Pergamon

Biochemical Pharmacology, Vol. 48, No. 6, pp. 1231–1243, 1994.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(94)00294-0

EXPRESSION OF A CANNABINOID RECEPTOR IN BACULOVIRUS-INFECTED INSECT CELLS

DENISE A. DOVE PETTIT, VINCENT M. SHOWALTER,* MARY E. ABOOD* and GUY A. CABRAL†

Departments of Microbiology/Immunology and *Pharmacology/Toxicology, Medical College of Virginia/Virginia Commonwealth University, Richmond, VA 23298-0678, U.S.A.

(*Received* 7 *February* 1994; *accepted* 4 *May* 1994)

Abstract—A cannabinoid receptor recombinant baculovirus (AcNPV-THCR) has been constructed and employed to express rat neural cannabinoid receptors. Northern analysis of total RNA from *Spodoptera frugiperda* (Sf9) insect cells infected with AcNPV-THCR revealed novel hyper-production of a 3.3 kb transcript when probed with nick-translated rat cannabinoid receptor cDNA. Optimal viral protein expression was observed in ^{35}S-metabolically labeled AcNPV-THCR-infected Sf9 cells at a multiplicity of infection of 2.5. Transmission electron microscopy of AcNPV-THCR-infected Sf9 cells showed extensive membrane perturbation and electron-dense cytoplasmic perinuclear accumulation, indicative of receptor glycoprotein expression. Immunofluorescence staining using antiserum produced to a fusion protein consisting of the external domain of the cannabinoid receptor and hepatitis B core antigen revealed cannabinoid receptor expression in AcNPV-THCR-infected Sf9 cells. Scatchard–Rosenthal analysis of [^{3}H]CP55,940 receptor binding indicated a K_d of 3.4 nM and a B_{max} equal to 3.17 pmol/mg protein. Western immunoblotting performed on AcNPV-THCR-infected Sf9 cell lysates revealed immunoreactive bands with relative molecular weights ranging from 45 to 79 kDa. The predominant species (55 kDa) exhibited a relative molecular weight consistent with that predicted for the translational product obtained from the cannabinoid receptor cDNA coding sequence. *In vitro* translation using AcNPV-THCR mRNA also yielded a 55 kDa immunoreactive species. These data indicate that the baculovirus expression system is a viable means of expressing relatively large quantities of cannabinoid receptor recombinant protein.

Key words: radioligand binding; cannabinoid receptor; G protein-coupled receptor; baculovirus expression system; delta-9-tetrahydrocannabinol; anti-cannabinoid receptor antiserum

THC‡, the major psychoactive component in marijuana, can produce a multiplicity of effects in humans, including alterations in mood, perception, cognition, memory, psychomotor activity, as well as analgesia, antiemesis, and immunosuppression [1, 2]. It is unlikely that one mechanism is responsible for the multiplicity of effects observed with THC exposure. Indeed, substantial evidence exists for two possible mechanisms for THC-induced cellular effects. The highly lipophilic nature of THC has suggested that some of the cannabinoid-induced effects are due to membrane perturbation [3–5]. Such disruption of cellular membranes may alter signal transduction pathways, stimulate or inhibit membrane-associated enzymes, and alter membrane-associated ion channels. However, the stereospecificity and structural requirements for physiological activity suggest that the cannabinoids also can act via a specific receptor [6–10].

Cannabinoid-induced inhibition of cAMP accumulation in a mouse neuroblastoma cell line, N18TG2, was the first definitive evidence for the presence of cannabinoid receptors in neural tissue [11]. Radioligand binding studies that utilized a potent tritiated THC analog, CP55,940, further substantiated the presence of cannabinoid receptors in neural tissue [12]. Anatomical distribution of the cannabinoid receptor in rat brain was characterized by receptor autoradiography using [^{3}H]CP55,940, and dense binding was observed in cerebellum, hippocampus, and basal ganglia [13, 14]. The serendipitous discovery of a cDNA clone, isolated from a rat cerebral cortex cDNA library, shown to bind and functionally interact with cannabinoids, has allowed for the molecular characterization of this receptor [5, 15]. Sequence analysis of this clone has revealed a high level of homology with GPCR in that it contains an external domain that possesses glycosylation sites, seven conserved transmembrane domains, and an internal or cytoplasmic domain that is thought to couple to a G-inhibitory protein complex.

† Corresponding author: Dr. Guy A. Cabral, Medical College of Virginia, Virginia Commonwealth University, Box 980678 MCV Station, Richmond, VA 23298-0678. Tel. (804) 828-2306; FAX (804) 828-9946.

‡ Abbreviations: THC, delta-9-tetrahydrocannabinol; AcNPV, *Autographa californica* multiply enveloped nuclear polyhedrosis virus; AcNPV-β-Gal, β-galactosidase recombinant baculovirus; BB, blocking buffer; THCR, cannabinoid receptor; AcNPV-THCR, cannabinoid receptor recombinant baculovirus; GPCR, G protein-coupled receptor; HBcAg, hepatitis B core antigen; HBcAg-THCR, hepatitis B core antigen cannabinoid receptor fusion protein; MOI, multiplicity of infection; PMSF, phenylmethylsulfonyl fluoride; pi, post-infection; Sf9, *Spodoptera frugiperda*; TCA, trichloroacetic acid; TBS, Tris-buffered saline; and PCR, polymerase chain reaction.

Biochemical and biophysical characterization of the THCR is particularly challenging since GPCRs are not expressed naturally in great abundance and are relatively unstable. This low level of expression makes studying the receptor's binding site, its functional domains, and conformation difficult since unrealistic amounts of tissue would be necessary to obtain ample native receptor. Furthermore, the role that post-translational modification, such as glycosylation, plays in receptor–ligand interactions cannot be studied *in vivo*. Therefore, it is desirable to establish an *in vitro* system that allows for production of relatively large amounts of recombinant receptor protein capable of binding cannabinoid analogs and for genetic manipulation and expression of structurally modified, recombinant receptor protein.

The baculovirus system is attractive for high level expression of eukaryotic proteins, particularly receptors, since processing of membrane glycoproteins is similar to that in mammalian cells. Indeed, many laboratories have reported a significant increase in GPCR production in infected insect cells when compared with GPCR production in other eukaryotic expression systems [16, 17]. Furthermore, the use of a transfer vector (plasmid) in the production of recombinant virus makes the genetic manipulations required for altering receptor structure feasible. For these reasons, the baculovirus expression system has been analyzed for its efficacy in the production of recombinant cannabinoid receptor.

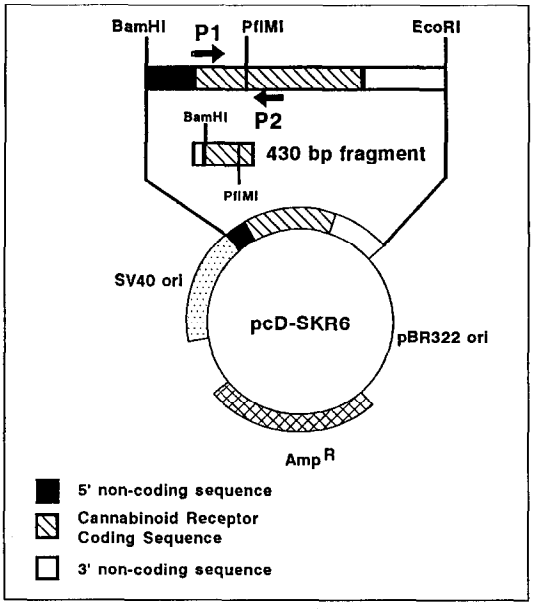

Fig. 1. PCR amplification of the start of the cannabinoid receptor coding sequence. PCR amplification using primers P1 and P2 allowed for the production of a 430 bp fragment representing the start of the cannabinoid receptor coding sequence. This fragment was employed to replace coding sequence that was extracted in the digestion utilized to remove the 5′ non-coding sequence.

MATERIALS AND METHODS

Cell culture and viral propagation. Sf9 cells (Invitrogen Corp., San Diego, CA) were cultured at 27° in Grace's insect medium (Sigma Chemical Co., St Louis, MO) supplemented with 1.25 g Yeastolate, 1.25 g lactalbumin hydrosylate (Difco Laboratories, Detroit, MI), and 10% fetal bovine serum (Biowhittaker, Walkersville, MD). Cell culture, viral propagation, plaque assay, virus purification, and metabolic labeling were carried out as previously described by others [18].

Construction of the THCR recombinant baculovirus. The plasmid, pCD-SKR6, containing the rat cannabinoid receptor cDNA was a gift from Dr. Lisa Matsuda (Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina). All plasmids were replicated in INV1 αF′ *Escherichia coli* (Invitrogen Corp.) and purified using the Qiagen Plasmid Kit (Qiagen, Charsworth, CA). To optimize protein expression, the 5′ non-coding sequence in the cannabinoid receptor cDNA was removed using a PCR subcloning strategy (Fig. 1). The following oligonucleotide primers (P1 and P2) were synthesized and used to amplify a 430 bp fragment containing the initiation codon of the cannabinoid receptor and the start of the cannabinoid receptor coding sequence:

P1: ATC AGG ATC CGA GGT TAT
P2: GAT GAC ACA CAG CAC CAG

P1 was engineered to contain a *Bam*HI site (underlined) to allow for ligation into the parent plasmid following *Bam*HI digestion. The P2 sequence was selected to allow for the amplification of a unique *Pfl*MI site within the coding sequence. The PCR reaction was carried out as previously described [19], using a DNA Thermal Cycler (Perkin–Elmer Corp., Norwalk, CT) that was programmed for 35 cycles in the following manner: 1 min denaturation at 94°, a 1 min annealing step at 52°, and a 1 min extension period at 72°.

Digestion of pCD-SKR6 with *Bam*HI and *Pfl*MI allowed for the removal of the 5′ non-coding sequence and of approximately 360 bp of coding sequence. Digestion of the amplified 430 bp fragment, using the same restriction enzymes, and subsequent ligation into the digested parent plasmid, allowed for the replacement of the removed coding sequence that resulted in the pcD-THCR construct. All restriction enzymes used were purchased from New England BioLaboratories (Beverly, MA). The sequence of the PCR amplified product was confirmed using two separate clones, the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corp., Cleveland, OH), and the P1 and P2 primers described above. The pcD-THCR construct then was used to produce a recombinant baculovirus transfer vector (Fig. 2A). Both pcD-THCR and pVL 1393 (Invitrogen Corp.) were digested with *Bam*HI and *Eco*RI. The 2.0 kb fragment corresponding to the cannabinoid receptor coding sequence from pcD-THCR was isolated using agarose gel electrophoresis and the GlassMAX DNA isolation spin cartridge system (Gibco BRL, Gaithersburg, MD). The fragment was ligated (T4

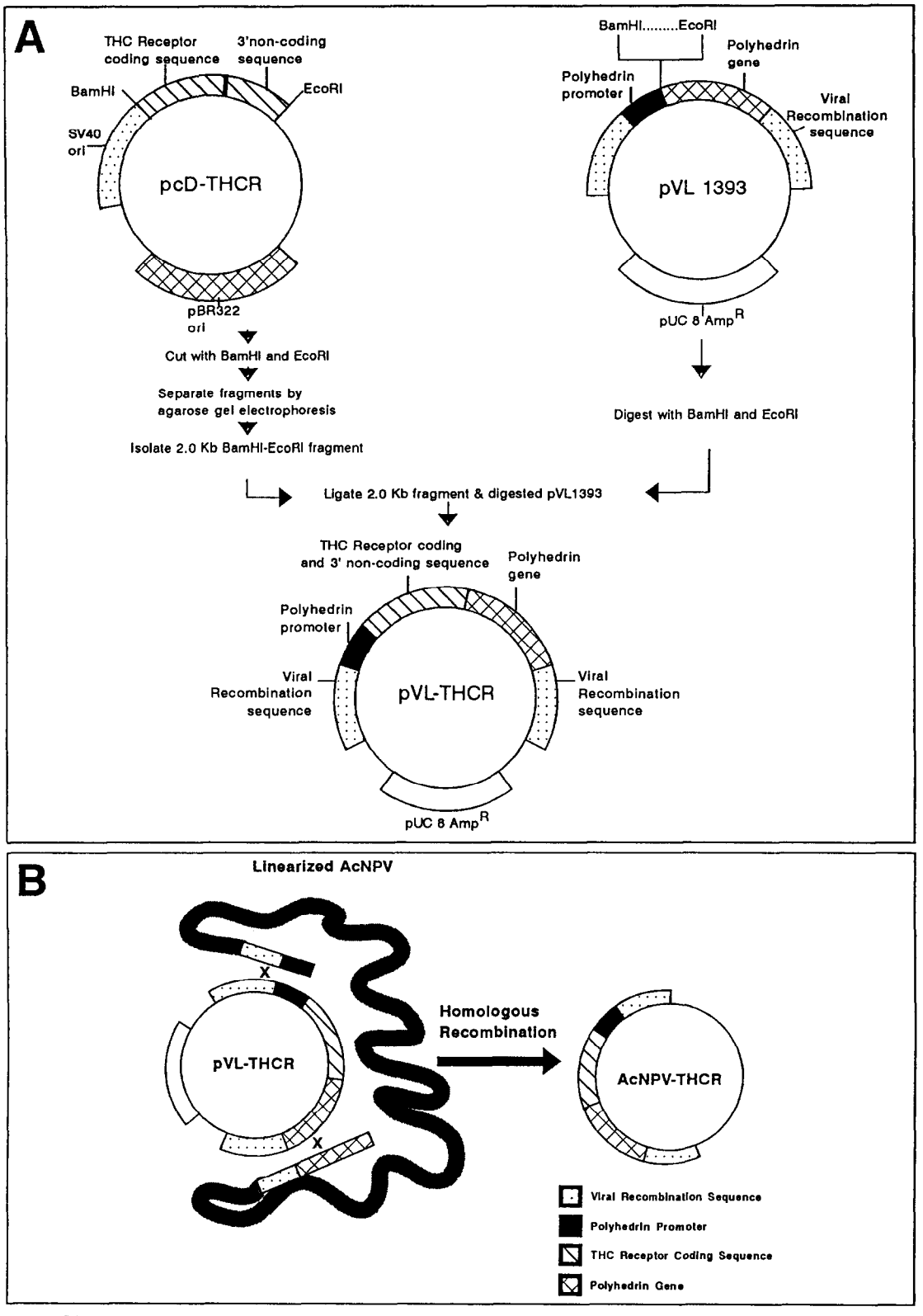

Fig. 2. Production of a cannabinoid receptor recombinant baculovirus. (A) The cannabinoid receptor cDNA found within pcD-THCR was subcloned into the baculovirus transfer vector pVL 1393 by *Bam*HI and *Eco*RI digestion. (B) The ligation resulted in the pVL-THCR construct that was used along with linearized AcNPV DNA to co-transfect Sf9 insect cells. Co-transfection resulted in homologous recombination and the production of a recombinant baculovirus containing the cannabinoid receptor cDNA.

Ligase, Gibco BRL) with digested pVL 1393, resulting in the construction of pVL-THCR.

The BaculoGold Transfection Kit (Pharmingen, San Diego, CA) was employed to produce a cannabinoid receptor recombinant baculovirus. Briefly, Sf9 cells were co-transfected with linearized AcNPV and pVL-THCR DNAs. Homologous recombination resulted in the production of AcNPV-THCR containing the coding sequence for the cannabinoid receptor (Fig. 2B).

Isolation and identification of recombinant baculovirus. Cell culture supernatant collected 4 days post-co-transfection was used as a source of AcNPV-THCR. The supernatant was quantitated for recombinant virus by plaque assay, and several plaques were purified three times and amplified to make viral stocks. Viral stocks were used to inoculate Sf9 cells at a MOI of 20, and infected cells were harvested 4 days pi. Total RNA from infected cells was isolated using the guanidinium isothiocyanate/cesium chloride method [20]. The RNA was separated by electrophoresis in a 1% agarose-formaldehyde gel and blot-transferred onto a nitrocellulose membrane. The blots were baked for 2 hr at 80° in a vacuum oven and then were incubated for 4 hr at 37° in prehybridization buffer containing 50% formamide, 5× SSC (20× SSC = 3.0 M NaCl, 0.3 M $Na_3C_6H_5O_4$, pH 7.0), 0.1% BSA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1 M NaH_2PO_4 and 20 μg/mL sheared and denatured salmon sperm DNA. Then blots were hybridized for 18 hr at 37° in hybridization buffer (prehybridization buffer containing 5% dextran sulfate and nick-translated cannabinoid receptor cDNA) and were washed using the following regimen: four 5 min washes in 2× SSC and 0.2% SDS at 37°; one 20 min wash in 2× SSC and 0.2% SDS at 37°; and a final 20 min wash in 0.5× SSC and 1% SDS at 37°. Autoradiography was carried out at room temperature for 6 hr using XRP-5 X-ray film (Eastman Kodak Co., Rochester, NY) and intensifying screens. Total RNA from rat brain, the predominant location for cannabinoid receptor expression, was obtained and processed as described above to serve as a positive quantitative control for cannabinoid receptor mRNA. Equivalent loading and quality of the RNA were determined by ethidium bromide staining of the gel before northern transfer.

The probe used in the hybridization buffer was generated from pcD-SKR6, which was digested with *Bam*HI and *Eco*RI. The resulting fragments were separated using gel electrophoresis and a 2.4 kb fragment containing the cDNA for the receptor was excised, eluted (Centrilutor, Amicon, Beverly, MA), and purified using organic solvent extractions. The receptor cDNA was ^{32}P-labeled ([^{32}P]dCTP; DuPont-New England Nuclear, Boston, MA) by nick-translation (Bethesda Research Laboratories, Life Technologies, Inc., Bethesda, MD) to yield a specific activity $>10^8$ dpm/μg. Autoradiograms were analyzed in a Shimadzu CS9000U Dual-Wavelength Flying-Spot Scanner (Shimadzu Corp., Kyoto, Japan) interfaced to a WIN 286 computer (Win Laboratories Ltd., Fairfax, VA) employing Quantascan 2D Analysis binary format and ASCII Conversion software (Shimadzu Corp.).

Viral protein expression and ^{35}S-metabolic labeling. Recombinant viral protein expression was monitored initially by metabolically labeling proteins synthesized in Sf9 cells infected with either AcNPV-THCR or a control AcNPV-β-Gal (Invitrogen Corp.) at a MOI of 20. To establish optimal conditions for recombinant cannabinoid receptor expression, Sf9 cells were infected at various multiplicities (MOI 2.5, 5, 10, 20), and metabolically labeled recombinant protein was analyzed. Briefly, Sf9 cells were seeded at 1×10^6 cells per 35 mm Primaria tissue culture dish (Becton Dickinson Labware, Lincoln Park, NJ). Cells were allowed to adhere for 1 hr prior to viral inoculation with either AcNPV-THCR or AcNPV-β-Gal. After a 1 hr exposure to virus, medium was removed and fresh medium was added to each dish. Following a 36 hr incubation, 100 μCi of [^{35}S]-Translabel (ICN Biomedicals, Inc., Costa Mesa, CA) was added to cultures for a period of 24 hr, and cells were harvested at 60 hr pi and solubilized in a 1% Triton X-100/PMSF buffer (0.14 M NaCl, 0.01 M Tris, pH 8.0, 0.1% NaN_3, 0.2 mM PMSF). Biosynthetic incorporation of radiolabel was quantitated by TCA precipitation using a Skatron Acid Precipitation Filtration Device (Skatron Instruments, Sterling, VA). A constant amount of protein was loaded into each sample well of a gradient SDS–polyacrylamide gel (7–20%), which was used to separate recombinant baculovirus-expressed proteins. ^{14}C-Labeled protein molecular weight standards (molecular weight range 14.3 kDa to 200 kDa; Gibco BRL) were included in each gel analysis. Gels were electrophoresed, fixed (30% methanol, 10% glacial acetic acid), vacuum-dried, and exposed to XRP-5 diagnostic film (Eastman Kodak Co.) to allow for determination of recombinant viral-specified protein expression.

Transmission electron microscopy. Sf9 cells were seeded at 1×10^6 cells per 35 mm tissue culture dish. Cells were allowed to adhere for 1 hr prior to viral inoculation. Medium was removed from the culture after a 1 hr exposure to virus. Cells were harvested at 12 hr intervals starting at 0 hr pi and ending at 72 hr pi and were washed two times in PBS (0.15 M NaCl, 0.2 M Na_2PO_4, pH 7.2). Infected cells were fixed for 1 hr at 27° in pre-warmed (27°) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Cells were stored in fixative at 4° until processed. Cells were pelleted by centrifugation and were washed gently two times with 0.1 M cacodylate buffer and post-fixed for 1 hr in 2% osmium tetroxide in 0.1 M cacodylate buffer. The cells were centrifuged, washed two times with cacodylate buffer, dehydrated through a graded series of ethanol, and imbedded in Poly-Bed 812 (Polyscience, Warrington, PA) resin. Thin sections were stained with saturated aqueous uranyl acetate and lead citrate, and were examined in a Zeiss EM10 transmission electron microscope (Carl Zeiss, New York, NY) operating at an accelerating voltage of 80 kV.

Polyclonal antiserum production and immunofluorescence staining. Polyclonal antiserum to the external domain of the cannabinoid receptor, amino acids 26–108, was generated by using a genetically engineered fusion protein*. Briefly, the coding

* Dove Pettit DA, Dassler CL, Peterson D and Cabral GA, Manuscript in preparation.

sequence corresponding to the external domain of the receptor was PCR-amplified and inserted into an expression plasmid that contained coding sequence for the highly immunogenic HBcAg [21]. The inserted sequence was placed in-frame at the 3′ end of the coding sequence for HBcAg, resulting in an expression plasmid that contained coding sequence for a HBcAg-THCR. HBcAg-THCR was expressed in TB-1 *E. coli*, purified to homogeneity, as previously described [21], and injected into New Zealand white rabbits. Antiserum was collected and purified by means of immunoabsorption with purified HBcAg. The resulting anti-THCR antiserum was shown by Laurell-rocket immunoelectrophoresis to be free of anti-HBcAg antibody and to contain detectable antibody to the external domain of the cannabinoid receptor. The purified anti-THCR antiserum then was employed in immunofluorescence and western immunoblotting studies.

AcNPV-THCR-infected Sf9 cells, 72 hr pi (MOI 2.5), or uninfected Sf9 cells, were harvested and collected onto microscope slides using a Cytospin 2 cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA). Slides were air-dried for 30 min, rinsed in PBS (two times), and fixed in absolute acetone for 10 min. The slides were stored at room temperature. For immunofluorescence staining, slides were rehydrated in PBS for 3 min followed by a 30 min incubation in blocking buffer (1% bovine serum albumin in PBS). The blocking solution was drained from slides and cells were incubated with a 1:15 dilution of anti-THCR antiserum in a humidifying chamber for 60 min. Following removal of the primary antiserum, slides were washed three times in PBS and were incubated with FITC-labeled goat IgG anti-rabbit IgG (H + L) (1:32 dilution in PBS) for 60 min at room temperature. The slides were rinsed three times in PBS, air-dried for 30 min, and mounted using Aquamount (Lerner Laboratories, New Haven, CT). Slides were examined using an Olympus BHA Microscope equipped with a model BH2RFL reflected fluorescence attachment and a model PM-10AD photomicrographic system (Olympus Corp., Lake Success, NY).

Radioligand binding assays. P_2 membranes were prepared from uninfected Sf9 cells and from cells infected with AcNPV-THCR using methods previously described by Felder *et al.* [5]. The methods for radioligand binding were those described by Compton *et al.* [22]. Briefly, membranes (40 μg) were added to siliconized tubes containing [^{3}H]-CP55,940 and a sufficient quantity of assay buffer to bring the total incubation volume to 1 mL. The concentration of [^{3}H]CP55,940 (102.9 Ci/mmol; DuPont-New England Nuclear, Boston, MA) ranged from 50 to 10,000 pM. Nonspecific binding was determined by the addition of 1 μM unlabeled CP55,940 (a gift from Dr. Lawrence Melvin, Central Pfizer Research, Pfizer, Inc., Groton, CT). After incubation at 30° for 1 hr, binding was terminated by vacuum filtration through Whatman GF/C filters. The B_{max} and K_d values generated from Scatchard–Rosenthal analysis were obtained using the KELL package of binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ).

In vitro *translation of mRNA obtained from virally infected Sf9 cells.* Messenger RNA was isolated from Sf9 cells 72 hr pi with AcNPV-THCR, using the QuickPrep micro mRNA Purification Kit (Pharmacia P-L Biochemicals, Inc., Milwaukee, WI). Then the isolated mRNA was *in vitro*-translated using the *In Vitro* Express Translation Kit (Stratagene, La Jolla, CA). Proteins were separated by SDS–PAGE, and western immunoblotting was performed as described below.

SDS–PAGE and western immunoblotting. Gradient SDS–PAGE was performed as previously described [23]. Proteins were mixed 1:1 with sample buffer (0.05 M Tris, pH 6.8, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.05% bromophenol blue) and were heated for 5 min at 100° prior to loading on a 0.75 mm thick gradient gel (7–20%). The samples were electrophoresed overnight (60 V) in a cooled chamber. Then proteins were transferred onto nitrocellulose at 90 mA overnight at room temperature using a Bio-Rad Blot Cell (Bio-Rad, Richmond, CA). The nitrocellulose blot was blocked with 5.0% nonfat dry milk in TBS (10 mM Tris, 0.9% NaCl, pH 7.4) overnight and incubated for 1 hr with absorbed rabbit antiserum containing THCR antibody (1:100 dilution in BB consisting of 2.0% bovine serum albumin, 10% normal goat serum, and 0.5% Tween-20) or with preimmune rabbit serum (1:100 dilution in BB). The blots were washed four times with TBS and were incubated for 2 hr in a 1:3000 dilution of blotting grade affinity-purified goat IgG anti-rabbit IgG (H + L) horseradish peroxidase conjugate (Bio-Rad). Following incubation with the peroxidase conjugate, blots were washed four times for 5 min in TBS and were developed with a horseradish peroxidase reaction mixture [60 mg HRP Color Development Reagent (Bio-Rad) in 20 mL methanol mixed with a solution containing 60 μL 30% H_2O_2 in 100 mL TBS].

RESULTS

Production of the AcNPV-THCR. A THCR recombinant virus was constructed by means of homologous recombination using a replication-deficient linearized baculovirus. Homologous recombination allowed for virus rescue, resulting in a replication-competent double-stranded circular viral genome. Replication-competent virus was plaque-purified, and several clones were isolated. Northern blot analysis was used to determine if mRNA for the cannabinoid receptor was present in Sf9 cells infected with various AcNPV-THCR clone isolates, and expression levels were compared with cannabinoid receptor mRNA levels in total RNA from rat brain. Infection of Sf9 cells with AcNPV-THCR clone 3 resulted in novel hyper-production of a 3.3 kb transcript that corresponds to the predicted size of the recombinant transcript (Fig. 3). The recombinant cannabinoid receptor transcript was approximately 30-fold more abundant than that observed in total RNA from rat brain, as determined by densitometry of autoradiograms. In addition, a faint hybridization signal was observed below the 3.3 kb transcript. This band most likely represents a product of alternative polyadenlyation since three

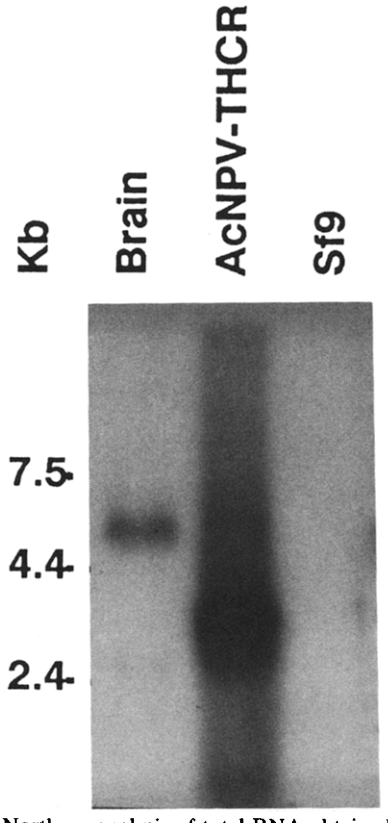

Fig. 3. Northern analysis of total RNA obtained from rat brain, uninfected Sf9 cells, and from Sf9 cells infected with plaque-purified AcNPV-THCR recombinant virus. Total RNA isolated from rat brain (left lane), AcNPV-THCR-infected Sf9 cells (center lane), or from uninfected Sf9 cells (right lanc) was separated by agarose gel electrophoresis and subjected to northern blot analysis using nick-translated cannabinoid receptor cDNA as a probe. The 3.3 kb transcript, corresponding to the predicted size of a recombinant transcript, observed in Sf9 cells infected with AcNPV-THCR was approximately 30-fold more abundant than that observed in total RNA isolated from rat brain. The AcNPV-THCR transcript is smaller in size than that obtained from rat brain since 5′ and 3′ non-coding sequence, found in the original cannabinoid receptor cDNA, was minimized to optimize protein expression. The faint hybridization signal running below the 3.3 kb transcript in AcNPV-THCR most likely represents a product of alternative polyadenylation since the pVL 1393 transfer vector used in homologous recombination for the production of recombinant virus contains three polyadenylation sites.

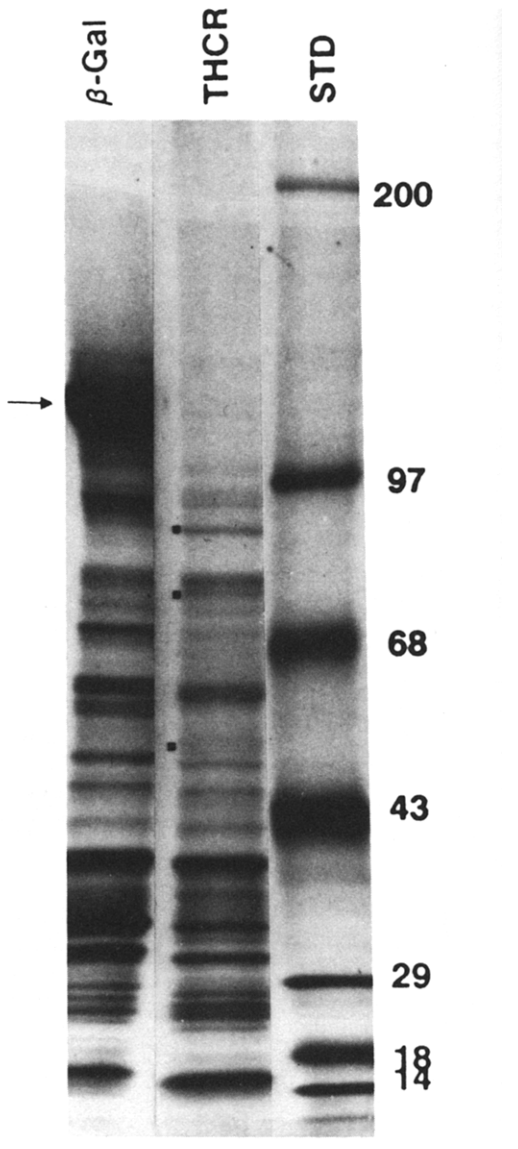

Fig. 4. Protein expression in AcNPV-β-Gal and AcNPV-THCR-infected Sf9 cells. Sf9 cells were metabolically labeled with [^{35}S]Translabel for 24 hr beginning at 36 hr pi. The virus-specified protein profile obtained from AcNPV-β-Gal-infected cells (left lane) was compared with that obtained from AcNPV-THCR-infected cells (center lane) in order to identify novel receptor recombinant protein expression in AcNPV-THCR-infected cells. The right lane depicts the electrophoresed molecular weight standards. Autoradiography of SDS–PAGE revealed novel AcNPV-THCR proteins having relative molecular weights of 55, 73, and 79 kDa, represented by (●). The large 115 kDa species observed in the AcNPV-β-Gal lysate (arrow) presents the hyper-production of recombinant β-galactosidase.

polyadenlyation sites are present in the pVL 1393 transfer vector used for homologous recombination in recombinant virus production. AcNPV-THCR clone 3 was used in subsequent experiments since it produced relatively large amounts of THCR mRNA.

Monitoring viral protein expression in recombinant baculovirus-infected Sf9 cells. Baculovirus-infected Sf9 cells were monitored for recombinant virus-specified protein expression by gradient SDS–PAGE of metabolically labeled proteins (Fig. 4). Analysis of the protein profile of AcNPV-β-Gal-infected Sf9 cells revealed the presence of a prominent protein band with a relative molecular weight of 115 kDa, consistent with the hyper-production of β-galactosidase. This profile, then, was compared with that obtained from AcNPV-THCR-infected Sf9 cells to allow for identification of recombinant cannabinoid

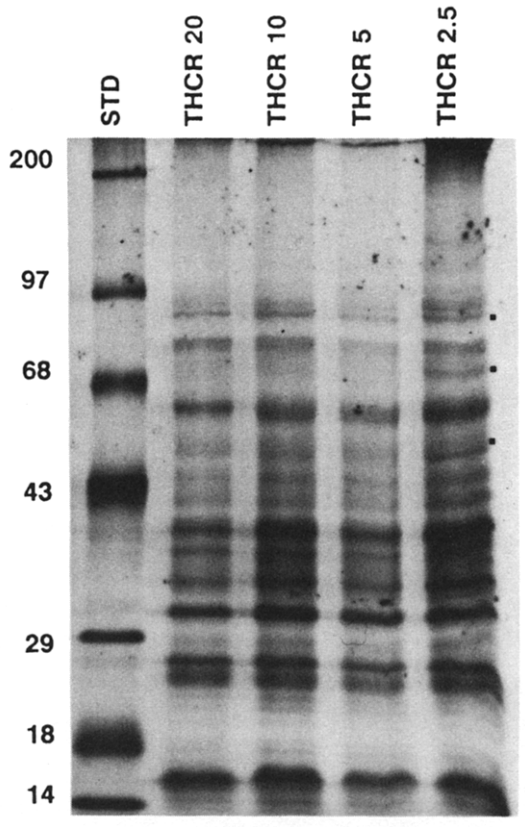

Fig. 5. Protein expression in Sf9 cells infected with AcNPV-THCR recombinant virus at various MOI. Sf9 cells were metabolically labeled with [^{35}S]Translabel for 24 hr beginning at 36 hr pi. Samples containing constant protein were subjected to analytical gradient SDS–PAGE (7–20%). Autoradiography of gradient SDS–PAGE revealed optimal production of protein species in the 55 to 79 kDa range (●) in Sf9 cells infected at a MOI of 2.5. Sf9 cells infected at higher multiplicities of 10 and 20 exhibited less expression of AcNPV-THCR-unique recombinant protein species. These data indicate that optimal recombinant virus-specified gene products were elicited following low multiplicity infection. The numbers shown at the left margin represent the molecular weights (kDa) of ^{14}C-labeled protein standards.

receptor proteins. Several apparently novel protein species having relative molecular weights of 55, 73, and 79 kDa were observed in Sf9 cells infected with AcNPV-THCR.

To determine the optimal conditions for AcNPV-THCR recombinant viral protein expression, Sf9 cells were infected with AcNPV-THCR at various MOI and were metabolically labeled. Equal volumes containing a constant protein concentration were analyzed. Autoradiograms of proteins separated by gradient analytical SDS–PAGE revealed that the highest level of recombinant protein expression occurred when Sf9 cells were infected at a MOI of 2.5 (Fig. 5). At this MOI, the 55, 73, and 79 kDa bands were readily observed. The expression of these species was shown to be inversely related to increased MOI. That is, maximal amounts of novel protein were expressed in cells infected at a MOI of 2.5 and minimal novel protein expression was observed in Sf9 cells infected at a MOI of 20. This inverse relationship of virus-specified protein expression versus MOI was not observed for Sf9 cells infected with AcNPV-β-Gal (data not shown).

Transmission electron microscopy. Transmission electron microscopy was performed in order to confirm that Sf9 cells were infected with AcNPV-THCR and to monitor the cellular effects associated with AcNPV-THCR infection. The ultrastructure of uninfected Sf9 cells was compared with that of cells infected with either AcNPV-β-Gal or with AcNPV-THCR (Fig. 6). Uninfected cells (Fig. 6A) exhibited intact nuclear and cytoplasmic membranes and a normal nuclear to cytoplasmic ratio (i.e. approximately 1:1 by area). Sf9 cells infected with AcNPV-β-Gal (Fig. 6B) were enlarged and balloon-shaped and exhibited a high nuclear to cytoplasmic ratio as early as 24 hr pi. By 36 hr pi, distinctive features of productive viral infection were seen. The 48 hr pi time point was selected for representation since both the distinctive features of productive viral infection and viral protein expression were apparent. These cells contained intranuclear electron-dense regions of apparent active viral transcription, multiple intranuclear viral particles, and large protein inclusions within the cytoplasm. These structural changes provided a baseline for assessing the cellular effects of expression of THCR in AcNPV-THCR-infected Sf9 cells. At 36 hr pi, these cells were enlarged, contained intranuclear electron-dense regions of active viral transcription, and exhibited a high nuclear to cytoplasmic ratio. However, relatively few intranuclear virus particles were observed within nuclei. Furthermore, AcNPV-THCR-infected Sf9 cells exhibited alterations in cell surface membranes, intracytoplasmic vacuolization, and electron-dense cytoplasmic perinuclear accumulations consistent with glycoprotein expression and accumulation (Fig. 6C). These membrane alterations were more pronounced in samples analyzed at later time points pi. At 72 hr pi, extensive cytoplasmic vacuolization and membrane disaggregation and dissolution were apparent in the majority of AcNPV-THCR-infected cells.

Immunofluorescence of AcNPV-THCR-Infected Sf9 cells. To confirm that AcNPV-THCR-infected cells expressed THCR, cells were examined by immunofluorescence using anti-THCR antiserum that was elicited to the extracellular amine terminus of the THCR. Intense intracytoplasmic and cell surface fluorescence, indicative of THCR expression, was observed in infected Sf9 cells (Fig. 7A). In addition, numerous cells were observed which exhibited a perinuclear fluorescence pattern that paralleled the intracytoplasmic perinuclear electron-dense accumulation pattern observed by electron microscopy. This perinuclear pattern of fluorescence was most prominent for infected cells examined at 48 and 72 hr pi. AcNPV-THCR-infected cells did not exhibit fluorescence when preimmune rabbit serum was employed. Furthermore, uninfected Sf9 cells did not exhibit fluorescence when incubated with anti-THCR antiserum (Fig. 7B).

Radioligand binding studies. To establish that

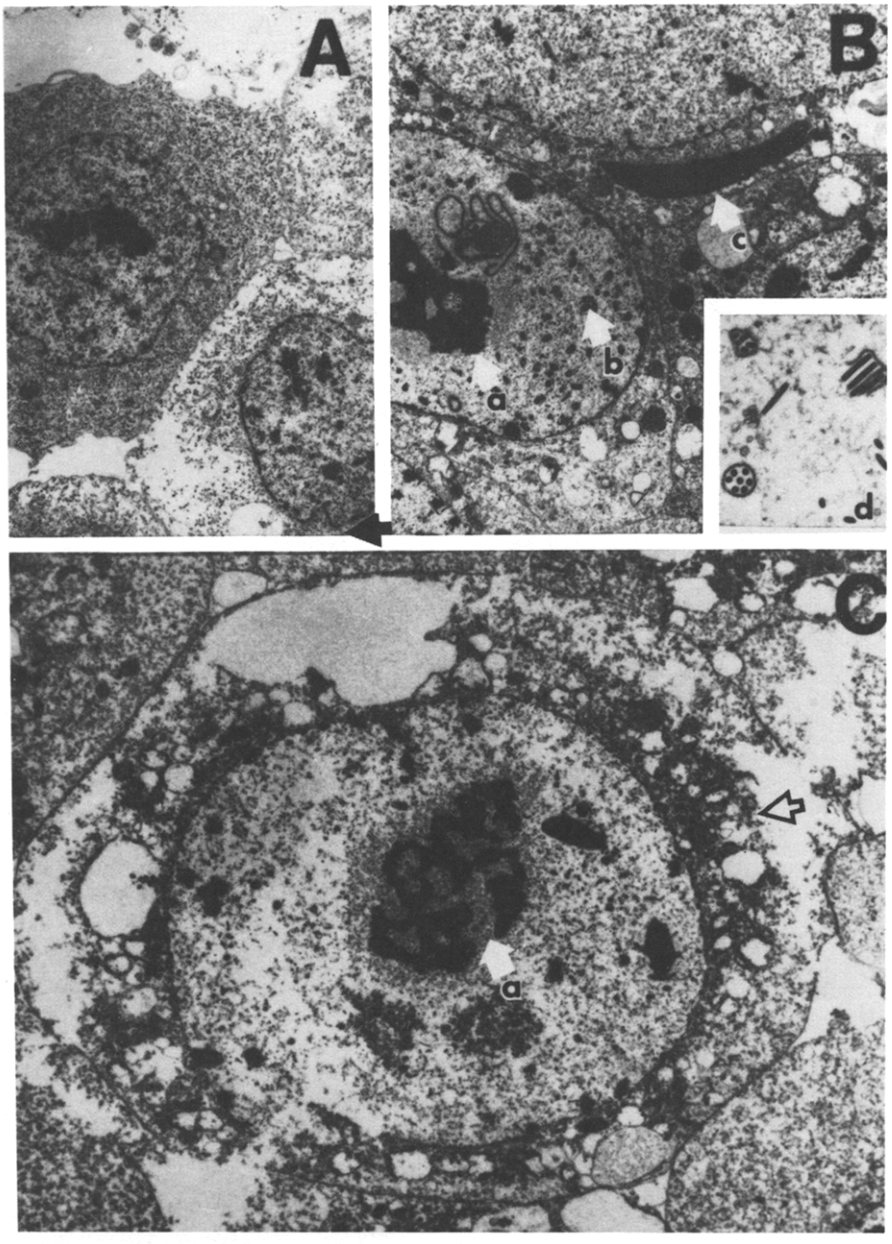

Fig. 6. Transmission electron microscopy of uninfected and recombinant virus-infected Sf9 insect cells. (A) Uninfected Sf9 cells exhibited a normal nuclear-to-cytoplasmic ratio (i.e. approximately 1:1 by area) and intact membrane structures. (B) Cells infected with the β-gal recombinant virus (48 hr pi) had a high nuclear-to-cytoplasmic ratio, active regions of intranuclear viral transcription (a), large numbers of intranuclear viral particles (b), and large protein inclusions within the cytoplasm (c). Inset d illustrates viral particles found within the nucleus of cells infected with the β-gal recombinant virus. (C) Cells infected with AcNPV-THCR recombinant virus (36 hr pi) had a high nuclear-to-cytoplasmic ratio and large regions of intranuclear viral transcription (a) indicative of viral infection. Although relatively few viral particles were observed within the nucleus, the extensive perturbation of cytoplasmic membranes and electron-dense perinuclear granular membrane accumulations (open arrow) are suggestive of glycoprotein (i.e. THCR) expression. These alterations in cell structure apparent at 36 hr pi were more pronounced at later time periods. Extensive membrane disaggregation and dissolution and cytoplasmic vacuolization were observed at 72 hr pi.

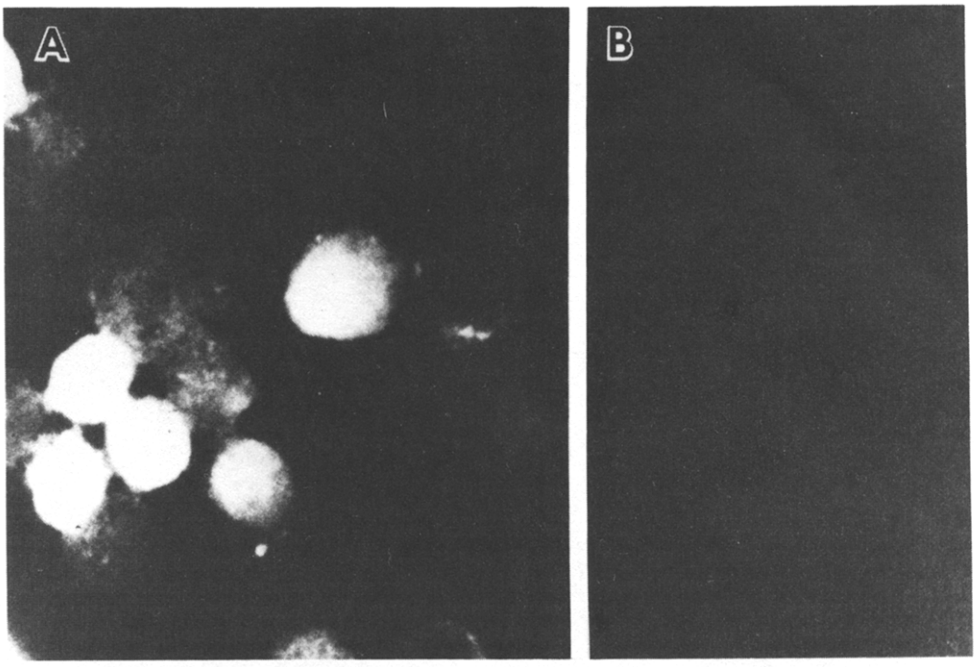

Fig. 7. Immunofluorescence staining in AcNPV-THCR-infected and uninfected Sf9 cells. AcNPV-THCR-infected Sf9 cells and uninfected Sf9 cells were incubated with antiserum to the external domain of the THCR (1:15 dilution). (A) Extensive cytoplasmic and cell surface immunofluorescence was observed in AcNPV-THCR-infected Sf9 cells. (B) Uninfected Sf9 cells did not exhibit fluorescence following incubation with the anti-THCR antiserum.

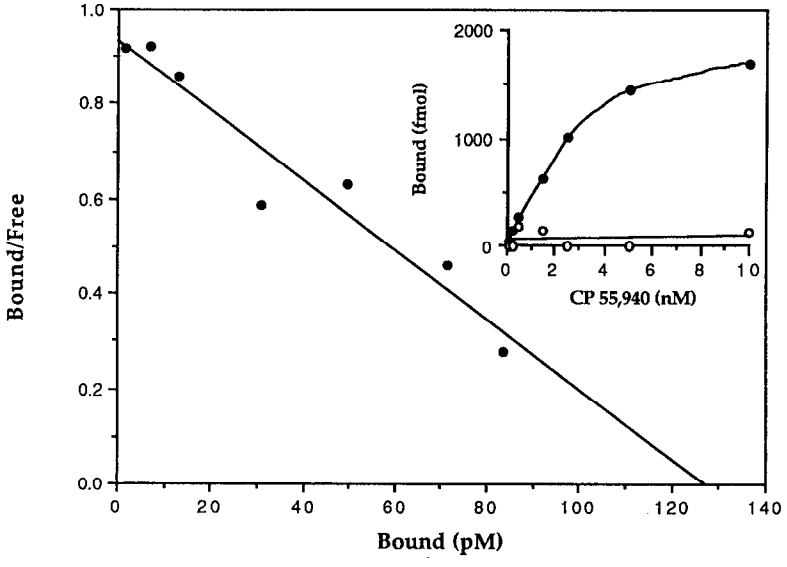

Fig. 8. Scatchard–Rosenthal analysis of [^{3}H]CP55,940 receptor binding. Inset: Saturation binding isotherms of specific binding from membranes prepared from Sf9 cells infected with AcNPV-THCR virus (●) or uninfected cells (○). These data are representative of seven experiments performed in triplicate. In this particular experiment, the K_d was found to be 3.4 nM and the B_{max} was equal to 3.17 pmol/mg protein.

Sf9 cells infected with AcNPV-THCR expressed cannabinoid receptors that exhibited the capacity to bind cannabinoid ligand, radioligand binding assays using [^{3}H]CP55,940 were performed. Membranes were prepared from infected cells (MOI = 3) 4 days pi. A Scatchard–Rosenthal analysis of a representative saturation binding experiment is shown in Fig. 8. EBDA/LIGAND analysis indicated

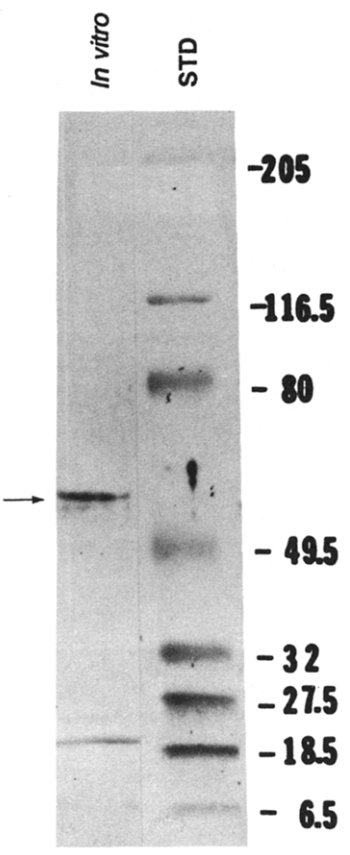

Fig. 9. Western immunoblotting of *in vitro*-translated protein. Messenger RNA was isolated from Sf9 cells 72 hr pi with AcNPV-THCR. The mRNA was *in vitro*-translated, and proteins were separated by SDS–PAGE and subjected to western immunoblotting using anti-THCR antiserum. A major immunoreactive band was observed with a relative molecular weight of 55 kDa (left lane, arrow), which corresponds to the molecular weight predicted for the cannabinoid receptor translational product. The right lane represents the electrophoresed molecular weight standards with their respective relative molecular weights in kDa.

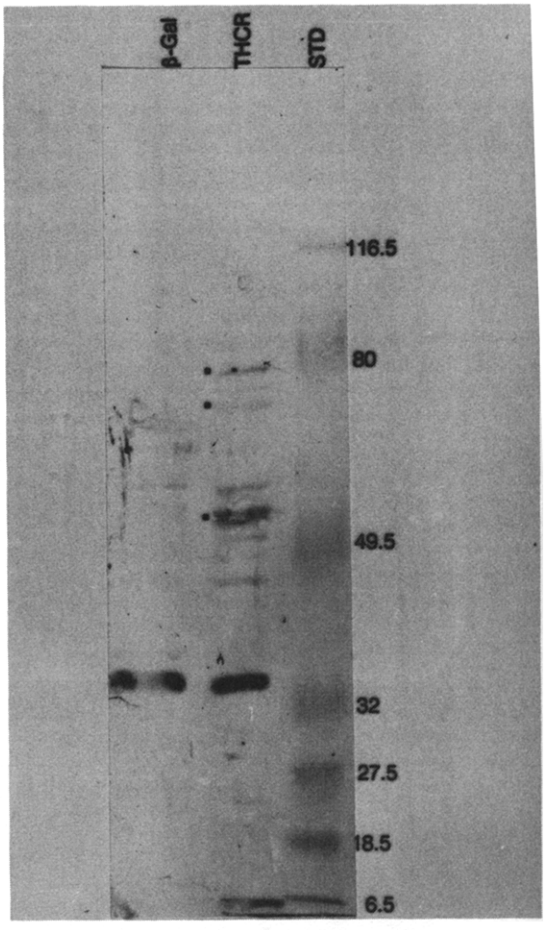

Fig. 10. Western immunoblotting of AcNPV-β-Gal and AcNPV-THCR recombinant protein using anti-THCR-antiserum. Sf9 cells inoculated with either AcNPV-β-Gal or AcNPV-THCR, 60 hr pi, were harvested and solubilized. Proteins were separated by SDS–PAGE, and western immunoblotting was performed using anti-THCR antiserum. Immunoreactive bands with relative molecular weights of 32, 45, 50, 55, 57, 73, 76, and 79 kDa were observed in AcNPV-THCR-infected Sf9 cell lysates (center lane). The 55, 73, and 79 kDa species were also apparent in metabolically labeled protein profiles and most likely represent the unglycosylated receptor and two glycosylated receptor forms, respectively. The 45 and 50 kDa species have a lower molecular weight than expected based on the predicted size of the translational product obtained from the cannabinoid receptor cDNA sequence. These proteins may represent a product of degradation or accumulation of truncated recombinant receptor protein. The 32 and 57 kDa species were also apparent in AcNPV-β-Gal-infected Sf9 cell lysates (left lane), suggesting that they are cross-reacting virus-specified proteins. The right lane represents relative molecular weight standards with their respective molecular weights in kDa.

a K_d of 3.4 nM, a B_{max} equal to 127 pM (3.17 pmol/mg protein), and a Hill coefficient of 0.99, similar to values previously reported for native and transfected cannabinoid receptors [5, 22]. Greater than 50% specific binding was observed in membranes prepared from infected cells. No significant binding to membranes prepared from uninfected Sf9 cells was observed (data not shown).

In vitro *translation.* Sf9 cells infected with AcNPV-THCR contained high levels of mRNA to the cannabinoid receptor at 72 hr pi. Therefore, mRNA was isolated from cells at this time point and was used for *in vitro* translation. The translated protein products were separated by analytical SDS–PAGE and were subjected to western immunoblotting using the anti-THCR antiserum. A major immunoreactive protein of approximately 55 kDa relative molecular weight was observed (Fig. 9). A 24-kDa band also was present and may be a product of incomplete translation or represent a non-specifically stained product.

Western immunoblotting of virally infected Sf9 cell lysates. Western immunoblotting was performed on lysates from Sf9 cells infected with either AcNPV-

β-Gal or AcNPV-THCR. The protein profiles obtained were compared to determine if immunoreactive species (ranging in relative molecular weight from 32 to 79 kDa) present in AcNPV-THCR-infected Sf9 cell lysates were specific to AcNPV-THCR infection (Fig. 10). Species of 55, 73, and 79 kDa relative molecular weight were identified. The relative molecular weights of these species are in agreement with those identified in the metabolic labeling experiments and are consistent with that predicted for a translational product obtained from a recombinant cannabinoid receptor transcript. These species were not present in profiles of AcNPV-β-Gal-infected cell lysates. Species of 45, 50, and 76 kDa also were observed. These products appear to be specific to AcNPV-THCR infection since comparable immunoreactive proteins were not seen in profiles of AcNPV-β-Gal-infected Sf9 cells. Species of 32 and 57 kDa were present in both the AcNPV-β-Gal-infected and the AcNPV-THCR-infected cell lysates and most likely represent cross-reacting viral-specified protein since lysates of uninfected Sf9 cells (data not shown) did not contain these immunoreactive protein species.

DISCUSSION

The serendipitous discovery of a rat cDNA clone coding for a receptor shown to functionally interact with THC has provoked many questions about the biochemical and biophysical properties of this protein. Since GPCRs are not typically found in tissue in great abundance and GPCRs are difficult to purify, it is of interest to develop a eukaryotic expression system that has the capability of expressing relatively high levels of receptor protein. The baculovirus system is a desirable expression system for functional and biochemical characterization of the cannabinoid receptor since it is a eukaryotic system that possesses protein processing systems similar to those found in other mammalian expression systems. Furthermore, this system has the potential to allow for the expression of relatively high quantities of recombinant receptor protein requisite for studies that attempt to identify the regions involved in receptor–ligand interactions and to elucidate the role that post-translational modification may play in receptor–ligand interactions.

To allow for optimal recombinant protein production in this system, a baculovirus transfer vector was constructed to contain only the coding sequence for the cannabinoid receptor and 100 bp of the 3′ non-coding region. The non-coding sequence present in the cannabinoid receptor cDNA (both 5′ and 3′) was minimized to circumvent translational regulation of protein expression [24]. Thus, eliminating it would most likely result in increased recombinant cannabinoid receptor expression.

Construction of a THCR recombinant baculovirus was accomplished using a replication-deficient linear baculovirus genome. This construct increased the rate of homologous recombination and circumvented the need for extensive plaque screening and purification, resulting in the rapid isolation of a replication-competent recombinant virus. Northern blot analysis was used to determine if any of the AcNPV-THCR clones were expressing mRNA for cannabinoid receptors. The hyper-production of a transcript smaller than that obtained from rat brain, our positive control for cannabinoid receptor expression, was expected since the native transcript possesses all of the 5′ and 3′ non-coding sequence present on native transcripts. These results indicate that Sf9 cells infected with AcNPV-THCR have the capability to express high levels of cannabinoid receptor mRNA.

To determine if Sf9 cells infected with AcNPV-THCR expressed novel virus-specified protein, metabolic labeling experiments were performed. This technique was employed because baculovirus infection results in the inhibition of host protein synthesis and thus allows for the incorporation of radiolabel into virus-specified protein. Novel expression of virus-specified protein species was minimal in Sf9 cells infected with AcNPV-THCR (MOI 20). Furthermore, cellular protein synthesis during viral infection was reduced in Sf9 cells infected with AcNPV-THCR (MOI 20) when compared with that observed in cells infected at the same MOI with AcNPV-β-Gal. The limited expression of THCR following high multiplicity infection with AcNPV-THCR apparently was not due to dysfunctional translation of the corresponding mRNA, since *in vitro* translation of mRNA isolated from AcNPV-THCR-infected cells yielded a single immunoreactive protein of 55 kDa relative molecular weight, as determined by analytical SDS–PAGE and western immunoblotting. This relative molecular weight is consistent with that predicted for the translational product obtained from the cannabinoid receptor cDNA sequence [15]. These data suggest that infection of Sf9 cells with AcNPV-THCR at high multiplicities (MOI 10 and 20) results in limited THCR expression.

To determine if high multiplicity infection with AcNPV-THCR resulted in unique alterations in cell structure, that could limit recombinant protein expression, ultrastructural studies were performed. Transmission electron microscopy observations indicated that the time kinetics for optimal virus-specified protein expression in AcNPV-THCR-infected Sf9 cells following high-multiplicity infection were distinctive from those of AcNPV-β-Gal-infected cells. Cells infected with AcNPV-THCR (MOI 20) exhibited extensive membrane perturbation and intracytoplasmic vacuolization, suggesting that recombinant receptor expression altered Sf9 cell membrane integrity. These alterations became progressively more pronounced at later time points, culminating in dissolution of the cell surface membrane at approximately 72 hr pi. This response is apparently not unique to AcNPV-THCR-infected Sf9 cells since similar alterations have been observed in cells infected with viruses that produce cell surface glycoproteins. Expression of virus-specified glycoproteins can cause membrane perturbations that alter membrane fluidity and affect membrane selective permeability. This series of events has been proposed as a means by which certain viruses effectively shutdown host cell macromolecular synthesis [25, 26]. Since the β-gal recombinant virus does not express cell surface glycoprotein, this

response was not apparent in cells infected with this recombinant virus. Thus, expression of relatively high levels of the cannabinoid receptor may place limitation on the total amount of receptor that can be expressed. It is important to note that expression of the cannabinoid receptor in tissue is most likely tightly regulated to limit toxicity. The non-coding sequence, which was removed in the construction of AcNPV-THCR, may be involved with the translational regulation of cannabinoid receptor mRNA. Therefore, removal of the non-coding sequence most likely allowed for increased expression of the cannabinoid receptor protein, thus altering cell structure and placing limitations on the hyper-production of viral proteins. Nevertheless, radioligand binding assays performed on crude membrane preparations suggest that the level of recombinant protein produced and inserted into the cellular membrane, even after high multiplicity infection, is comparable to that expressed in neural tissue and in stably transfected CHO cells.

Since high multiplicity infection (i.e. MOI 20) resulted in limited recombinant viral protein synthesis, it was desirable to determine if lower multiplicity infection would increase recombinant protein expression. Therefore, Sf9 cells were infected with AcNPV-THCR at various multiplicities ranging from 2.5 to 20, and infected cell lysates were subjected to ^{35}S-metabolic labeling. The amount of recombinant protein produced exhibited an inverse relationship to MOI based on image analysis of autoradiograms of analytical SDS–PAGE-separated virus-specified proteins. Optimal receptor expression was observed at a MOI of 2.5. Unique proteins were shown to have relative molecular weights of 55, 73, and 79 kDa. These data suggest that, at lower MOI, Sf9 cell viability was altered less extensively, which allowed for gradual expression and accumulation of the recombinant cannabinoid receptor protein.

To confirm that recombinant baculovirus-infected Sf9 cells expressed cannabinoid receptor protein, immunofluorescence staining of AcNPV-THCR-infected Sf9 cells and western immunoblotting of AcNPV-THCR-infected cell lysates were performed. The intense cytoplasmic fluorescence observed in AcNPV-THCR infected cells was expected since the antiserum used was generated to the amine terminus of the cannabinoid receptor. Thus, proteins at all stages of biosynthesis should be recognized. Recombinant virus-infected cells examined at later periods (i.e. 48 and 72 hr) exhibited a perinuclear cytoplasmic fluorescence pattern that paralleled the perinuclear electron-dense pattern seen by transmission electron microscopy.

To determine if cannabinoid receptor recombinant protein was capable of binding cannabinoids, radioligand binding studies using [^{3}H]CP55,940 were performed on P_2 membranes prepared from Sf9 cells infected with AcNPV-THCR. The K_d value obtained for AcNPV-THCR-infected Sf9 cells was higher than the K_d values reported in rat brain [12, 22], but similar to that reported for transfected cells [5]. Since Sf9 cells have been reported to lack $G_{\alpha i}$ proteins [27], the expressed cannabinoid receptor may not be properly coupled to G proteins, which would be reflected as a high K_d value. The B_{max} value obtained for AcNPV-THCR-infected Sf9 cells is similar to that obtained from rat brain membrane preparations. Since binding data provide a means of quantitating receptor expression, it is important to note that these studies were performed on crude membrane preparations. Therefore, receptor expression in recombinant virus-infected cells occurred in the context of cellular membranes. The binding data corroborate the immunofluorescence observations, indicating that the infected Sf9 cells expressed cannabinoid receptor.

To confirm that the unique radiolabeled virus-specified protein products (i.e. 55, 73, and 79 kDa) observed in autoradiograms of AcNPV-THCR infected cell lysates were specific for recombinant cannabinoid receptor, western immunoblotting was performed. The presence of immunoreactive bands with relative molecular weights ranging from 32 to 79 kDa is not unanticipated since whole cell homogenates of infected Sf9 cells should harbor intracellular pools of recombinant-virus-specified protein at different stages of biosynthesis and/or post-translational modification. In contrast, western immunoblotting of electrophoresed *in vitro*-translated protein, generated from mRNA isolated from AcNPV-THCR-infected Sf9 cells, revealed one major immunoreactive band with a relative molecular weight of 55 kDa. The presence of one major immunoreactive band is anticipated since (1) *in vitro* translation does not allow for post-translational modifications as occur *in vivo* and (2) *in vitro* conditions were optimized to drive THCR synthesis to a fully translated unglycosylated product. Therefore, the predominant 55 kDa band observed in AcNPV-THCR-infected Sf9 cell lysates most likely represents the unglycosylated receptor recombinant protein, whereas the 73, 76, and 79 kDa species may represent fully glycosylated cannabinoid receptor. Furthermore, the lack of these protein species (i.e. 73, 76, and 79 kDa), and the presence of only one immunoreactive band in the *in vitro*-translated preparation, strongly suggest that these proteins are indeed post-translationally modified cannabinoid receptor protein since the mRNA was isolated from AcNPV-THCR-infected Sf9 cells. A predominance of the unglycosylated species is expected since glycosylation is not an efficient process in virally infected Sf9 cells. The 45 and 50 kDa AcNPV-THCR specific proteins have a lower relative molecular weight than that predicted for a translational product obtained from the receptor cDNA sequence. Since these species were not observed in AcNPV-β-Gal-infected Sf9 cells, they may represent products of incomplete biosynthesis or of degradation. These data are in general agreement with those obtained from the autoradiography of analytical SDS–PAGE where bands having relative molecular weights of 55, 73, and 79 kDa were observed. The 32 and 57 kDa species that were observed in AcNPV-THCR-infected cell lysates were also seen in lysates from AcNPV-β-Gal-infected cells. These results suggest that the two proteins are cross-reacting virus-specified proteins unrelated to THCR since they were not observed in western immunoblots of uninfected Sf9 cells.

The results of this investigation indicate that Sf9 infection with the recombinant baculovirus, AcNPV-THCR, results in the expression of a protein that reacts with an antibody elicited to the amine-terminus of the rat cannabinoid receptor, has a relative molecular weight consistent with that extrapolated from the rat cannabinoid receptor cDNA [15], is expressed in association with cell membranes, and binds the potent THC analog CP55,940. Thus, this study constitutes the first report on the initial biochemical characterization of a recombinant cannabinoid receptor protein.

Furthermore, these data indicate that the baculovirus expression system may be suitable for the expression of unmodified or genetically modified cannabinoid receptor. Preparative levels of receptor protein will be useful for studies directed toward elucidating the basic biochemical and biophysical properties of the receptor and for defining the amino acids that are essential for receptor–ligand interactions. Most importantly, the baculovirus system may provide a means for identifying endogenous ligands that interact with the cannabinoid receptor.

Acknowledgements—The authors thank Dr. Darrell Peterson for providing the vector pKK223 for fusion protein expression and for his assistance in the purification of the fusion protein used to generate polyclonal antiserum to the external domain of the cannabinoid receptor and Dr. Lisa Matsuda for her gift of the pcD-SKR6 clone. We also thank Charlene Johnson for technical assistance. This research was supported by National Institute on Drug Abuse/NIH Grants R01-DA05832 and R01-DA05274 and by National Service Award F31-DA05518 to D. A. Dove Pettit.

REFERENCES

1. Dewey WL, Cannabinoid pharmacology. *Pharmacol Rev* **38**: 151–178, 1986.
2. Munson AE and Fehr KO, Immunological effects of cannabis. In: *Cannabis and Health Hazards* (Eds. Fehr KO and Kalant H), pp. 257–353. Alcoholism and Drug Addiction Research Foundation, Toronto, 1983.
3. Hillard CJ, Harris RA and Bloom AS, Effects of the cannabinoids on physical properties of brain membranes and phospholipid vesicles: Fluorescence studies. *J Pharmacol Exp Ther* **232**: 579–588, 1985.
4. Martin BR, Cellular effects of cannabinoids. *Pharmacol Rev* **38**: 45–74, 1986.
5. Felder CC, Veluz JS, Williams HL, Briley EM and Matsuda LA, Cannabinoid agonists stimulate both receptor and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Mol Pharmacol* **42**: 838–845, 1992.
6. Martin BR, Balster RL, Razdan RK, Harris LS and Dewey WL, Behavioral comparisons of the stereoisomers of tetrahydrocannabinols. *Life Sci* **29**: 565–574, 1981.
7. Howlett AC, Cannabinoid inhibition of adenylate cyclase relative activities of marihuana constituents and metabolites. *Neuropharmacology* **26**: 507–512, 1987.
8. Johnson MR and Melvin LS, The discovery of non-classical cannabinoid analgetics. In: *Cannabinoids as Therapeutic Agents* (Ed. Mechoulam R), pp. 121–145. CRC Press, Boca Raton, FL, 1986.
9. Little PJ, Compton DR, Johnson MR, Melvin LS and Martin BR, Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. *J Pharmacol Exp Ther* **247**: 1046–1051, 1988.
10. Biduat-Russell M, Devane WA and Howlett AC, Cannabinoid receptors and modulation of cyclic AMP accumulation in the rat brain. *J Neurochem* **55**: 21–26, 1990.
11. Howlett AC, Cannabinoid inhibition of adenylate cyclase: Biochemistry of the response in neuroblastoma cell membranes. *Mol Pharmacol* **27**: 429–436, 1985.
12. Devane WA, Dysarz FA III, Johnson MR, Melvin LS and Howlett AC, Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **34**: 605–613, 1988.
13. Herkenham M, Lynn AB, Little MD, Ross Johnson MR, Melvin LS, de Costa BR and Rice KC, Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA* **87**: 1932–1936, 1990.
14. Herkenham M, Lynn AB, Ross Johnson MR, Melvin LS, de Costa BR and Rice KC, Characterization and localization of cannabinoid receptors in rat brain: A quantitative *in vitro* autoradiographic study. *J Neuroscience* **11**: 563–583, 1991.
15. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI, Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346**: 561–564, 1990.
16. Parker EM, Kameyama K, Higashijima T and Ross EM, Reconstitutively active G protein-coupled receptors purified from baculovirus-infected insect cells. *J Biol Chem* **266**: 519–527, 1991.
17. George ST, Arabian MA, Ruoho AE, Kiely J and Malbon CC, High efficiency expression of mammalian β-adrenergic receptors in baculovirus-infected insect cells. *Biochem Biophys Res Commun* **163**: 1265–1269, 1989.
18. O'Reilly DR, Miller LK and Lucklow VA, *Baculovirus Expression Vectors: A Laboratory Manual*. W. H. Freeman & Co., New York, 1992.
19. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Erlich HA, Primer directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* **239**: 487–491, 1988.
20. Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299, 1979.
21. Zheng J, Schödel F and Peterson DL, The structure of hepadnaviral core antigens. Identification of free thiols and determination of the disulfide bonding pattern. *J Biol Chem* **267**: 9422–9429, 1992.
22. Compton DR, Rice KC, De Costa BR, Razdan RK, Melvin LS, Johnson MR and Martin BR, Cannabinoid structure–activity relationships: Correlation of receptor binding and *in vivo* activities. *J Pharmacol Exp Ther* **265**: 218–226, 1993.
23. Laemmli UK, Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
24. Melefors O and Hentze MW, Translational regulation by mRNA/protein interactions in eukaryotic cells: Ferritin and beyond. *Bioessays* **15**: 85–90, 1993.
25. Carrasco L and Smith AE, Sodium ions and the shut-off of host cell protein synthesis by picornaviruses. *Nature* **264**: 807–809, 1976.
26. Garry RF, Ulug ET and Bose HR, Membrane-mediated alterations of intracellular Na^+ and K^+ in lytic-virus-infected and retrovirus-transformed cells. *Biosci Rep* **2**: 617–623, 1982.
27. Quehenberger O, Prossnitz ER, Cochrane CG and Ye RD, Absence of G_i proteins in the Sf9 insect cell. Characterization of the uncoupled recombinant *N*-formyl peptide receptor. *J Biol Chem* **267**: 19757–19760, 1992.