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Characterization and Purification of Human *fos* Protein Generated in Insect Cells with a Baculoviral Expression Vector

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We generated recombinant baculoviruses that contained the human *fos* gene and that, upon infection of insect cells, synthesized *fos* protein. The quantity of *fos* protein produced was at least 10 to 20 times higher than that observed in any mammalian cells reported so far. The *fos* protein made in insect cells manifested most of the characteristics of mammalian *fos* protein, which include (i) 55-kilodalton size, (ii) nuclear localization, (iii) phosphoesterification at serine residues, (iv) identical ^{35}S tryptic peptide maps, (v) ability to make heterodimers with the nuclear *jun* oncoprotein, and (vi) cooperation with the *jun* protein to bind to a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element. A 100- to 150-fold purification of the *fos* protein from infected insect cells was achieved in a single step by immunoaffinity chromatography. Availability of authentic *fos* protein made by baculoviral vectors in insect cells should allow a more rigorous analysis of its biochemical and biological properties.

The proto-oncogene *fos* encodes a 55- to 72-kilodalton (kDa) nuclear phosphoprotein that is associated with chromatin (7, 31, 32). Its viral homolog, v-*fos*, the resident transforming gene of FBJ-murine osteosarcoma virus, can induce transformation in vitro and bone tumors in vivo (8, 14, 24, 42). The precise function of the *fos* protein is not understood, but recent data support the notion of it being a modulator of transcription: (i) it is found in the nucleoprotein complex involved in regulation of expression of the adipocyte gene aP2 (12, 30), (ii) it can repress transcription from its own promoter as well as that from the heat-shock promoter (36, 43), and (iii) it activates transcription from a promoter containing a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) (5, 21, 34, 37). The function of the *fos* protein is mediated through the formation of a complex with the product of another nuclear oncogene, *jun*, which encodes transcription factor AP-1 (1, 3, 5, 34).

Biochemical studies of the *fos* protein have been hampered by the relatively limited amounts synthesized in mammalian cells. In cells induced with optimal concentrations of platelet-derived growth factor, the maximal levels of the *fos* protein are only 0.005% of the total cellular protein level (19). The relative paucity of the *fos* protein is further compounded by its very short half-life (2, 7). Attempts to generate large amounts of *fos* protein by traditional procedures such as expression in bacteria and *Saccharomyces cerevisiae* have met limited success (23, 33). In both cases the amount of the *fos* protein generated was at best fivefold higher than that in the best eucaryotic system. Moreover, the extensive posttranslational modification of the *fos* protein may not be adequately carried out in these systems. We have also observed that overproduction of *fos* protein may be toxic for yeast cell growth (J. Barber, G. Thill, and I. Verma, manuscript in preparation). With these limitations in mind, we decided to express *fos* protein in insect cells by using baculoviral vectors.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been shown to be suitable as a helper-independent viral expression vector for the high-level production of recombinant proteins in *Spodoptera frugiperda* (fall army worm) cells (25). Cloning and overexpression of proteins through the baculoviral system is based on the utilization of the strong viral polyhedrin gene promoter to which the gene to be expressed is linked by homologous recombination in vivo. The polyhedrin gene product, representing over 50% of the total cellular protein during late infection, is dispensable for extracellular infectious virus production. Loss of expression of the polyhedrin gene results in lack of formation of occlusion bodies. A large number of eucaryotic genes placed under the transcriptional control of the polyhedrin promoter have been expressed in insect cells via infection with recombinant AcNPV (13, 22).

We report the successful use of baculoviral host-vector system to generate and purify high amounts of proto-oncogene *fos* protein. The protein thus synthesized manifests most of the properties characterized to date of mammalian *fos* protein, i.e., (i) nuclear localization, (ii) extensive phosphorylation, (iii) association with in vitro-synthesized mammalian *jun* (AP-1) protein, and binding of the complexes thus formed to TRE.

MATERIALS AND METHODS

Cells and viruses. *S. frugiperda* insect cells (sf9) were propagated either as a suspension or as a monolayer culture in TNM-FH medium (39), a modification of Grace medium (16) (Hazzleton) supplemented with yeastolate and lactalbumin hydrolysate (Difco Laboratories; 3.3 g of each per liter) and 10% fetal bovine serum. The transfer vector pAc373 and the wild-type AcNPV (E2 strain) were obtained from M. Summers, Texas A&M University.

Viral infections were performed at a multiplicity of infection of 1 for virus production or 10 for protein production.

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Unless otherwise specified, infection proceeded for 24 h before cellular extracts were prepared.

The rat transformed cell line MMV constitutively produces murine c-*fos* protein (2). HeLa cells were induced by addition of 75 ng of phorbol-12-myristate-13-acetate (PMA) per ml of medium 30 to 45 min before the cells were lysed.

Transfection and selection of recombinant baculoviruses. Details concerning the construction of pAcc316, the recombinant transfer vector that contains c-*fos* cDNA, are given in the Results section. Cotransfection experiments with AcNPV DNA and pAcc316 and screening for recombinant viruses were performed as described by Summers and Smith (39). For plaque hybridization, the *Nae*I restriction fragment containing the human c-*fos* cDNA was radiolabeled with [^{32}P]dCTP by nick translation.

Radiolabeling of cells and immunoprecipitation of *fos* proteins. Unless otherwise specified, labeling of cells was performed in 0.5 ml for 1.5×10^6 cells per 35-mm tissue culture dish or in 1.5 ml for 5×10^6 cells per 60-mm dish. Times of infection for sf9 cells include time of labeling. For ^{35}S labeling, cells were incubated in methionine- and cysteine-free medium (Dulbecco modified Eagle medium [DMEM] for HeLa and MMV cells, TNM-FH for sf9 cells) for 30 min and then incubated for 1 h (HeLa and MMV cells) or 3 h (sf9 cells) in the same medium containing 200 μCi of Trans-label (70% [^{35}S]methionine, 20% [^{35}S]cysteine; ICN Pharmaceuticals Inc.) per ml and 1% dialyzed fetal calf serum. For ^{32}P labeling, sf9 cells were incubated for 1 h in phosphate-free TNM-FH medium, followed by 12 h in the same medium supplemented with 1% dialyzed fetal calf serum and 1 mCi of $^{32}P_i$ (Dupont, NEN Research Products) per ml. HeLa cells were labeled in phosphate-free DMEM for 1 h in the presence of 1 mCi of $^{32}P_i$ per ml.

After labeling, cells were washed with Tris-saline buffer, solubilized in lysis buffer (50 mM Tris hydrochloride, 0.5% sodium dodecyl sulfate [SDS], 70 mM β-mercaptoethanol [pH 8.0]), and then heated at 100°C for 3 min. All further steps were performed at 4°C. Four volumes of RIPA buffer (10 mM Tris hydrochloride [pH 7.5], 0.5 M NaCl, 1% deoxycholate, 1% Nonidet P-40, 1 mM Aprotinin [Sigma Chemical Co.], 1 mM phenylmethylsulfonyl fluoride [Sigma]) was added, and the lysates were centrifuged for 20 min at 20,000 × *g* to remove insoluble debris. Supernatants were precleared with a 10% solution of Pansorbin (Calbiochem) in RIPA and then incubated for 1 h with *fos* 18H6 monoclonal ascites fluid (11) (typically 1 μl per 10^6 cells). The immune complexes were immunoprecipitated by the addition of Pansorbin (10 μl per 1 μl of antibody). The pellets were washed three times with RIPA, and immunoprecipitated proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE) as described previously (20). When ^{35}S-labeled proteins were analyzed, the signal was enhanced by incubating 30 min in 1 M salicylic acid (Sigma) before drying.

Nuclear extracts and immunoblotting. Infected sf9 cells or PMA-induced HeLa cells were incubated for 10 min in hypotonic buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.5], 1 mM dithiotreitol, 1% aprotinin, 1% phenylmethylsulfonyl fluoride) at 4°C. Cells were lysed by the addition of 0.3% Nonidet P-40 and homogenized by 25 strokes in a tissue grinder with a glass pestle (type B). Nuclei were pelleted by centrifugation at 2,000 rpm for 10 min in an IEC tabletop centrifuge and then suspended in RIPA buffer containing 5 mM $MgCl_2$, 50 μg of deoxyribonuclease I (Worthington Diagnostics) per ml, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. When nuclear extracts were prepared for DNA binding experiments, deoxyribonuclease I was omitted. Insoluble debris were removed by centrifugation for 1 h at 35,000 × *g*. Proteins were separated on a 10% SDS–polyacrylamide gel (20) and transferred onto nitrocellulose paper (Schleicher & Schuell, Inc.) with a Bio-Rad blotting apparatus. Transfer proceeded overnight at 100 mA in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Nitrocellulose sheets were incubated for 1 h in MTA (phosphate-buffered saline containing 5% nonfat dry milk, 0.3% Tween 20, and 3 mM NaN_3) at room temperature. Blots were washed in phosphate-buffered saline containing 0.2% Tween 20 and 150 mM NaCl incubated for 2 to 3 h at 37°C in a 1:250 dilution of monoclonal 18H6 ascites fluid in MTA, washed as above, and then incubated with ^{125}I-labeled sheep anti-mouse immunoglobulin (Dupont, NEN) diluted in MTA (10^6 cpm/ml). Filters were washed, air dried, and analyzed by autoradiography.

Immunofluorescent staining. For the immunofluorescence procedure, sf9 cells were seeded and infected on cover slips. The whole procedure was performed at room temperature. All washes were performed in buffer A (10 mM glycine in phosphate-buffered saline). At 24 h postinfection, cells were fixed in 3% paraformaldehyde in phosphate-buffered saline for 20 min and then washed twice for 10 min. Fixed cells were permeabilized in buffer A containing 1% Nonidet P-40, washed twice, and incubated for 1 h at room temperature in a 1:250 dilution of 18H6 monoclonal ascites fluid in buffer A containing 0.3% Nonidet P-40. Cells were washed again and incubated for 1 h with the secondary antibody, a fluorescein-conjugated goat anti-mouse immunoglobulin (Pharmacia) diluted 1:200 in buffer A. After three washes, cells were mounted in glycerol containing 1% paraphenylenediamine and photographed at ×100 on a Zeiss microscope equipped for epifluorescence.

Two-dimensional tryptic peptide analysis. Immunoprecipitated ^{35}S-labeled *fos* protein was eluted from gel slices, oxidized, and digested with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington) as described previously (6). Samples were spotted onto precoated cellulose thin-layer chromatography plates (20 by 20 cm; EM reagents) and subjected to electrophoresis in pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid) for 27 min at 1 kV. Second-dimension thin-layer chromatography was performed for 8 h in a buffer containing *n*-butanol, pyridine, acetic acid, and water (6.5:5:1:4). Plates were air dried and exposed to preflashed X-ray film for 5 days.

Phosphoamino acid analysis. ^{32}P-labeled proteins were extracted from gel and hydrolyzed in 6 N HCl at 110°C for 1 h as described previously (6). Amino acids were separated by two-dimensional electrophoresis on precoated cellulose thin-layer chromatography plates (first dimension, 1.5 kV for 20 min in pH 1.9 buffer; second dimension, 1.3 kV for 16 min in pH 3.5 buffer [5% acetic acid, 0.5% pyridine]).

In vitro association of *fos* and *jun*. Human c-*fos* and murine c-*jun* proteins translated by reticulocytes lysate in vitro were kindly provided by L. J. Ransone. The samples used in our experiments were taken out of a 100-μl translation reaction as described previously (28). The in vitro association of *fos* and *jun* proteins was performed as described previously (35). ^{35}S-labeled proteins were incubated for 30 min at 30°C and then immunoprecipitated by using 18H6 monoclonal antibody. *fos* protein expressed in baculovirus was eluted from polyacrylamide gel as described by Briggs et al. (4) before use in in vitro association with *jun* protein.

Gel shift analysis. For gel retardation assays, proteins were incubated for 20 min at room temperature in a 20-μl final

volume containing 3 μg of poly(dI-dC) (Boehringer Mannheim Biochemicals) in TM buffer (50 mM Tris hydrochloride [pH 7.9], 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol). A synthetic 18-base-pair oligonucleotide containing the human metallothionein IIA TRE was end labeled with [α-^{32}P]ATP with T4 polynucleotide kinase. Approximately 0.1 ng of labeled DNA (<10,000 cpm) was added to the preincubated proteins. The DNA-protein complexes were resolved on a 4% polyacrylamide gel (39:1 acrylamide-bisacrylamide) in 0.25× TBE (1× TBE is 50 mM Tris borate–1 mM EDTA). The gels were dried and autoradiographed with intensifying screens at −70°C.

Immunoaffinity purification. Coupling of monoclonal antibody to protein A-Sepharose was done as follows: 3 ml of ascites fluid containing 18H6 antibody was incubated with 1 ml of protein A-Sepharose (Repligen) for 1 h at room temperature. Beads were washed with 0.2 M borate buffer (pH 9) and then suspended in 10 ml of the same buffer; dimethylpimelimidate (Sigma Chemical Co.) was added to a final concentration of 20 mM. After 30 min of incubation at room temperature, beads were washed with 0.2 M ethanolamine (pH 8) for 2 h at room temperature. Immunoaffinity chromatography was performed as follows. ^{35}S-labeled nuclear extract from 8 × 10^7 infected insect cells was mixed with cold extract from 5 × 10^8 cells and incubated for 1 h at 4°C with protein A-Sepharose. The beads were pelleted at 2,000 rpm in an IEC centrifuge, and the supernatant was incubated with 18H6 antibodies coupled with protein A-Sepharose. After 1 h of incubation at 4°C, the beads were washed in a column with 150 ml of buffer containing 0.5 M LiCl, 1 mM EDTA, 50 ml Tris hydrochloride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (pH 8). The beads were then washed with 100 ml of buffer containing 5 mM NaCl, 1 mM EDTA, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (pH 7.4). Bound proteins were eluted with 6 ml of 6 M guanidine, and 0.5-ml fractions were collected. Fractions were dialyzed against TM buffer. Samples of the fractions were counted in Econofluor (Dupont, NEN) and were analyzed by SDS-PAGE followed by autoradiography.

RESULTS

Construction and selection of recombinant baculoviruses containing human c-*fos* cDNA. The strategy followed for the construction of the vector containing c-*fos* human cDNA is shown in Fig. 1. A 1.27-kilobase-pair DNA fragment of human c-*fos* cDNA containing a 100-base-pair 5′-untranslated sequence, the complete coding sequence, and 40 base pairs of 3′-untranslated sequence was isolated by *Nae*I digestion of plasmid pWJD444 (W. W. Lamph, unpublished data). *Bam*HI linkers were ligated, and the fragment was cloned into the *Bam*HI site of the baculoviral transfer vector pAc373 (39). The resulting recombinant plasmid pAcc316 was cotransfected into sf9 cells together with wild-type AcNPV DNA. After 48 h, the supernatant of transfected cells was recovered and utilized to infect sf9 cells. The presence of infected cells that may be expressing *fos* protein was initially judged by the absence of occlusion bodies. However, definitive proof was obtained by immunofluorescent staining with 18H6 monoclonal anti-*fos* as the first antibody and fluorescein-conjugated goat anti-mouse immunoglobulin as the fluorescent antibody.

Cells infected with the wild-type virus were readily detectable under the phase-contrast microscope due to the presence of refringent polyhedrin occlusion bodies in their nuclei (Fig. 2A, arrows no. 1). Since the infection was carried out with a mixture of wild-type and recombinant viruses, cells exhibiting no occlusion bodies (arrows no. 2 and 3) are either uninfected or likely to represent infection with *fos*-expressing baculoviruses (Acfos). Figure 2B shows the immunofluorescence staining pattern of sf9 cells infected with *fos*-recombinant baculovirus. There were a number of cells which showed extensive immunofluorescent staining with *fos* antibodies (arrow no. 3), confirming the production of the *fos* protein. No immunofluorescence was observed in cells containing occlusion bodies (arrow no. 1) or uninfected cells (arrow no. 2). Interestingly, the fluorescent staining of insect cells infected with recombinant baculoviruses expressing *fos* appeared to be mainly nuclear (Fig. 2B). Because the nucleus in infected cells occupies a very large portion of the cell, it was difficult to see the cytoplasm; however, cell fractionation data gave similar results (data not shown). Thus, *fos* protein expressed in insect cells retains its characteristic of nuclear localization (7), indicat-

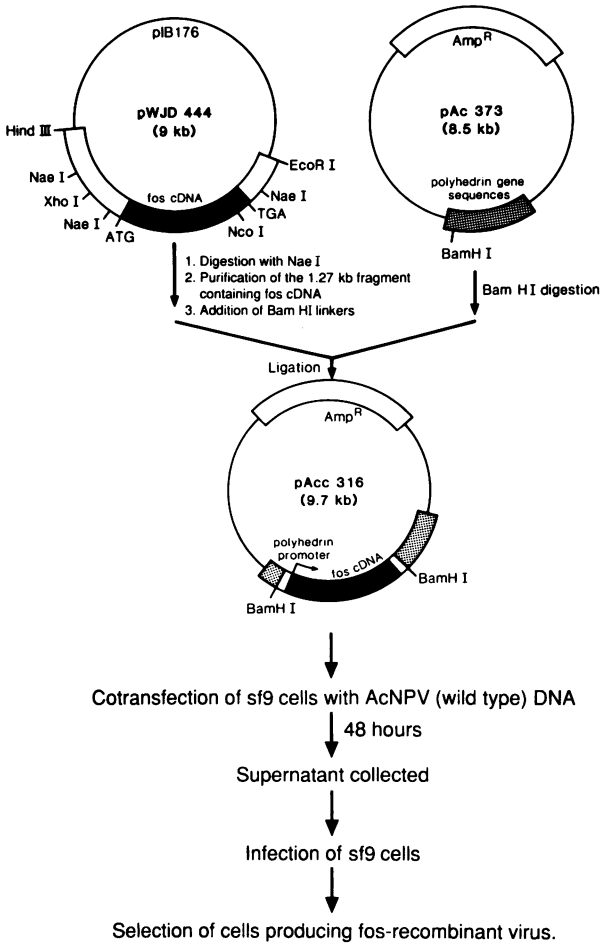

FIG. 1. Strategy for construction of the baculovirus expression vector for human c-*fos* expression. In pWJD444 (kindly provided by W. W. Lamph), solid and open bars indicate *fos* coding and noncoding sequences, respectively. In pAC373, the open bar indicates plasmid pUC8 sequences that include the gene for ampicillin resistance. The remaining sequences in the plasmid pAc373 represent baculoviral sequences, which include polyhedrin gene sequences (dotted bar). In pAcc316, the arrow indicates start and direction of translation.

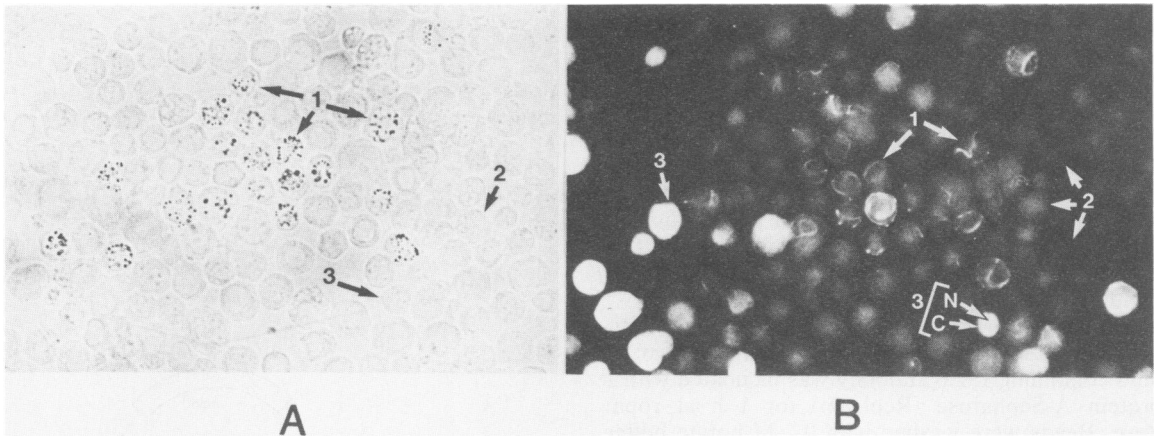

FIG. 2. Immunofluorescent staining of sf9 cells infected with nonpurified Acfos. Immunofluorescent staining was performed 24 h after infection. Arrows numbered 1 point to cells showing polyhedrin occlusions due to infection with wild-type virus. Arrows numbered 2 and 3 point to uninfected cells and cells infected with Acfos, respectively. (A) Phase-contrast observation. (B) Immunofluorescent staining. N, Nuclei; C, cytoplasm. Magnification ×100.

ing that the signal involved in *fos* nuclear translocation in mammalian cells is recognized by insect cells.

During cotransfection of wild-type DNA and recombinant pAcc316, homologous recombination occurs with a low frequency (0.1 to 5%). To isolate recombinant viruses expressing *fos* from the bulk of wild-type viruses, three rounds of visual selection for occlusion-negative plaques as well as plaque hybridization with a nick-translated human c-*fos* cDNA probe were performed (data not shown). The viral preparations were considered free of wild-type virus and homogenous for Acfos production when infection of sf9 cells resulted in occlusion-negative plaques only.

Characterization and quantification of *fos* protein synthesized in insect cells. To characterize *fos* protein produced by baculoviral vectors, insect cells were infected for various times with purified recombinant viruses and labeled with [^{35}S]methionine for 3 h. Total cell extracts were prepared, and immunoprecipitation was carried out by using the *fos* monoclonal antibody 18H6 (11). As a control, sf9 cells not infected or infected with wild-type AcNPV were treated in a similar manner. The proteins immunoprecipitated were analyzed by 10% SDS–PAGE (Fig. 3A). An intense broad band corresponding to *fos* protein and migrating with a molecular mass of approximately 55 kDA was observed in Acfos-infected cells 24 h postinfection (lane 4) but not in noninfected (lane 1) or wild-type-infected (lane 2) cells. No *fos* protein is detected at 12 h postinfection (lane 3). This result is not unexpected since polyhedrin, whose promoter drives *fos* gene expression in recombinant viruses, is not synthesized until 15 to 20 h after viral infection (25). Maximal expression was observed at 24 h postinfection (lane 4). Lower-molecular-mass bands (46 and 25 kDa) were detected at 36 and 48 h postinfection (lanes 5 and 6). These bands probably represent degradation products of the *fos* protein. It is noteworthy that the 46-kDa band was also visible at 24 h postinfection, suggesting that *fos* protein might be rapidly degraded in insect cells as it is in mammalian cells, where it is known to have a very short half-life (2, 7). The quantity of *fos* protein produced in 2×10^6 sf9 cells 24 h after infection appears to be 10 to 20 times higher than that detected in 5×10^6 MMV cells (Fig. 3A, lane 7), a high constitutive producer of murine c-*fos* protein (2). Labeled proteins immunoprecipitated with *fos* antibodies represented about 0.2% of the total cellular proteins precipitated with trichloroacetic acid. The high ratio of *fos* protein versus total protein in insect cells is probably due partly to overexpression and partly to the decrease in cellular proteins synthesized after baculoviral infection. This quantity might even be an underestimate, since ^{35}S labeling only detects the amount of protein synthesized during the time of incorporation of the radioactive compound. To determine the actual amount of the *fos* protein present in infected sf9 cells, nuclear extracts from cells infected with different recombinant viruses were prepared 24 h postinfection and analyzed by immunoblotting (Fig. 3B). Accurate quantitation by densitometer scanning of a shorter exposure of the autoradiogram presented in Fig. 3B was performed. It indicated that 10 to 20 times more protein was made in infected sf9 cells (lanes 3 to 8) than in TPA-treated HeLa cells (lane 9), depending on the recombinant virus used and taking into account that the extracts from HeLa cells shown in Fig. 3B were two times more concentrated than extracts from sf9 cells. Thus the baculovirus-infected cells offer an excellent source for high production of *fos* protein, up to 1 to 5 μg per 10^6 cells. It is remarkable that the immunoblotting and ^{35}S labeling techniques led to similar estimation of the quantity of *fos* protein produced in insect cells compared with mammalian cells. This result indicates that no large accumulation of *fos* protein occurs during late baculoviral infection, in contrast to what is observed for polyhedrin and other proteins expressed through the baculoviral system (13). This observation argues for a short half-life of *fos* protein in insect cells, as is the case in mammalian cells (2, 7).

Structural comparison of the *fos* proteins synthesized in insect and mammalian cells. Our ultimate aim in expressing *fos* protein in insect cells is to purify the protein and study its interaction with other transcription factors. Therefore, it is essential to assess the integrity of *fos* protein made through a baculoviral system as compared with that made in mammalian cells. We performed two-dimensional peptide mapping on proteins immunoprecipitated from insect and mammalian cell extracts labeled with [^{35}S]methionine and [^{35}S]cysteine. The peptides generated by tryptic digestion of the *fos* protein made in insect cells (Fig. 4B) were identical to those arising from the *fos* protein made in mammalian cells (Fig. 4A). A mix of the two proteins before electrophoretic separation showed a similar pattern (Fig. 4C). Both the positions and relative intensities of the spots a through h are

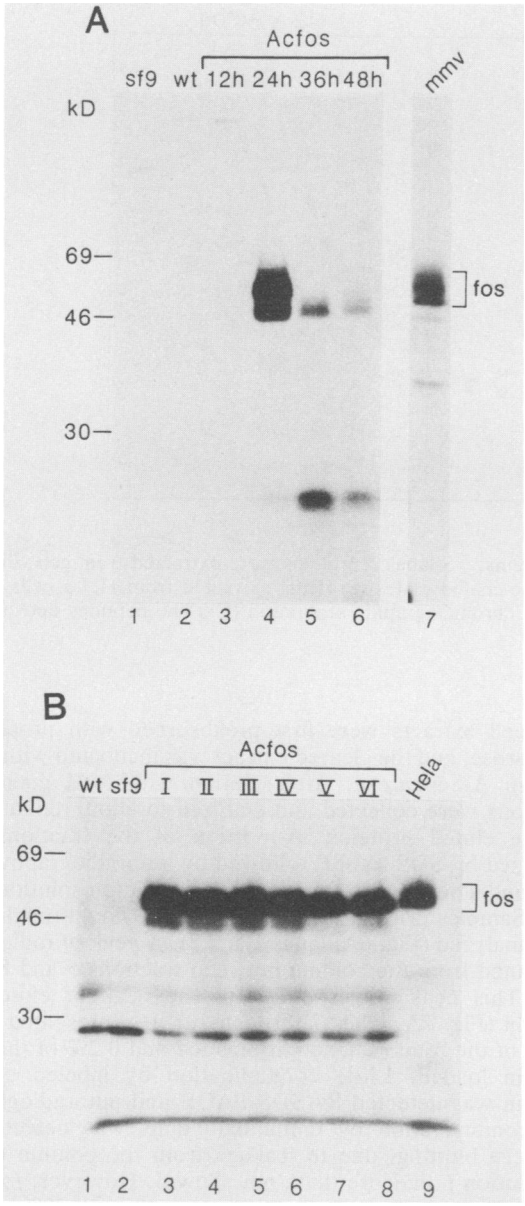

FIG. 3. Characterization of *fos* protein expressed in sf9 cells infected with Acfos. (A) Immunoprecipitation of ^{35}S-labeled *fos* protein from 1×10^6 sf9 cells that were not infected (lane 1), infected for 24 h with wild-type virus (lane 2), or infected with Acfos for 12, 24, 36, and 48 h (lanes 3, 4, 5, and 6, respectively) and from 5×10^6 MMV cells (lane 7). Labeling was as described in Materials and Methods. (B) Western blot (immunoblot) on nuclear extracts from 5×10^6 sf9 cells infected for 24 h with wild-type AcNPV (lane 1), noninfected (lane 2), or infected for 24 h with different clones of Acfos (lanes 3 through 8) or from 1×10^7 HeLa cells treated for 1 h with PMA (lane 9). The positions of migration of molecular mass standards are indicated.

conserved. This result, taken together with the expected apparent molecular weight of baculoviral *fos* protein, suggests that the protein expressed in insect cells is structurally very similar, if not identical, to the one made in mammalian cells.

Identity of maps also suggests that any possible modification, such as phosphorylation, glycosylation, methylation, or acetylation, that would modify the migration of a given peptide must be accomplished in insect cells as observed in the mammalian cells. However, none of the peptides detected seemed to be a substrate for phosphorylation, since alkaline phosphatase treatment of the protein before peptide mapping did not modify the position of any spot (data not shown). This is in agreement with previous reports showing that the main target for TPA-dependent phosphorylation is the tryptic peptide constituted by the 20 C-terminal amino acids within which no cysteine or methionine is encountered (2, 41).

Phosphorylation of the baculoviral c-*fos* protein. c-*fos* protein is phosphorylated when expressed in mammalian cells (7, 9). The carboxy terminus of the c-*fos* protein appears to be the main target for TPA-stimulated phosphorylation (2). This domain of the protein has also been involved in transactivation of heterologous promoter and transrepression of its own promoter (36, 43). It is thus conceivable that phosphorylation of the *fos* protein might play a key role in modulating its activity. Therefore, we wanted to investigate whether the baculoviral *fos* protein is modified by phosphorylation as is the mammalian protein.

Uninfected sf9 cells or cells infected with wild-type virus or with two different Acfos viruses were labeled overnight with $^{32}P_i$. As a control, HeLa cells were labeled for 1 h in the presence or absence of PMA. Total cell extracts were prepared, immunoprecipitated, and analyzed by SDS-PAGE (Fig. 5A). c-*fos* protein was detected in sf9 cells infected with Acfos I or II (lanes 3 and 4) but not in mock-infected cells (lane 5) or cells infected with wild-type AcNPV (lane 6). The c-*fos* protein made in sf9 cells displayed a heterogeneity in size also found in *fos* protein from TPA-induced mammalian cells (compare lanes 2 and 3). Our results show that baculoviral *fos* protein undergoes modification in insect cells as observed in mammalian cells. Phosphoamino acid analysis of the labeled proteins showed that phosphorylation of both baculoviral and mammalian cells occurred mainly at serine residues (Fig. 5B), in agreement with previously published results (2).

It is worth noting that a 150-kDa phosphoprotein was detected in all sf9 cell extracts, regardless of whether the cells were infected with Acfos (Fig. 5, lanes 3 through 6). Its identify remains obscure, but it could represent a *fos*-related antigen.

Activity of *fos* protein synthesized in insect cells. Although the function of the *fos* protein has not yet been precisely defined, an accumulated body of evidence suggests its likely involvement in transcriptional regulation through interaction with other transcription factors. It has long been observed that a 39-kDa protein is always coprecipitated with *fos* protein (7, 8, 10). Recently, this associated protein was identified as the product of the oncogene *jun* (1, 3, 5, 29, 34). This tight association of *fos* and *jun* results in over a 100-fold increase in binding to TRE (17, 27, 35). Therefore, we wanted to determine whether the *fos* protein produced in insect cells also has the ability to form a complex with mammalian *jun* protein.

Association of *fos* and *jun* was investigated by incubating the proteins together for 30 min at 30°C (35), followed by immunoprecipitation with *fos* monoclonal antibody 18H6 (Fig. 6). The ^{35}S-labeled *jun* protein used in these experiments was synthesized in vitro as described previously (28). ^{35}S-labeled baculoviral *fos* protein was purified by extraction from polyacrylamide gels (4). As a control, the association of *jun* with in vitro-translated *fos* was also tested (Fig. 6A, lane 3). In the absence of *fos*, no *jun* protein could be immunoprecipitated with *fos* antibodies (Fig. 6, lane 6). When

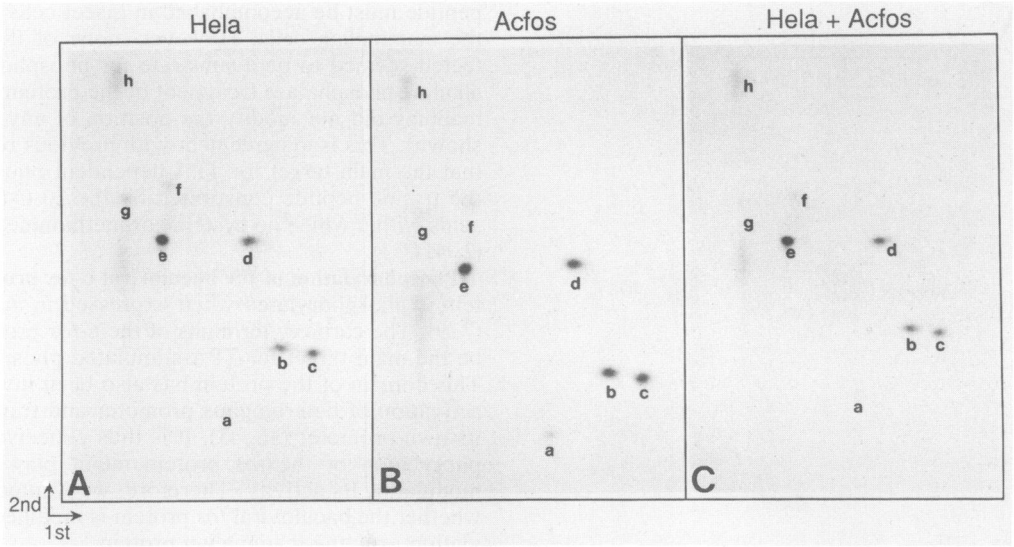

FIG. 4. Two-dimensional peptide maps of [^{35}S]methionine-labeled *fos* proteins. ^{35}S-labeled proteins were extracted from gels, digested with trypsin, and separated by two-dimensional chromatography on cellulose-coated plates. Proteins extracted from HeLa cells (A) or Acfos-infected insect cells (B) were run separately or mixed together before chromatographic separation (C). The peptides detected are designated a through h.

baculoviral *fos* protein and in vitro-translated *jun* protein were mixed together and immunoprecipitated with *fos* antibodies, *jun* protein was coimmunoprecipitated (Fig. 6A, lane 5), suggesting that the *fos* protein made in insect cells is capable of binding to mammalian *jun* protein.

We next wanted to study the possible cooperativity in DNA binding of *fos* protein produced by recombinant baculovirus with the in vitro-synthesized *jun* protein. Nuclear extracts from cells infected with Acfos were mixed with increasing amounts of in vitro-synthesized *jun* protein, and binding was then tested on a ^{32}P-labeled TRE oligonucleotide. The two proteins cooperated in binding (Fig. 6B, lanes 3 and 4). The addition of increasing quantities of *jun* protein to constant amount of *fos* protein led to increasing quantities of complex formed (lanes 2 through 4), showing that *fos* protein is in excess as compared with *jun* protein. Although two nucleoprotein complexes were detected, only the slower-migrating band represented the *fos-jun* protein complex. The faster-moving complex was due to nonspecific binding of protein(s) present in the reticulocyte lysate (35). Specificity of the upper complex was demonstrated by inhibition of complex formation when *fos* protein was preincubated with M2 antibody (Fig. 6B; lanes 6 through 8), a polyclonal antibody directed against amino acids 127 through 153 of *fos* protein (10, 11). This inhibition is due to the proximity of this epitope to the leucine zipper domain, a region of the protein involved in association of *fos* and *jun* proteins (15, 18, 28, 35, 38, 40). Thus it appears that the baculoviral *fos* protein not only can associate with in vitro-translated *jun* protein but also augments the binding of the resultant complex to TRE.

Purification of the *fos* protein from recombinant baculovirus-infected sf9 cells. To purify *fos* protein from infected sf9 cells, we used an immunoaffinity chromatography procedure where the monoclonal antibody 18H6 was covalently coupled to protein A-Sepharose. Extracts were prepared from Acfos-infected cells 24 h after infection. To monitor the *fos* protein during purification, about 10% of the extract was labeled in vivo with ^{35}S-labeled amino acids before cell lysis. The cell extracts were first preabsorbed with protein A-Sepharose, and the cleared extract was incubated with 18H6 protein A-Sepharose. After elution with 6 M guanidine, fractions were collected and dialyzed to allow renaturation of the eluted proteins. A portion of the fractions was analyzed by SDS–PAGE followed by autoradiography (Fig. 7A), and a portion was counted in scintillation solution (Fig. 7B). Samples of the total extract and the flowthrough were also analyzed (Fig. 6A, lanes 1 and 2). A peak of radioactivity eluted from the column between fractions 5 and 8 (Fig. 7B). This peak corresponded to the peak of eluted *fos* protein (Fig. 7A, lanes 7 through 10). It represented about 0.1% of the total radioactivity loaded and 0.2% of the total protein loaded. Little contamination by labeled cellular protein was detected by SDS-PAGE and autoradiography, but contamination by immunoglobulins was detected by Western blotting, due to leakage from the column during the elution procedure (data not shown). However, *fos* protein appeared to represent between 20 and 30% of the proteins eluted, and therefore an enrichment of 100- to 150-fold was achieved by this one-step immunoaffinity chromatography.

DISCUSSION

We report expression, characterization, and purification of a biochemically active human *fos* protein from insect cells infected with recombinant baculoviruses. The baculoviral *fos* protein has the following features in common with *fos* protein expressed in mammalian cells: (i) a molecular mass of approximately 55 kDa, (ii) nuclear localization, (iii) post-translational modification by serine phosphesterification, (iv) identical ^{35}S tryptic peptide maps on two-dimensional gel electrophoresis, (v) ability to associate with in vitro-synthesized mammalian *jun* protein, and (vi) cooperativity with *jun* in binding to TRE. The level of expression of *fos* protein was 10- to 20-fold higher in insect cells as compared with that in PMA-induced mammalian cells or a cell line constitutively making *fos* protein. Maximal levels of *fos* protein accumu-

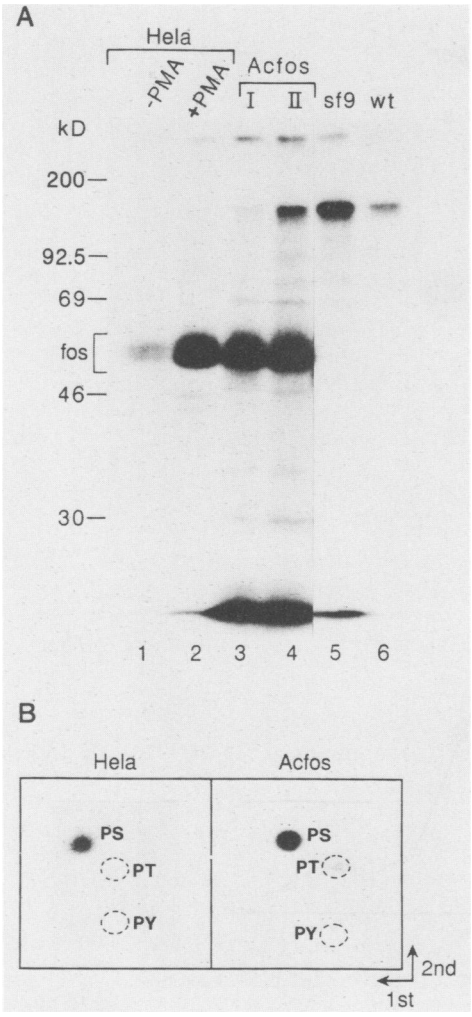

FIG. 5. Metabolic incorporation of $^{32}P_i$ into c-*fos* protein expressed in insect and HeLa cells. Cells (2×10^6 sf9 cells or 5×10^6 HeLa cells) were metabolically labeled with 1 mCi of $^{32}P_i$ per ml. Nuclear proteins were extracted and immunoprecipitated with *fos* monoclonal antibodies. (A) SDS-PAGE analysis: HeLa cells uninduced (lane 1) or incubated for 45 min with 75 ng of PMA per ml of medium (lane 2) were labeled for 1 h (including time of induction). sf9 cells infected for 24 h with two different isolates of Acfos (lanes 3 and 4), uninfected (lane 5), or infected for 24 h with the wild-type virus (lane 6) were labeled for 12 h during the course of infection. The positions of migration of molecular mass markers are indicated. (B) Phosphoamino acid analysis: ^{32}P-labeled *fos* proteins were extracted from gels, hydrolyzed with 6 N HCl at 110°C, and then separated by two-dimensional electrophoresis. The positions of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) are indicated.

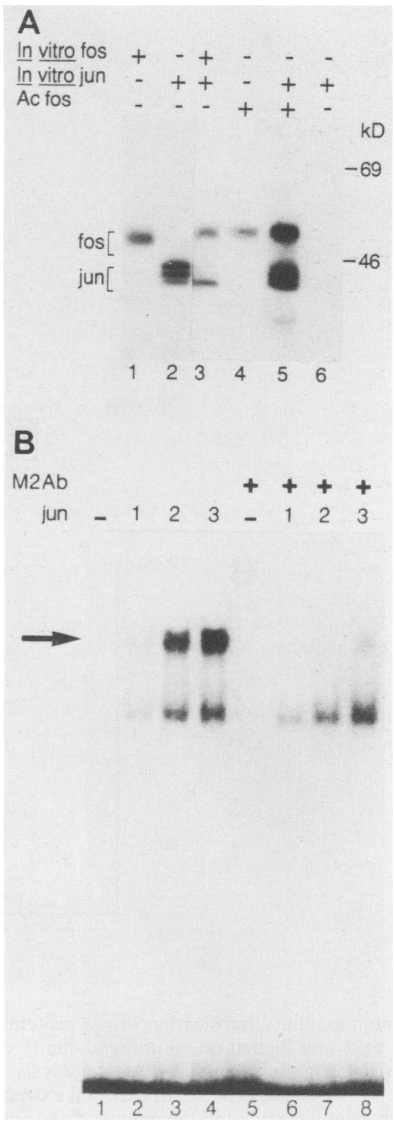

FIG. 6. Biological activity of *fos* protein expressed in insect cells. (A) Association of baculoviral *fos* protein with *jun* protein. Samples of 2 μl from a 100-μl reaction mixture of in vitro-synthesized *fos* and *jun* proteins were run separately (lanes 1 and 2) or mixed and immunoprecipitated with *fos* monoclonal antibodies (lane 3). *fos* protein gel purified from Acfos-infected cells was immunoprecipitated alone (lane 4) or after mixing with 2 μl of in vitro-synthesized *jun* protein (lane 5). Immunoprecipitation of *jun* protein alone (2 μl) with *fos* antibodies is shown in lane 6. Exposures were for 2 h for lanes 1 and 2 and 24 h for lanes 3 to 6. (B) Baculoviral *fos* protein cooperates in binding to a TRE with in vitro-translated *jun* protein. A sample (equivalent to 10^5 cells) of a sf9 nuclear extract containing the recombinant *fos* protein was mixed with approximately 0.1 ng of labeled TRE oligonucleotide and 3 μg of poly(dI-dC) in the absence (lane 1) or presence of 1 to 3 μl of in vitro-synthesized *jun* protein (lanes 2 to 4). Similar experiments were performed with prior incubation of *fos* protein with M2 antibodies (lanes 5 to 8).

late 24 h postinfection with considerable reduction by 36 to 48 h, indicating that, like its mammalian counterpart, the baculoviral *fos* protein has a short half-life. The fact that the amount of the immunoprecipitated [^{35}S]methionine-labeled *fos* protein is identical to the amount of *fos* protein detected by immunoblotting further confirms lack of accumulation of *fos* protein. Thus it appears that signals for rapid degradation of mammalian proteins are recognized by the cellular machinery of insect cells.

The baculoviral *fos* protein is nuclear in localization like its mammalian homolog. Thus, the nuclear transport signal in the *fos* protein that is operative in mammalian cells is also recognized in insect cells. Various mammalian nuclear proteins expressed in insect cells using a baculoviral expression vector, including *myc*, simian virus 40 large T antigen, and other transcription factors (22, 26) have also been shown to be nuclear in insect cells, suggesting that mammalian nuclear translocation signals are recognized and functional in insect cells.

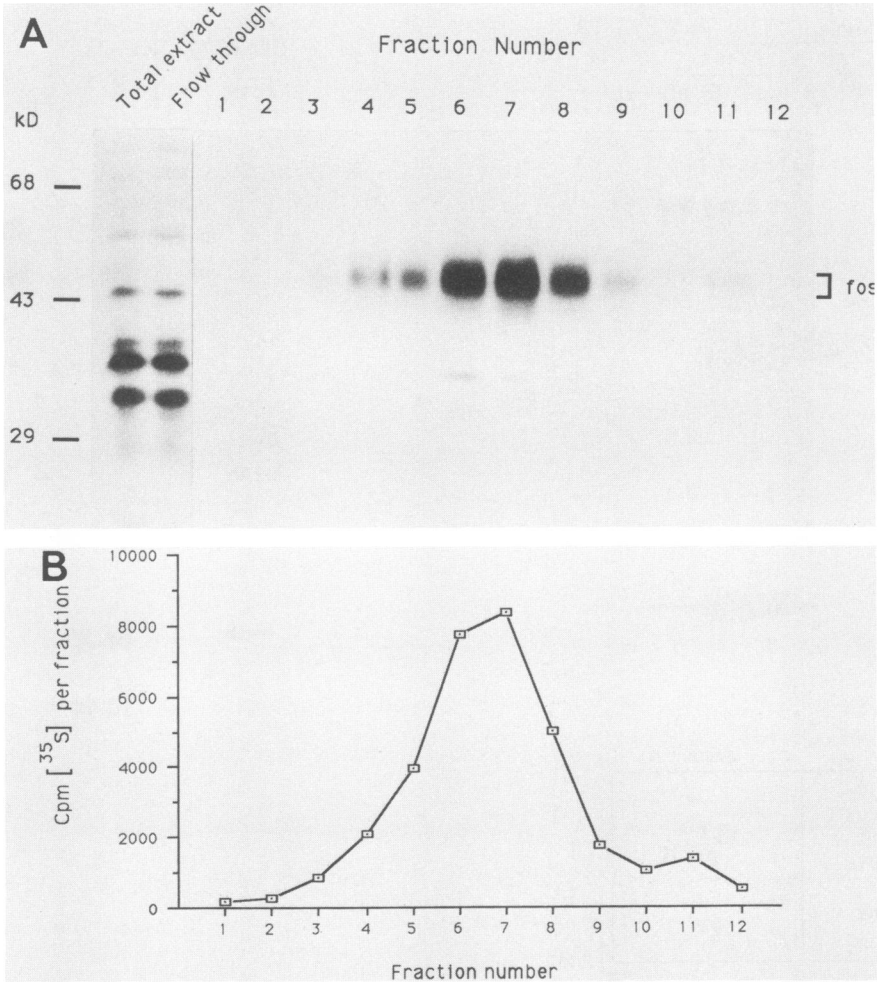

FIG. 7. Immunoaffinity purification of *fos* protein from sf9-infected cells. Unlabeled and ^{35}S-labeled extracts (ratio, 9:1) were prepared from 5×10^8 cells and loaded on an immunoaffinity column as described in Materials and Methods. Fractions of 0.5 ml were collected and dialyzed against TM buffer. (A) SDS-PAGE analysis: 20-μl samples of each fraction (lanes 1 to 12) were analyzed by 10% SDS–PAGE along with samples (0.1%) of the proteins of the total extract after preclearing and of the flowthrough after loading. After migration, the signal was enhanced with salicylic acid, and the gel was dried and autoradiographed. (B) ^{35}S counts eluted of the column: 10-μl samples of each fraction were counted in scintillation solution.

Because *fos* protein is extensively posttranslationally modified, it was a concern whether the protein made in insect cells would also undergo similar modifications. Phosphoesterification of serine residues is the modification of the *fos* proteins that has been best characterized (2, 9). Labeling with $^{32}P_i$ showed (Fig. 5) that *fos* protein in insect cells is extensively phosphorylated on serine residues and, based upon its mobility on SDS-PAGE, exhibits a heterogeneity in size comparable to that of PMA-induced mammalian *fos* protein from HeLa cells. We did not determine the sites of phosphorylation, but when the ^{35}S-labeled protein was treated with alkaline phosphatase before tryptic peptide mapping, there was no shift in the electrophoretic mobility of any of the spots detected (data not shown). This suggests that none of the ^{35}S-labeled peptides are phosphorylated, which is consistent with our previous observation that the majority of the phosphorylation of mammalian *fos* protein occurs in the 20 carboxy-terminal amino acids, which are devoid of either methionine or cysteine residues (2).

Overall, these data show that the *fos* protein overexpressed in insect cells is structurally very similar to the protein synthesized in mammalian cells. More importantly, the ability of the baculoviral *fos* protein to associate with *jun* nuclear oncoprotein (AP-1) testifies to its functional integrity. Indeed, not only is the baculoviral *fos* protein able to associate with *jun* protein, but the resulting complex manifests avid binding to TRE (Fig. 6B). Therefore, the *fos* protein synthesized in insect cells appears to be a good substrate for purification. Immunoaffinity purification (Fig. 7) allowed a 100- to 150-fold enrichment in a single-step purification procedure. Due to the high affinity of the monoclonal antibody for its antigen, we had to use a denaturing agent at a high concentration to recover the protein bound to the immunoaffinity support. However, dialysis of the fractions after elution allowed renaturation of the eluted protein as judged by its ability to associate with *jun* protein and subsequent binding of the complex to DNA (data not shown). We have also generated baculoviral *jun* protein and are currently pursuing studies to determine whether the two proteins can activate transcription from TRE in an in vitro

transcription system. The availability of large amounts of purified *fos* and *jun* proteins should allow a more rigorous analysis of their molecular interactions.

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