Drosophila Krüppel gene product produced in a baculovirus expression system is a nuclear phosphoprotein that binds to DNA

(regulatory proteins/embryogenesis/segmentation gene/gap gene/zinc finger)

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ABSTRACT The product of the *Drosophila* segmentation gene Krüppel was produced in cultured insect cells using the baculovirus expression system. When a cloned Krüppel cDNA sequence was inserted into the viral genome downstream from the promoter of the polyhedrin gene, a polypeptide with an apparent molecular weight of $\approx 72,000$ was observed in the nuclei of infected cells. Antibodies were raised against this protein and used to detect Krüppel in *Drosophila* embryos. Characterization of the Krüppel protein extracted from infected cells showed that it is tightly bound to the nucleus, it binds to calf thymus DNA-cellulose, and it is phosphorylated. These results support the hypothesis that Krüppel is a regulatory protein that acts by binding DNA.

Genes involved in the segmentation of *Drosophila melanogaster* embryos have been grouped into three phenotypic classes: gap, pair rule, and segment polarity (1–4). Embryos homozygous for deletions in the Krüppel (*Kr*) locus lack three contiguous thoracic and the five anterior abdominal segments. On the basis of this phenotype, *Kr* has been designated a gap gene (1, 5–7). The defects in the thoracic and abdominal segments in *Kr* mutants can be partially corrected by injection of cytoplasm from wild-type embryos (8). Analysis of *Kr* gene transcription by *in situ* hybridization revealed the presence of *Kr* RNA in regions of the blastula and gastrula that correspond, at least in part, to the regions affected in *Kr* embryos (8). The distribution of Krüppel protein during early embryogenesis has not yet been reported. Although the function of the *Kr* gene product is not known, the possibility that it acts through binding to DNA is suggested by the observation that the *Kr* protein contains “zinc-finger” domains similar to those found in the *Xenopus* transcription factor IIIA (9, 10).

Further insights into the role of the *Kr* gene product in early development could be achieved by studying the structure and function of the protein. However, isolation of sufficient amounts of *Kr* protein for detailed biochemical studies is not feasible because of the low abundance of the protein in embryos. This problem could be circumvented by introducing the coding sequence of the *Kr* gene into a bacterial expression vector, an approach that has been used to study the DNA binding properties of the *Drosophila* engrailed gene protein (11). Unfortunately, analysis of such proteins could be misleading if post-translational modifications, which occur only in eukaryotic cells, are required for the normal function of the protein (12, 13). In addition, extraction of the protein from bacteria sometimes results in incorrect folding or denaturation of the protein. An alternative approach is to express the cloned gene in eukaryotic cells.

A potentially useful vector for expressing high levels of biologically active *Drosophila* regulatory proteins is the

baculovirus system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) (14–16). This virus propagates in cultured insect cells and has strong temporally regulated promoters, and the coding sequence of interest can be easily inserted by *in vivo* recombination. Proteins expressed in insect cells may be correctly folded and contain appropriate posttranslational modifications. To establish the feasibility of expressing functional *Drosophila* regulatory proteins, we have introduced the cDNA corresponding to the major open reading frame of Krüppel into an AcNPV expression vector and have propagated the recombinant virus in insect cells. We find that the *Kr* gene protein is produced at high levels in this host-vector system ($\approx 1\%$ of the total protein), that the protein is phosphorylated, that it accumulates in the nucleus of the cultured cells, and that it binds to DNA.

MATERIALS AND METHODS

Construction of Recombinant Plasmids and Vectors. Constructions were carried out using plasmids pc2kb and A13kr obtained from H. Jackle (Max-Planck Institut für Entwicklungsbiologie, Tübingen, F.R.G.), Sp6Kr405 obtained from G. Struhl (Harvard University), and pev55 obtained from D. W. Miller (Genetics Institute, Cambridge, MA).

Cells and Virus. AcNPV stocks were grown, and titers were determined in *Spodoptera frugiperda* cells using TC 100 medium (GIBCO) plus 10% (vol/vol) fetal bovine serum (16). The procedure used for transfection and selection of recombinant virus was carried out as described (16). Radiolabeling of infected cells and analysis of polypeptides were carried out as described by Miyamoto *et al.* (17).

Fractionation and Preparation of Nuclear Extracts. Nuclear and cytoplasmic fractions were prepared from virus infected *S. frugiperda* cells by hypotonic Dounce homogenization and low-speed centrifugation. The soluble cytoplasmic fraction was discarded, and the pellet was washed once with the same hypotonic buffer (10 mM Hepes, pH 7.8/3 mM $MgCl_2$ /10 μM $ZnCl_2$ /50 mM KCl/0.5 mM phenylmethylsulfonyl fluoride). The nuclear pellet was extracted with the same buffer containing 0.5 M or 2 M NaCl as described by Dignam *et al.* (18). Alternatively, in the acetonitrile procedure, the extraction buffer used was 50% (vol/vol) acetonitrile and 0.3 M ammonium carbonate (pH 9). The resulting crude nuclear extract was centrifuged 4–8 hr at $30,000 \times g$ to remove insoluble components.

DNA Binding Assay. A nuclear extract from [^{35}S]methionine-labeled cells infected with AcNPV-Kr was prepared using the acetonitrile procedure and applied to a mini gel-filtration column (Pharmacia) to change the buffer. The protein fraction in the binding buffer (20 mM Hepes, pH 7.8/0.1 M NaCl/3 mM $MgCl_2$ /10 μM $ZnCl_2$ /0.2 mM phenylmethylsulfonyl fluoride) was chromatographed on a small

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Abbreviation: AcNPV, *Autographa californica* nuclear polyhedrosis virus.

column containing a mixture of single-strand and double-strand DNA-cellulose (Pharmacia), which was equilibrated with the binding buffer (19, 20). The flow-through was applied a second time to the same column and then collected. The column was extensively washed with binding buffer, and proteins bound to the column were eluted in one step with a buffer composed of 20 mM Hepes, pH 8.4/1 M NaCl/3 mM MgCl₂/10 μ M ZnCl₂/0.2 mM phenylmethylsulfonyl fluoride. Aliquots from the flow-through and the eluted fraction were precipitated with acetone and used for immunoblot analysis.

Preparation of the Antiserum to Krüppel Protein and Affinity Purification of Anti-Kr Antibodies. Two rabbits were inoculated subcutaneously with gel-purified Kr protein from infected insect cells in an equal volume of Freund's complete adjuvant (Difco). The rabbits were given a booster injection after 4 weeks and bled at 6 weeks. Antibodies were characterized using AcNPV cellular extracts from cells infected with AcNPV-Kr or AcNPV-Del. Sera from both rabbits were positive for anti-Krüppel antibodies, which were affinity purified using Kr nuclear extracts coupled to Affi-Gel 10 (Bio-Rad). The cross-reacting antibodies were depleted using extracts prepared from AcNPV-Del-infected cells.

RESULTS

Expression of Krüppel Protein in the Baculovirus System.

The polyhedrin promoter was fused to BAL-31 exonuclease deletions of the 5'-untranslated region of Kr cDNA clone pc2kb (10). Two clones, pKr4 and pKr9, contain 7 and 37 base pairs of sequence upstream of the presumed ATG translational initiation site, respectively. The full-length cDNA was excised from these plasmids and recombined into the "transplacement" vector pev55 (16), to obtain pev55-Kr7 and pev55-Kr37. As shown in Fig. 1, the resulting transplacement vectors encode an RNA that contains the majority (55 base pairs) of the polyhedrin 5'-leader sequences fused to the 7 or 37 base pairs of the 5'-noncoding region of the Krüppel cDNA followed by the entire coding sequence. These fusion

FIG. 1. Construction of recombinant baculoviruses containing polyhedrin promoter-Krüppel cDNA fusions. The Krüppel cDNA cloned in the transplacement vector pev55-Kr was mixed with wild-type AcNPV DNA and used to cotransfect *S. frugiperda* cells. Recombinants containing the polyhedrin promoter fused to Krüppel cDNA were identified by the absence of polyhedrin inclusion bodies in viral plaques. The 5' and 3' regions of the polyhedrin gene that are present in the wild-type virus and the transplacement vector pev55 are designated by the solid and stippled boxes, respectively. The polyhedrin- and Krüppel-coding regions are indicated by the open and hatched boxes, respectively. The wild-type virus DNA is $\approx$ 130 kilobases long, while pev55-Kr7 is about 10 kilobases long. The recombinant virus AcNPV-Kr7, which is generated by double-homologous recombination, contains the polyhedrin promoter fused to the Kr cDNA.

genes contain no ATG codons upstream of the authentic Krüppel initiation site and thus encode the entire Kr protein.

To transfer the Kr gene into AcNPV genome, *S. frugiperda* cells were cotransfected with the plasmid pev55-Kr7 or pev55-Kr37 and the genomic DNA of wild-type AcNPV. Recombinant viruses, produced by allelic replacement of the polyhedrin gene by homologous recombination, were identified by plaque morphology. Plaques produced by the recombinant viruses do not contain polyhedrin inclusion bodies (14, 15). The recombinant virus clones designated AcNPV-Kr7 and AcNPV-Kr37 contain the expected polyhedrin promoter-Krüppel cDNA fusions, as indicated by the restriction map of purified recombinant viral DNA.

Whole-cell extracts were prepared from cells labeled with [³⁵S]methionine and infected with the recombinant viruses or with AcNPV-Del, a virus in which the polyhedrin gene is deleted. Analysis of these extracts by NaDodSO₄/PAGE revealed the presence of a protein of $\approx$ 72 kDa in extracts from cells infected with recombinant viruses containing the Krüppel cDNA (Fig. 2A, lane 1). This protein, which represents $\approx$ 1% of the total cellular protein, was not detected in extracts from cells infected with AcNPV-Del (Fig. 2, lane 2).

FIG. 2. Identification of Krüppel protein in AcNPV-Kr-infected cells. Cells infected with AcNPV-Kr or AcNPV-Del were labeled with [³⁵S]methionine, and total cell extracts were analyzed by electrophoresis on a 6–13% NaDodSO₄/polyacrylamide gradient gel. (A) Total extract from cells infected with AcNPV-Kr (lane 1) and a AcNPV-Del (lane 2). The arrow to the left of lane 1 indicates the position of a protein that is only found in the cells infected with AcNPV-Kr, while the arrow on the right of lane 2 indicates the position of a 69-kDa size marker in a parallel lane. (B) Comparison of Krüppel protein produced in AcNPV-Kr-infected cells (lane 1) with ³⁵S-labeled *in vitro* translation products of an SP6 RNA transcript from the Kr cDNA Sp6-Kr405 plasmid (lane 2). The arrow on the right indicates the position of the *in vitro*-translated Krüppel polypeptide.

The size of the putative Kr polypeptide determined by NaDodSO₄/PAGE is somewhat larger than that predicted from the open reading frame of the cDNA sequence (50 kDa). To confirm that the polypeptide made in AcNPV-infected cells is the Kr protein, we used a rabbit reticulocyte *in vitro* translation system and Sp6 Krüppel RNA derived from the clone Sp6Kr405 to produce a ³⁵S-radiolabeled protein (21). A comparison of the Kr *in vitro* translation product and the Kr protein produced in AcNPV-Kr-infected cells is shown in Fig. 2B. Both proteins have similar mobilities on NaDodSO₄/PAGE, although the band observed with the *in vivo*-produced protein is more diffuse.

Detection of Kr in Cultured Cells and Embryos Using Anti-Kr Antibodies. Gel-purified Kr protein prepared from AcNPV-Kr-infected *S. frugiperda* cells was used to raise a rabbit polyclonal antiserum against Kr protein. The sera obtained from the injected rabbits were screened for antibodies against Kr protein by immunoblot analysis. The sera that tested positive were first passed through an affinity column containing an extract from cells infected with AcNPV-Del. The Kr antibodies were then affinity purified using partially purified Kr protein cross-linked to Affi-Gel 10. The affinity-purified antibodies recognize a 72-kDa polypeptide in extracts from *S. frugiperda* cells infected with AcNPV-Kr viruses but not in extracts from cells infected with AcNPV-Del (data not shown).

The affinity-purified antibodies were used in immunoblot studies to compare the Kr protein produced in AcNPV-Kr-infected cells and in staged *Drosophila* embryos. As shown in Fig. 3, a protein with the same electrophoretic mobility is observed in both cell types. A doublet was detected in extracts from infected cells, but not in the extracts from embryos, suggesting that the Krüppel protein is clipped by a protease present in infected cells.

Post-Translational Modification of Kr Protein in AcNPV-Kr-Infected Insect Cells. To determine whether the Kr protein produced in insect cells infected with the recombinant virus is post-translationally modified, we carried out experiments to determine whether the protein is glycosylated or phosphorylated. A potential glycosylation site was noticed in the Kr protein sequence deduced from the DNA sequence of Kr cDNA (10). If this site is glycosylated *in vivo*, it should be

FIG. 3. Identification of Krüppel protein in cells infected by recombinant viruses and in staged *Drosophila* embryos. NaDodSO₄ cell lysates of AcNPV-Kr-infected cells (lanes 1 and 5) or of staged *Drosophila* embryos (lane 2, 2–4 hr; lane 3, 4–6 hr; lane 4, 6–8 hr) were electrophoresed on a 8% NaDodSO₄/polyacrylamide gel and analyzed by immunoblotting using anti-Kr antibodies. Approximately 5 times less material was loaded on lane 5 compared to lane 1. The upper arrow to the right of lane 5 shows a 72-kDa band that is present in *Drosophila* embryos and in the infected insect cells. The lower arrow with a star indicates a presumptive degradation product of the 72-kDa polypeptide observed only in infected embryos. The arrow to the left of lane 1 indicates the position of a 69-kDa marker.

possible to detect a difference in the mobility of the modified and unmodified proteins by polyacrylamide gel electrophoresis. ³⁵S-labeled Kr protein was produced in infected insect cells in the presence or absence of tunicamycin, a drug shown to prevent glycosylation in insect cells (22). We found that the mobility of the 72-kDa polypeptide produced in cells treated with tunicamycin was indistinguishable from that of the control (data not shown). Inhibition of glycosylation in the tunicamycin-treated cells was indicated by the fact that bands corresponding to viral polypeptides known to be glycosylated were sharpened (data not shown). We conclude that the Kr protein produced in infected insect cells does not contain detectable N-linked carbohydrate. In addition, the Kr protein does not appear to contain O-linked carbohydrate, since the protein does not bind to a lens culinaris lectin (data not shown).

The possibility that the Kr protein is phosphorylated in infected insect cells was examined by incubating AcNPV-Kr- or AcNPV-Del-infected cells with ³²P in a phosphate-free medium for 2 hr at 40 hr after infection. Total cell extracts from the infected cells were then analyzed by NaDodSO₄/PAGE. As shown in Fig. 4A, a phosphoprotein of 68–72 kDa was detected in extracts from cells infected with AcNPV-Kr but not with those infected with AcNPV-Del. In addition, this band was not observed in extracts from cells infected with the wild-type AcNPV virus or uninfected cells (data not shown). Finally, the ³²P-labeled 72-kDa protein could be immunoprecipitated using antibodies against Kr (Fig. 4B). These observations show that the Kr protein produced in insect cells infected with the recombinant virus is phosphorylated. We do not know whether the protein produced in the *Drosophila* embryo is similarly modified.

Nuclear Localization and DNA Binding Activity of the Kr Protein. Analysis of nuclear and cytoplasmic extracts from cells infected with the recombinant AcNPV-Kr virus by NaDodSO₄/PAGE revealed that the Kr protein is present only in the nuclear fraction (data not shown). We found that the Kr protein is tightly bound to nuclear components, as most of the protein could not be separated from the chromatin by treatment with high salt or nonionic detergents. Similar observations were made when the human MYC (17) or

FIG. 4. Phosphorylation of Kr protein produced in AcNPV-Kr-infected insect cells. Infected cells were labeled with ³²P in phosphate-free medium, extracted with NaDodSO₄, and analyzed by electrophoresis on a 7.5% NaDodSO₄/polyacrylamide gel. (A) Extracts from cells infected with AcNPV-Kr (lane 1) or AcNPV-Del (lane 2). (B) Immunoprecipitation of ³²P-labeled extracts of cells infected with AcNPV-Del (lane 3) or AcNPV-Kr (lane 4) using affinity-purified anti-Kr antibodies. The arrowhead shows the 72-kDa band corresponding to the Kr polypeptide, while the arrow indicates the 69-kDa marker.

Drosophila fushi tarazu (our unpublished results) proteins were produced in the baculovirus system.

The nuclear localization of the Kr protein was confirmed by indirect immunofluorescent staining of insect cells infected with AcNPV-Kr. As shown in Fig. 5 A and C, *S. frugiperda* cells infected with baculoviruses contain unusually large nuclei. When cells infected with AcNPV-Del were incubated with anti-Krüppel antibodies, no binding was observed (Fig. 5B). However, intense immunofluorescent staining is observed exclusively over the nuclei of cells infected with AcNPV-Kr (Fig. 5A).

A variety of extraction conditions were investigated to establish conditions for recovering native Krüppel protein from nuclear extracts in a soluble form. For example, the nuclear fraction was extracted with 0.2–2.0 M NaCl or ammonium sulfate in the presence or absence of nonionic detergents. In addition these extraction conditions were used in combination with DNase treatment. We also varied the temperature, pH, buffer, and concentration of chelating agents or metal ions. In addition, we extracted the nuclear fraction with 8 M urea or 6 M guanidinium. Unfortunately, none of these conditions resulted in efficient recovery of Kr protein in a soluble form. However, a systematic examination of the effect of varying the salt concentration and pH in the presence of acetonitrile led to the establishment conditions for the efficient recovery of Kr protein. We found that most of the Kr protein could be recovered in a soluble form by extracting with 50% (vol/vol) acetonitrile in a 0.3 M ammonium carbonate buffer at pH 9 (Fig. 6).

To determine whether the solubilized Kr protein binds to DNA it was chromatographed on a DNA-cellulose column containing double- and single-stranded calf thymus DNA (19, 20). The acetonitrile-extracted Kr protein was desalted by passage through a small gel-filtration column and then loaded onto DNA-cellulose. The column was extensively washed, and the bound proteins were eluted in one step with 1 M NaCl. The fractions were concentrated by acetone precipitation and then analyzed by immunoblotting using the anti-Kr antibodies. As shown in Fig. 7 Kr protein was not present in the flow-through, but was present in the fraction eluted with 1 M NaCl. We conclude that the solubilized Kr protein binds to single- or double-stranded DNA.

FIG. 5. Intracellular localization of Krüppel protein expressed in infected cells by indirect immunofluorescence using anti-Kr antibodies. Phase-contrast view of cells infected with AcNPV-Del (A) or AcNPV-Kr (B). Indirect immunofluorescence of cells infected with AcNPV-Del (C) or AcNPV-Kr (D).

FIG. 6. Acetonitrile extraction of Krüppel protein from AcNPV-Kr-infected cells. AcNPV-Kr-infected cells were fractionated. The nuclear pellet was extracted and analyzed by electrophoresis on a 6–13% NaDodSO₄/polyacrylamide gradient gel. Nuclei were prepared from [³⁵S]methionine-labeled cells infected with AcNPV-Kr and extracted with 3% (wt/vol) NaDodSO₄ (lane 1) or 50% (vol/vol) acetonitrile/0.3 M ammonium carbonate, pH 9.0 (lane 2). The arrowheads to the right of lanes 1 and 2 show the bands corresponding to Kr protein, and the arrows to the left of lane 1 indicate the position of molecular size markers: 92 kDa, 69 kDa, 43 kDa, and 25 kDa. Also shown is an immunoblot of acetonitrile-extracted nuclei from cells infected with AcNPV-Kr (lane 3) or AcNPV-Del (lane 4). The arrowhead to the left of lane 3 shows the acetonitrile-extracted Kr protein detected with anti-Kr antibodies (≈72 kDa). The arrow to the right of lane 4 indicates the position of a 69-kDa marker.

DISCUSSION

We have shown that the *Drosophila* regulatory protein Krüppel can be produced in relatively large amounts in

FIG. 7. DNA-cellulose chromatography of Krüppel protein. An immunoblot of the flow-through fractions (lane 1) and the fractions eluted from a DNA-cellulose column with 1 M NaCl (lane 2), electrophoresed on a 7.5% NaDodSO₄/polyacrylamide gel and detected with anti-Kr antibodies. An aliquot of the acetonitrile-extracted nuclei that was applied to the column was run in lane 3. The arrowhead indicates the position of the Kr protein, and the arrow to the left indicates the position of a 69-kDa marker.

cultured insect cells using the baculovirus vector system. We find that the Krüppel protein produced in this system represents $\approx 1\%$ of the total proteins in infected cells. Furthermore, we have established conditions for the efficient recovery of soluble protein from nuclear extracts that involve extraction with acetonitrile in high salt. The Krüppel protein recovered in this manner is likely to be in its native form, since the acetonitrile has been used routinely in the purification of native proteins (P. Ferrara, personal communication; ref. 23).

The electrophoretic mobility of the Krüppel protein produced in infected insect cells, in *Drosophila* embryos, or by *in vitro* translation of SP6-Krüppel RNA corresponds to a molecular weight of $\approx 72,000$. In contrast the molecular weight predicted from the open reading frame of the Krüppel cDNA sequence is 50,000 (10). This apparent discrepancy may be a consequence of an unusual feature of the primary structure of the protein. Similar discrepancies have been reported for other DNA binding proteins including the simian virus 40 tumor antigen (24) and the *Drosophila* fushi tarazu gene product (unpublished results and M. Scott, personal communication). The nature of the sequences responsible for these anomalies is not understood. In the case of Krüppel, it is unlikely that the anomaly is a consequence of protein modification, since we have shown that the protein is not glycosylated and phosphorylation does not result in major changes in the mobility of the protein on NaDodSO₄/PAGE. In addition, the electrophoretic mobility of the Krüppel protein produced by *in vitro* translation is similar to that produced *in vivo*.

The significance of the phosphorylation of Krüppel observed in insect cells infected with the recombinant Kr virus is not known. We do not know whether Krüppel produced in *Drosophila* embryos is phosphorylated. However, we note that the human MYC protein, which is phosphorylated in human cells, is also phosphorylated when produced using the baculovirus system. Phosphorylation has been shown to modulate the function of DNA binding proteins. For example, phosphorylation of simian virus 40 large tumor antigen results in changes in the activity of the protein in *in vitro* DNA synthesis assays, and it alters the affinity of the protein for specific binding sites on simian virus 40 DNA (25).

Although the mechanism by which Kr protein functions is not known, the presence of a zinc-binding finger domain suggested that this mechanism may involve binding to DNA. Similar suggestions have been made for the proteins encoded by the serendipity δ and β genes (9, 10, 26). Analysis of the Kr protein produced with the baculovirus vector system is consistent with this hypothesis. First, Krüppel is found in the nuclear fraction of insect cells infected with the Kr virus. Second, indirect-immunofluorescent staining experiments using anti-Kr antibodies show that Kr protein accumulates in the nucleus of insect cells infected with the Kr baculovirus. These antibodies also stain the nuclei in a band of cells located 45–55% of the distance from the posterior pole of the embryo (unpublished results). The same region of the embryo has been shown to contain localized Krüppel mRNA by *in situ* hybridization (8). Finally, we find that the Kr protein produced in the baculovirus system binds to single- and double-stranded DNA-cellulose. The availability of large amounts of soluble Kr protein should provide the opportunity to search for DNA sequences with which Krüppel specifi-

cally interacts. Candidates for such sequences are those that flank the gap or pair-rule genes, since the expression of these genes may be regulated directly or indirectly by Krüppel (27).

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