27) and ^{13}C -isotopes of tryptophan (27), histidine (27) and glutamate (28), all through expression in HEK293 cells. ^{15}N -lysine, ^{13}C -glycine/serine double labeling has also been shown for rhodopsin (25). For example, multiple combinations of ^{15}N - and ^{13}C -isotope-labeled rhodopsin samples were used to assign ^{15}N -tryptophan resonances in the NMR spectrum of rhodopsin, shown in Fig. 1 (29). Since the protocol for such a labeling method has been optimized and works very well at least for rhodopsin, we are providing a step-by-step protocol below. The first step is the creation of an inducible stable cell line of rhodopsin in HEK293 cells. Scaling up the expression level is accomplished by growing these cells in a suspension culture, thereby producing the milligram quantities required for NMR studies. The yield of rhodopsin from such a transient transfection is on the order of 50 $\mu\text{g}/15\text{ cm}^2$ plate. After establishing the stable cell line with the opsin (apo form of rhodopsin) gene, the next step is to transfer these cells to suspension culture and grow them in isotope-labeled medium containing unlabeled amino acids along with the labeled amino acid(s) of interest. Up to 10 mg of rhodopsin can be obtained from such suspension cultures when amino acid supplements are provided (30), but typically 2 mg of rhodopsin are obtained from isotope labeled medium in which amino acid mixtures cannot be supplemented, to avoid dilution of the isotope-labeled amino acids provided in the medium.

2. Materials

2.1. Expression of Isotope-Labeled Recombinant Proteins

1. Plasmid containing the gene of interest (see Note 1).
2. HEK293 cells.
3. Dulbecco's modified Eagle medium (DMEM F-12).
4. Blasticidin (500 µg/mL stock solution prepared in DMEM F-12).
5. Geneticin, G418 (100 mg/mL stock solution prepared in DMEM F-12).
6. 0.05% Trypsin–EDTA (Gibco).
7. Penicillin–streptomycin (PS): 100 U/mL of each.
8. Fetal bovine serum (FBS).
9. Phosphate buffer saline (PBS): Autoclaved.
10. Tetracycline: 200 µg/mL (100× stock).
11. Sodium butyrate: 500 mM (100× stock), filtered.
12. Complete medium: DMEM F-12, 10% FBS, 1% PS.
13. Induction medium: Complete medium containing 2 µg/mL tetracycline and 5 mM sodium butyrate.
14. Selection medium: Complete medium containing 5 µg/mL blasticidin and 1, 2, or 3 mg/mL of G418.
15. Cryo medium: Complete medium containing 10% DMSO.
16. 2.5 M CaCl₂.
17. BES: 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, pH 7.2, 250 mM NaCl, 1.5 mM Na₂HPO₄, 1 M NaOH used to adjust pH.
18. 15- and 10-cm² Tissue culture plates.
19. 6- and 24-Well cell culture plates.
20. Sterile forceps.
21. Cloning rings.
22. Vacuum grease.
23. Dodecyl maltoside.

2.2. Specific Isotope Labeling of Proteins

Suspension DMEM medium:

1. Table 1 gives the individual components of suspension DMEM in their final concentrations based on the composition of commercially available DMEM. 100× stock solutions are prepared for most components (see Note 2). Each component is dissolved in distilled water.

¹A tetracycline (tet)-regulated mammalian expression vector should be used.

²All solutions were prepared as 100× concentrated stock solutions, except glucose, NaCl, glutamine, and the isotope-labeled amino acid(s), which were added as solids. Typically, 500 mL of stock solutions were prepared and filtered to maintain sterility. Sterile stock solutions were kept at 4°C and were stable for several weeks.

2. 0.05% trypsin–EDTA (Subheading 2.1).
3. FBS (Subheading 2.1).
4. PS (Subheading 2.1).
5. Dialyzed FBS: Heat inactivated, dialyzed against PBS and then filtered.
6. 10% Pluronic F-68 (100× stock), filtered.
7. 5 mg/mL Heparin (100× stock), filtered.
8. 15-cm² tissue culture plates.
9. 2-L spinner flasks.
10. 100× tetracycline (Subheading 2.1).
11. 100× sodium butyrate (Subheading 2.1).
12. 20% (w/v) glucose, sterile filtered.
13. 8% (w/v) NaHCO₃.
14. Complete medium (Subheading 2.1).
15. Selection medium (Subheading 2.1).

3. Methods

3.1. Expression of Isotope-Labeled Recombinant Proteins

Following is a protocol for generating an inducible stable cell line of HEK293 (30–32).

1. Wake up HEK293 cells from cryostocks and maintain them in 15 cm² plates in 25 mL of complete medium containing 5µg/mL blasticidin.
2. Split the cells into a 10-cm² dish at 80% confluency.
3. Split the cells 1:10 or 1:8 (~1–2 million cells per plate) into complete medium containing 5µg/mL blasticidin the day before transfection.
4. On the day of transfection, the cells should be 30–40% confluent. To transfect the cells, prepare the following cocktail, adding components in the order given (volumes are for one 10-cm² plate): In a falcon tube, mix 30µg of plasmid DNA, 50µL of 2.5 M CaCl₂, 500µL of BES, and sterile water to 1 mL. Incubate the mixture for exactly 1 min and gently add it to the cells. After 1 h, verify the efficiency of transfection by the presence of calcium phosphate precipitate which appears as fine sand particles between cells.
5. Incubate the plates at 35°C and 3% CO₂ for 19 h.
6. After 19 h, wash the plates twice with complete medium containing 5µg/mL blasticidin and add fresh complete medium containing 5µg/mL blasticidin.
7. Incubate the cells at 37°C and 5% CO₂ overnight.
8. Split the cells 1:10 (0.5–1 million cells/plate) into nine plates, three plates each for selection with G418 (1, 2, and 3 mg/mL).
9. Replace the medium after 20 h with selection medium.
10. Replace the selection medium every 2–3 days until colonies of workable size are formed.

11. On the day of picking the clones:
 - a. Circle the colonies that are to be picked such that the circles are big enough to place the cloning rings on its perimeter.
 - b. Aspirate the medium and look for more colonies on the empty plate by eye. Wash with 8 mL of PBS.
 - c. Using sterile forceps, dip one end (base) of each cloning ring in vacuum grease and place it over each clone (see Note 3). Make sure there is no grease on the inner walls of the rings. Make sure that the rings form a well around the clones with a leak proof seal at the bottom.
 - d. Add 40 μ L of 0.05% trypsin–EDTA to each well and incubate for 1 min. Then, add 80 μ L of fresh selection medium and gently pipette up and down. Transfer the cells (100 μ L) to a 24-well plate containing 1 mL of selection medium per well.
 - e. Repeat for all the clones.
12. Replace the selection medium every 2–3 days until the cells are confluent. Make a note of medium changes and transfers to 6-well plates in a chart (see step 13 for details).
13. As the cells become confluent, transfer them to two 6-well plates at different cell densities. For the transfer:
 - a. Pipette 3 mL of selection medium into each well of a 6-well plate.
 - b. Aspirate the medium from the cells (24-well plate) and add 300 μ L of 0.05% trypsin–EDTA to each well and incubate for 1 min.
 - c. Add 1,200 μ L (use a 1-mL pipette and add 600 + 600 μ L) of selection medium and gently pipette up down.
 - d. Remove 300 μ L and add it to one well and place the remaining (1,200 μ L) in another well. The well with higher cell density will be induced in the future to screen the clones while the one with lower density will be maintained and used for making glycerol stocks.
14. Change the selection medium on the cells every 2–3 days.
15. As soon as the cells in the higher density well reach confluence, induce with induction medium.
16. Harvest the cells 48-h post induction.
 - a. After harvesting, solubilize the cells in a detergent suitable for the membrane protein of interest (e.g., 1% dodecyl maltoside).
 - b. Centrifuge the solubilized cells at $126,000 \times g$ for 20 min at room temperature and collect the supernatant.
17. Check for levels of expression by performing a Western dot blot on serial dilutions of the supernatant in PBS, containing 1% of the detergent used for solubilization, versus protein samples of known concentrations (see Note 4).

³Cloning rings, vacuum grease, and forceps should be autoclaved.

⁴PVDF membranes should not be used.

18. When the cells from the low density well reach confluence, split 1:5 into two 10-cm² cell culture dishes. Add 0.5 mL of 0.05% trypsin–EDTA to a 6-well plate, and then add 1.5 mL of selection medium. Add 1 mL of this cell suspension to each of the two 10-cm² plates.
19. After the cells reach confluence on the 10-cm² plate, prepare three 1-mL glycerol stocks from each plate. To prepare glycerol stocks:
 - a. Wash 70–90% confluent plates twice with PBS and trypsinize with 1 mL of 0.05% trypsin–EDTA for 1 min.
 - b. Add 10 mL of complete medium (no selection) and collect the cells in a 15-mL falcon tube.
 - c. Centrifuge the cells at $600 \times g$ for 10 min at 4°C.
 - d. Aspirate the medium and gently resuspend the cells in 3 mL of cryo medium.
 - e. Transfer the cells to cryo vials in 1-mL aliquots and place the tubes in cryo boxes. Incubate at –20°C for 1 h, and then –80°C overnight.
 - f. On the following day, move the tubes to liquid nitrogen storage tanks.

3.2. Specific Isotope Labeling of Proteins

After estimating the yield from above (Subheading 3.1) and identifying the highest yield clone, the following procedure is used to prepare a protein sample, in suspension culture, in which particular amino acids are specifically isotope labeled (30–32).

1. Grow the highest yielding stable cell line clone in complete medium.
2. Split the cells 1:5 into 10-cm² dishes after ~3 days using 15 mL of selection medium.
3. Split the cells until a sufficient number of plates are obtained for setting up spinner flasks. For reference, 1 mg of protein per liter of suspension culture is usually obtained for rhodopsin and 3–4 confluent 15-cm² plates are used to inoculate a 500-mL flask (see Notes 5 and 6).
4. Prepare labeled suspension DMEM medium from 100× stocks of the individual components (Table 1, Subheading 2.2), except for the isotope labeled amino acid(s). Supplement with 10% dialyzed FBS and 1% PS or obtain isotope-labeled medium from a commercial source.
 - a. Add glucose, NaCl, glutamine, isotope-labeled amino acid(s) as solids.
 - b. Lower the glutamine concentration to half when starting the suspension culture.
 - c. Add Pluronic F-68 and heparin to 1× concentration.
 - d. Add isotope-labeled amino acid(s) as solids.
5. Inoculate the suspension cultures and grow at 37°C, spinning at 47 rpm for 6 days. To inoculate, add 2 mL of 0.05% trypsin–EDTA to each plate and extract the cells with 8 mL of unlabeled suspension medium. Collect cells from each plate (10-mL

⁵Cells should be counted before adding them to spinner flasks so that the appropriate number of cells (in the range between 60 and 90 million cells/500 mL) can be transferred.

⁶A 2-L spinner flask is ideal for a 500-mL suspension culture.

volume) and centrifuge at $1,000 \times g$ for 10 min at 4°C. Aspirate the medium and add 10 mL of labeled suspension DMEM medium to each plate and resuspend the cells. Add the cell suspension to the suspension culture in the spinner flask.

6. After 6 days (see Note 7), supplement the growth medium with 6 mL of 20% (w/v) glucose and 4 mL of 8% (w/v) NaHCO_3 and induce expression with 5 mM sodium butyrate and 2 $\mu\text{g/mL}$ of tetracycline.
7. After the 6th day, feed the cells with 6 mL of 20% glucose every 24 h.
8. Grow the cells for 2 more days after the 6th day (total 8 days in suspension).
9. Harvest at the end of 8 days.

4. Conclusions

A variety of expression systems have been developed that can be used to express a protein of choice in isotope-labeled form, each system having advantages and disadvantages. These systems include bacterial, yeast, insect, and mammalian cells. The choice of the system largely depends on the type of the protein to be expressed. Other factors are the costs involved, the need for modifications and yield of protein required. Bacterial expression systems are most commonly used for soluble protein expression due to ease of use and cost-effectiveness in cloning and expression. However, this system is not the system of choice for proteins that require posttranslational modification for their activity. In such cases, eukaryotic expression systems are required. Yeast being both eukaryotic and a microorganism is a simple system to use, allowing relatively inexpensive complete isotope labeling of proteins for NMR studies. Although it can carry out some posttranslational modifications, these are often not sufficient and deficiency in folding machinery can be a problem for some proteins, especially membrane proteins. For these reasons, higher eukaryotic systems, such as insect cells have been in use where posttranslational modifications are very similar to those found in mammalian cells. The greatest advantage of insect cell expression is the very large amounts of proteins that can be expressed, in most cases more than that in mammalian cells. However, disadvantages of expensive isotope-labeled media, incomplete labeling and intolerance to perdeuteration that hamper mammalian cell expression are also prevalent in insect cells. Hence, efforts are ongoing toward further developing mammalian expression systems since these are the only systems in which the full complement of folding and posttranslational modification machineries are available, optimally supporting the activity of the expressed protein.

References

1. Nettleship JE, Assenberg R, Diprose JM, Rahman-Huq N, Owens RJ. Recent advances in the production of proteins in insect and mammalian cells for structural biology. *J Struct Biol.* 2010; 172:55–65. [PubMed: 20153433]
2. Aricescu AR, Lu W, Jones EY. A time- and cost-efficient system for high-level protein production in mammalian cells. *Acta Crystallogr D Biol Crystallogr.* 2006; 62:1243–1250. [PubMed: 17001101]
3. Lee JE, Fusco ML, Ollmann Saphire E. An efficient platform for screening expression and crystallization of glycoproteins produced in human cells. *Nat Protoc.* 2009; 4:592–604. [PubMed: 19373230]
4. Nettleship JE, Rahman-Huq N, Owens RJ. The production of glycoproteins by transient expression in mammalian cells. *Methods Mol Biol.* 2009; 498:245–263. [PubMed: 18988030]

⁷The color of the medium should turn yellow after 6 days.

5. Reeves PJ, Callewaert N, Contreras R, Khorana HG. Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. *Proc Natl Acad Sci USA*. 2002; 99:13419–13424. [PubMed: 12370423]
6. Scimanti CR, Calos MP. Epstein-Barr virus vectors for gene expression and transfer. *Curr Opin Biotechnol*. 1998; 9:476–479. [PubMed: 9821275]
7. Sambrook J, Rodgers L, White J, Gething MJ. Lines of BPV-transformed murine cells that constitutively express influenza virus hemagglutinin. *EMBO J*. 1985; 4:91–103. [PubMed: 2990897]
8. Grossi MP, Caputo A, Rimessi P, Chiccoli L, Balboni PG, Barbanti-Brodano G. New BK virus episomal vector for complementary DNA expression in human cells. *Arch Virol*. 1988; 102:275–283. [PubMed: 2849391]
9. Piechaczek C, Fetzner C, Baiker A, Bode J, Lipps HJ. A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells. *Nucleic Acids Res*. 1999; 27:426–428. [PubMed: 9862961]
10. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*. CSH Laboratory Press; Cold Spring Harbor, NY: 1989.
11. Doerfler W, Schubert R, Heller H, Kammer C, Hilger-Eversheim K, Knoblauch M, Remus R. Integration of foreign DNA and its consequences in mammalian systems. *Trends Biotechnol*. 1997; 15:297–301. [PubMed: 9263477]
12. Xia W, Bringmann P, McClary J, Jones PP, Manzana W, Zhu Y, Wang S, Liu Y, Harvey S, Madlansacay MR, McLean K, Rosser MP, MacRobbie J, Olsen CL, Cobb RR. High levels of protein expression using different mammalian CMV promoters in several cell lines. *Protein Expr Purif*. 2006; 45:115–124. [PubMed: 16289982]
13. Van Craenenbroeck K, Vanhoenacker P, Haegeman G. Episomal vectors for gene expression in mammalian cells. *Eur J Biochem*. 2000; 267:5665–5678. [PubMed: 10971576]
14. Urlaub G, Chasin LA. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc Natl Acad Sci USA*. 1980; 77:4216–4220. [PubMed: 6933469]
15. Grabenhorst E, Schlenke P, Pohl S, Nimtz M, Conradt HS. Genetic engineering of recombinant glycoproteins and the glycosylation pathway in mammalian host cells. *Glycoconj J*. 1999; 16:81–97. [PubMed: 10612409]
16. Suttie, JW. *Thromb Res; Report of Workshop on expression of vitamin K-dependent proteins in bacterial and mammalian cells; Madison, Wisconsin, USA. April 1986; 1986. p. 129-134.*
17. Schlaeger EJ, Kitas EA, Dorn A. SEAP expression in transiently transfected mammalian cells grown in serum-free suspension culture. *Cytotechnology*. 2003; 42:47–55. [PubMed: 19002927]
18. Takahashi H, Shimada I. Production of isotopically labeled heterologous proteins in non-*E. coli* prokaryotic and eukaryotic cells. *J Biomol NMR*. 2010; 46:3–10. [PubMed: 19787297]
19. Hansen AP, Petros AM, Mazar AP, Pederson TM, Rueter A, Fesik SW. A practical method for uniform isotopic labeling of recombinant proteins in mammalian cells. *Biochemistry*. 1992; 31:12713–12718. [PubMed: 1463742]
20. Lustbader JW, Birken S, Pollak S, Pound A, Chait BT, Mirza UA, Ramnarain S, Canfield RE, Brown JM. Expression of human chorionic gonadotropin uniformly labeled with NMR isotopes in Chinese hamster ovary cells: an advance toward rapid determination of glycoprotein structures. *J Biomol NMR*. 1996; 7:295–304. [PubMed: 8765736]
21. Shindo K, Masuda K, Takahashi H, Arata Y, Shimada I. Backbone ^1H , ^{13}C , and ^{15}N resonance assignments of the anti-dansyl antibody Fv fragment. *J Biomol NMR*. 2000; 17:357–358. [PubMed: 11014603]
22. Archer SJ, Bax A, Roberts AB, Sporn MB, Ogawa Y, Piez KA, Weatherbee JA, Tsang ML, Lucas R, Zheng BL, et al. Transforming growth factor beta 1: NMR signal assignments of the recombinant protein expressed and isotopically enriched using Chinese hamster ovary cells. *Biochemistry*. 1993; 32:1152–1163. [PubMed: 8424942]
23. Werner K, Richter C, Klein-Seetharaman J, Schwalbe H. Isotope labeling of mammalian GPCRs in HEK293 cells and characterization of the C-terminus of bovine rhodopsin by high resolution liquid NMR spectroscopy. *J Biomol NMR*. 2008; 40:49–53. [PubMed: 17999150]

24. Arata Y, Kato K, Takahashi H, Shimada I. Nuclear magnetic resonance study of antibodies: a multinuclear approach. *Methods Enzymol.* 1994; 239:440–464. [PubMed: 7830594]
25. Klein-Seetharaman J, Reeves PJ, Loewen MC, Getmanova EV, Chung J, Schwalbe H, Wright PE, Khorana HG. Solution NMR spectroscopy of (α - ^{15}N) lysine-labeled rhodopsin: The single peak observed in both conventional and TROSY-type HSQC spectra is ascribed to Lys-339 in the carboxyl-terminal peptide sequence. *Proc Natl Acad Sci USA.* 2002; 99:3452–3457. [PubMed: 11904408]
26. Klein-Seetharaman J, Yanamala NV, Javeed F, Reeves PJ, Getmanova EV, Loewen MC, Schwalbe H, Khorana HG. Differential dynamics in the G protein-coupled receptor rhodopsin revealed by solution NMR. *Proc Natl Acad Sci USA.* 2004; 101:3409–3413. [PubMed: 14990789]
27. Patel AB, Crocker E, Reeves PJ, Getmanova EV, Eilers M, Khorana HG, Smith SO. Changes in interhelical hydrogen bonding upon rhodopsin activation. *J Mol Biol.* 2005; 347:803–812. [PubMed: 15769471]
28. Han M, Smith SO. High-resolution structural studies of the retinal–Glu113 interaction in rhodopsin. *Biophys Chem.* 1995; 56:23–29. [PubMed: 7662866]
29. Werner K, Lehner I, Dhiman HK, Richter C, Glaubitz C, Schwalbe H, Klein-Seetharaman J, Khorana HG. Combined solid state and solution NMR studies of α , ϵ - ^{15}N labeled bovine rhodopsin. *J Biomol NMR.* 2007; 37:303–312. [PubMed: 17318366]
30. Reeves PJ, Kim JM, Khorana HG. Structure and function in rhodopsin: a tetracycline-inducible system in stable mammalian cell lines for high-level expression of opsin mutants. *Proc Natl Acad Sci USA.* 2002; 99:13413–13418. [PubMed: 12370422]
31. Reeves PJ, Callewaert N, Contreras R, Khorana HG. Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line 452. *Proc Natl Acad Sci USA.* 2002; 99:13419–13424. [PubMed: 12370423]
32. Reeves PJ, Thurmond RL, Khorana HG. Structure and function in rhodopsin: high level expression of a synthetic bovine opsin gene and its mutants in stable mammalian cell lines. *Proc Natl Acad Sci USA.* 1996; 93:11487–11492. [PubMed: 8876162]

Fig. 1. Dephased (red line) and nondephased (blue line) ^{15}N detected $^{13}\text{C}/^{15}\text{N}$ CP REDOR spectra of selectively labeled rhodopsin in DOPC lipid bilayers at 220 K. (a) $\alpha,\epsilon\text{-}^{15}\text{N-Trp}$; (b) $^{13}\text{C}'\text{-Leu}/\alpha,\epsilon\text{-}^{15}\text{N-Trp}$; (c) $^{13}\text{U-Thr}/\alpha,\epsilon\text{-}^{15}\text{N-Trp}$; (d) $^{13}\text{C}'\text{-Cys}/\alpha,\epsilon\text{-}^{15}\text{N-Trp}$; (e) $^{13}\text{C}'\text{-Pro}/\alpha,\epsilon\text{-}^{15}\text{N-Trp}$; (f) $^{13}\text{C}'\text{-Gly}/\alpha,\epsilon\text{-}^{15}\text{N-Trp}$. Figure reproduced with permission from (29).

Table 1

Essential amino acid	mg/L	Nonessential amino acid	mg/L	Vitamins	mg/L	Inorganic salts and other	mg/L
Arginine ·HCl	84	Alanine	None	D-Ca pantothenate	4	CaCl ₂	50
Histidine ·HCl·H ₂ O	42	Asparagine	None	Choline Chloride	4	Fe(NO ₃) ₃ ·9H ₂ O	0.1
Isoleucine	105	Aspartate	None	Folic Acid	4	MgSO ₄	97.7
Leucine	105	Cystine ·2HCl	63	i-Inositol	7.2	KCl	400
Lysine ·HCl	146	Glutamate	None	Niacinamide	4	NaCl	6,400
Methionine	30	Glutamine	584	Pyridoxal ·HCl	4	NaH ₂ PO ₄ ·H ₂ O	125
Phenylalanine	66	Glycine	30	Riboflavin	0.4		
Threonine	95	Proline	None	Thiamine ·HCl	4		
Tryptophan	16	Serine	42			Phenol Red ·Na ⁺	15
Valine	94	Tyrosine ·2Na ⁺ ·2H ₂ O	104			Glucose	4,500