MORPHOLOGICAL CHANGES OF THE INSECT CELLS IN THE BACULOVIRUS SYSTEM AS A FUNCTION OF *V-MYB* AND *C-MYB* INSERTS EXPRESSION AND TOPOGRAPHIC LOCALIZATION OF V-MYB AND C-MYB PROTEINS

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Summary. – Structural changes of insect cells *Spodoptera frugiperda* in the baculovirus expression system after expression of *v-myb* oncogene and *c-myb* protooncogene inserts were studied by means of electron microscopy. Expression of *v-myb* gene insert was accompanied by extensive changes in cell structure, when compared with those of the noninfected and wild-type virus-infected cells. Enormous increase in nuclear content was apparent within 48 hrs after infection, along with changes in nucleolus appearance. Large ring-shaped nucleoli, compact nucleoli and nucleoli with nucleolonemas were detected together with dense nucleolus of normal appearance and small nucleolar structures localized in the nuclear periphery. The cytoplasm practically disappeared 72 hrs after infection. Morphological changes of insect cells expressing the c-myb gene were significantly less distinct, but more frequent unraveling of nucleoli was observed. Both v-Myb and c-Myb proteins were localized in the nucleus of infected cells as was revealed by fluorescence microscopy and electron microscopy. c-Myb marker decorated distinctly the ring-shaped area of nucleolus with some less intensive labelling of the inner part of nucleolus and proximal area on nuclear membrane. v-Myb protein revealed predominantly more compact and homogeneous distribution inside the nucleolus but a small proportion of it was also detected outside the nucleolus in the nuclear compartment. The data obtained on insect cells suggest that Myb proteins may participate also in the processes in which the nucleolus plays a role.

Key words: insect cells; morphology; v-Myb protein; c-Myb protein; topographic localization

Introduction

AMV is an acutely oncogenic virus causing myeloblastic leukemia in chickens (Moscovici, 1975). It contains the *v-myb* oncogene, which is responsible for the transformation of myelomonocytic haematopoietic cells *in vitro* and represents a truncated and point-mutated version of the cellular proto-oncogene *c-myb* (Lipsick and Baluda, 1986; Shen-Ong, 1990). The protein product of this proto-oncogene, $p75^{c\text{-}myb}$, is involved in the regulation of differentiation and proliferation of haematopoietic cells (Gewirtz and Callabretti, 1988). The protein product of the *v-myb* oncogene, $p48^{v\text{-}myb}$, possesses the characteristics of transcription factors. Both c-Myb and v-Myb products are nuclear transactivating proteins containing three (c-Myb) and two (v-Myb) repeats of 52 amino acids, highly conserved, which are responsible for their DNA-binding activity (Klempnauer and Sippel, 1987; Biedenkapp *et al.*, 1988). They contain also several domains responsible for the regulation of their transactivating function (Klempnauer *et al.*, 1989; Garrido *et al.*, 1992). However, only few targets of v-Myb and c-Myb regulation in vertebrate cells are known (Ness *et al.*, 1989; Nicolaides *et al.*, 1991; Cogswell *et al.*, 1993). The exact mechanism of action of the *v-myb* oncogene in malig-

Abbreviations: AMV = avian myeloblastosis virus; BSA = bovine serum albumin; FITC = fluorescein isothiocyanate; PBS = phosphate buffered saline; TRITC = tetramethylrhodamine isothiocyanate

nant transformation and of *c-myb* proto-oncogene in normal cell development still remains to be elucidated.

One of the approaches to full understanding of the function of *v-myb* oncogene and *c-myb* proto-oncogene is a precise determination of subcellular distribution of their products and study of interactions of these products with cellular structures and their components. Previous studies have shown that a nuclear fraction isolated from AMV-transformed myeloblasts contains the $p48^{v\text{-}myb}$ protein (Boyle *et al.*, 1984) which is attached to the nuclear matrix-lamina complex (Boyle *et al.*, 1985). However, in AMV-transformed myeloblasts a fraction of this protein was detected also in the perinuclear region of the cytoplasm and if the myeloblasts were induced to differentiate to macrophages, most of v-Myb was detected in the cytoplasm (Klempnauer *et al.*, 1984).

Recombinant baculovirus expression system containing *v-myb* oncogene and *c-myb* proto-oncogene inserts was constructed at this institute (Vranovský *et al.*, 1992). Obtained protein products were electrophoretically identical to those of the authentic c-Myb and v-Myb proteins from chicken cells.

Our preliminary studies have shown some changes in appearance and morphology of insect cells as a consequence of the expression of *v-myb* and *c-myb* genes (Korb *et al.*, 1994). The aim of this work was to analyze in more detail the effect of *c-myb* proto-oncogene and *v-myb* oncogene expression on the structural changes of insect cells and to study the topographic localization of their products in the insect cells by means of both fluorescence microscopy and immunoelectron microscopy.

Materials and Methods

Viruses and cells. Recombinant baculoviruses NPV *v-myb* and *c-myb* carrying *v-myb* and *c-myb* genes, respectively, were obtained as described elsewhere (Vranovský *et al.*, 1992). Experiments were performed with Sf9 (*Spodoptera frugiperda*) cells infected with these viruses. Non-infected cells and those infected with wild-type virus were used as a control.

Antibodies. Anti-v-Myb antibody (rabbit, polyclonal) recognizing v-Myb and c-Myb protein products and antibody IL19 (rabbit, polyclonal) recognizing 19 amino acids of v-Myb and c-Myb protein products were obtained from Dr. M. Dvořák of this institute. Anti-fibrillarin antibody (S4 human autoantibody) recognizing fibrillar structures of the nucleolus and nucleus (Ochs *et al.*, 1985) and anti-DNA antibody (mouse, monoclonal IgG) recognizing DNA structures were a gift from Dr. I. Raška (Institute of Experimental Medicine AS CR, Prague).

Immunofluorescence. Cells grown on poly-L-lysine-treated (Roth, 1986) glass coverslips were fixed with methanol-acetone. Fixed cells were then incubated with primary antibody (rabbit anti-v-Myb antibody and S4 human anti-fibrillarin autoantibody or mouse anti-DNA monoclonal IgG), washed thoroughly in PBS pH 7.2 and stained with secondary antibody (FITC-conjugated goat anti-rabbit IgG (Sigma) and TRITC-conjugated anti-human polyvalent IgG (Sigma), or TRITC-conjugated goat anti-mouse IgG (Sigma)).

Electron microscopy. Ultrastructural analysis was performed on ultrathin sections of material fixed in 3% glutaraldehyde in 0.1 mol/l cacodylate buffer pH 7.5, postfixed in 1% osmium tetroxide in veronal buffer and embedded in Epon 812, or fixed in 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/l cacodylate buffer pH 7.5, and embedded in Lowicryl K4M resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate.

Immunoelectron microscopy. Fixed cells (3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/l cacodylate buffer pH 7.5) were dehydrated at low temperature in a graded series of ethanols and embedded in Lowicryl K4M resin. Ultrathin sections mounted on nickel grids were pretreated on drops of blocking solution containing 1% BSA and 5% normal goat serum in PBS prior to immunostaining with primary antibody (anti-v-Myb). After washing in PBS containing 1% BSA, the grids were stained with secondary antibody (GAR G5) (Agar Scientific) in PBS containing 1% BSA. The grids were rinsed with PBS, followed by distilled water, and stained with uranyl acetate and lead citrate. The samples were observed with a JEM 1200 EX electron microscope.

Results and Discussion

Effect of v-myb and c-myb gene expression on the cell structure

Expression of both *v-myb* oncogene and *c-myb* proto-oncogene in the baculovirus expression system was accompanied by extensive changes in the cell structure of insect cells. Although infection of the cells with wild-type baculovirus (without *v-myb* or *c-myb* gene inserts) reflects some structural changes, the effect of *v-myb* oncogene and *c-myb* proto-oncogene expression on the cellular structure was quite distinct.

The morphology of non-infected insect cells *Spodoptera frugiperda* is shown in Fig. 1. Structural changes observed after infection of cells with wild-type virus resemble the effect of some pathogens and are apparent namely on the chromatin and nucleolus appearance (Fig. 2). Infected cells do not show any regression changes. It is well known that nucleoli of plant and invertebrate cells differ in their morphology from that of the mammalian cells. The large nucleus frequently reveals the presence of typical fibrillar structures (Fig. 2, arrows) which are accumulated also in the cytoplasm of Sf9 cells. Such large fibrillar masses have been described to be associated with *Autographa californica* nuclear polyhedrosis virus p10 protein (van der Wilk *et al.*, 1987; Williams *et al.*, 1989).

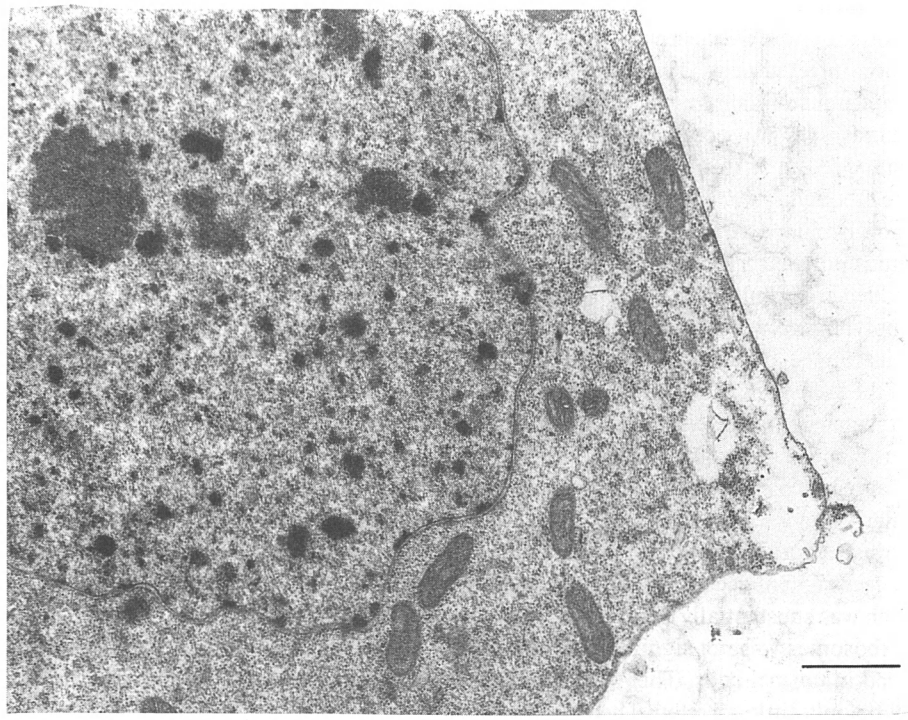

Fig. 1
Morphology of intact non-infected insect cells *Spodoptera frugiperda*
Bar = 1 μm.

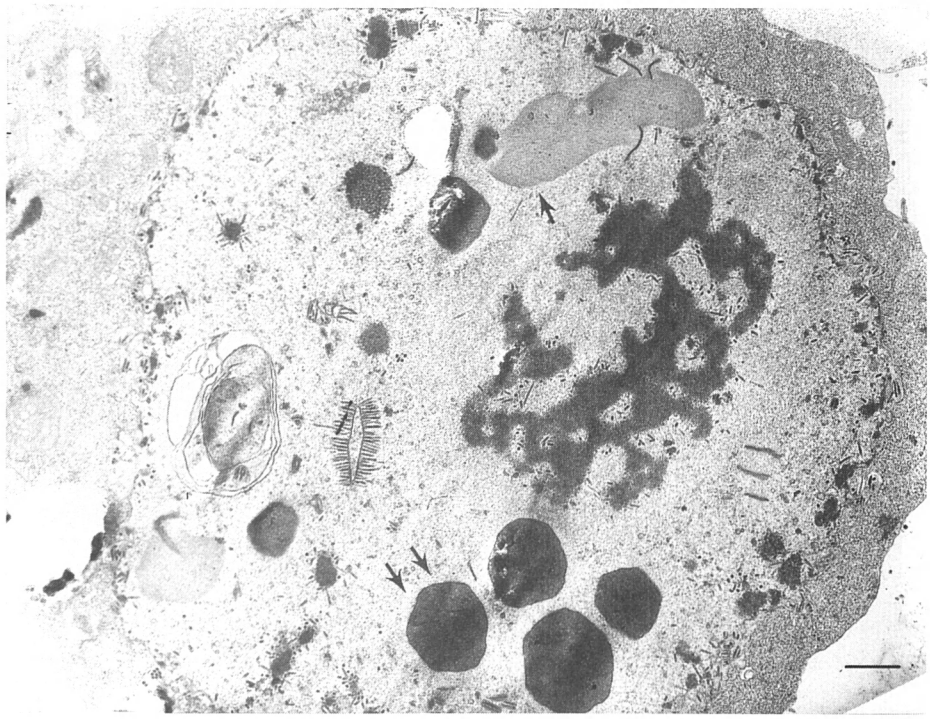

Fig. 2
Morphology of insect cell *Spodoptera frugiperda* in the baculovirus expression system infected with wild-type virus
Arrow indicates typical fibrillar structures, double arrow indicates polyhedra (bar = 1 μm).

Chromatin was in some cases higly condensed and its perinuclear localization was more frequently observed, when compared with the non-infected cells. The nucleolus usually has a compact appearance containing numerous granular components, but unravelling was occassionally observed, a property typical of the effect of endotoxins. Nucleolonemas were frequently very distinct. Many viral nucleocapsids and non-occluded virus were present in the nuclear area. Numerous polyhedra (occlusion bodies) containing virions embedded in crystalline protein matrix, composed primarily of polyhedrin, were the typical structures present in cell nucleus (Fig. 2, double arrow).

The expression of the *v-myb* oncogene was accompanied by extensive structural changes in the cells when compared with those of the non-infected and wild-type infected cells. These changes were apparent as soon as 48 hrs after infection (Fig. 3), particularly a cytotoxic effect on the cells was usually observed. Ultrastructural analysis revealed an enormous increase in nuclear content accompanied by changes in the cytoplasm which was substantially diminished. However, the amount of ribosomes was not significantly different from that observed in normal cells. This suggests that proteosynthesis may be only little affected. The condensation of chromatin as a marker of functional inactivation was seldom observed. A typical feature was the accumulation of interchromatin granules.

Particularly extensive changes in the structure of nucleoli were found by both electron and light microscopy (using specific staining with toluidine blue in McIlvain buffer pH 5.2) (Fig. 4). In most of the cells a large ring-shaped nucleolus was seen together with a dense nucleolus of normal appearance. Besides, number of small nucleolar structures were localized in the periphery of nuclear membrane. Unravelling of nucleoli was seldom observed. This suggests that the transport pathway from nucleoli to the cytoplasm was significantly reduced. Only a small proportion of unravelled nucleoli was found in which also the presence of microfibrillar structures was apparent. Usually the interstitial area was dilated and fibrillar centers were found with many viral particles accumulated in nucleolar interstices. In contrast to cells infected with wild-type baculovirus, where the fibrillar structures were localized in both the nucleus and cytoplasm, localization of these structures after *v-myb* gene expression was quite different. In this case the fibrillar bodies were markedly enlarged and exclusively localized in the nuclear area.

Our observation of nucleolar appearance is in accord with previous findings that morphology of nucleoli is often aberrant in cancer cells. Nucleolar size has been found to be greater in progressing malignant tumor cells of mammals (Wachtler and Stahl, 1993). Also treatment of experimental animals with azo-dyes, acetylaminofluorene or ethionine

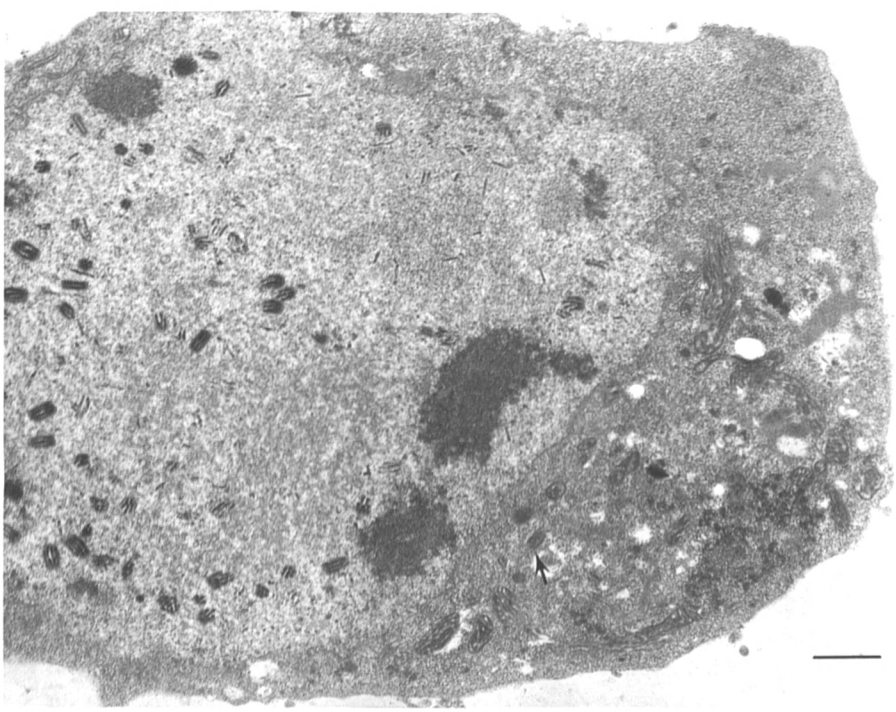

Fig. 3
Morphology of insect cell of baculovirus expression system containing *v-myb* gene insert 48 hrs after infection
Arrow indicates enveloped nucleocapsid in cytoplasm (bar = 1 μm).

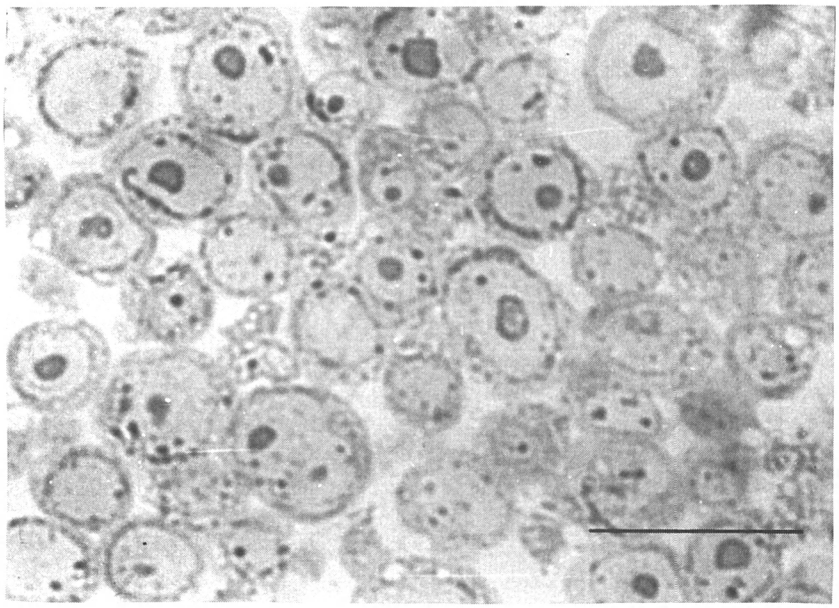

Fig. 4
Insect cells after *v-myb* gene expression selectively stained for RNA-containing structures
Bar = 10 μm.

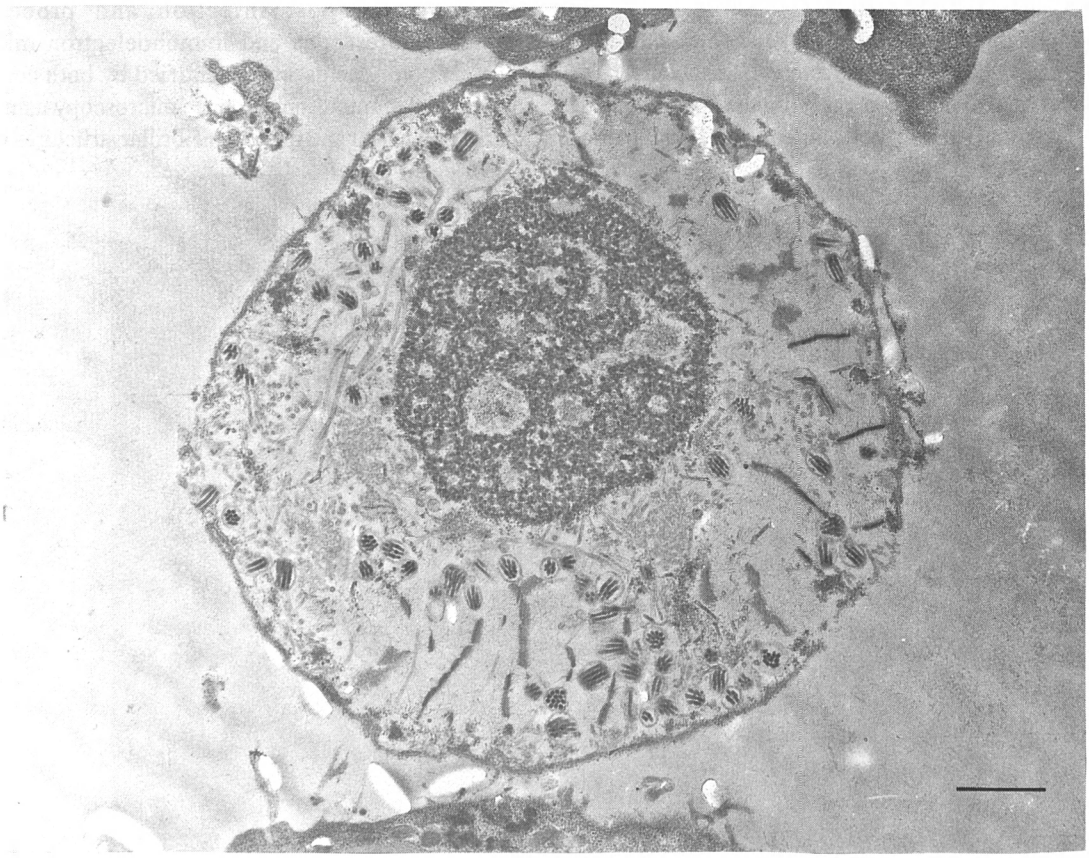

Fig. 5
Morphology of insect cell of baculovirus expression system containing *v-myb* gene insert 72 hrs after infection
Bar = 1 μm.

resulted in enlargement of the nucleoli in liver cells and induced hepatomas (Karasaki, 1969). The presence of small nucleolar bodies in the nuclear periphery is not clear, but it may be related to changes in RNA synthesis.

In later stages of infection (72 hrs), the cytoplasm practically disappeared and numerous enveloped viral particles were present, prevalently in the nucleus in both the nucleoplasm and nucleolus (Fig. 5, arrows). In contrast to cells infected with wild-type virus, a cytotoxic effect and heavy destructive and regression changes were seen on the cells in the later stages of infection. On the other hand, the nucleolus usually revealed a compact structure and unravelling was seldom observed even in later stages of infection. In most cases, the nuclear membrane was well preserved. It has already been described that compact nucleoli are found very rarely in normal cells, but they are quite frequently found in malignant cells (Wachtler and Stahl, 1993).

On the other hand, the effect of *c-myb* proto-oncogene expression was different. Morphological changes of the insect cells expressing the *c-myb* gene were significantly less distinct in both the nucleus and cytoplasm (Fig. 6). In this case, in contrast to the *v-myb* gene expression, unravelling of the nucleolus was more frequently observed with many viral inclusions in these areas (Fig. 7) and so was the presence of extremely large fibrillar structures in the nuclei (arrows). However, also in cells expressing the *c-myb* gene the presence of unusual ring-shaped nucleoli was found in most cases. Also in later stages of infection (72 hrs) the structural changes of the cells were not more distinct when compared with those after 48 hrs in contrast to the effect of *v-myb* gene insert.

Expression of the *v-myb* oncogene in the baculovirus expression system was about three times higher than that of the *c-myb* proto-oncogene, as was revealed by immunoblot analysis (data not shown). Also c-Myb protein was distinctly less stabile in insect cells and was faster degraded than v-Myb protein. However, the observed differences in cell structure of the cells expressing the *v-myb* and *c-myb* gene, respectively, are apparently due to different interactions of the gene products with cell structures instead of their different amounts. The *v-myb* gene product apparently interacts with cell components and affects the cell growth to an extent that the morphology of the cells is basically changed.

Localization of v-myb oncogene and c-myb proto-oncogene products

To study the interaction of *v-myb* and *c-myb* gene products with insect cell structures, the cells infected with baculovirus carrying *v-myb* or *c-myb* gene inserts were collected 48 hrs post infection and processed for immunofluorescence and immunoelectron microscopy. Nuclear components were identified by both conventional optical and immunofluorescence microscopy using specific markers. Nuclear and nucleolar fibrillar structures were iden-

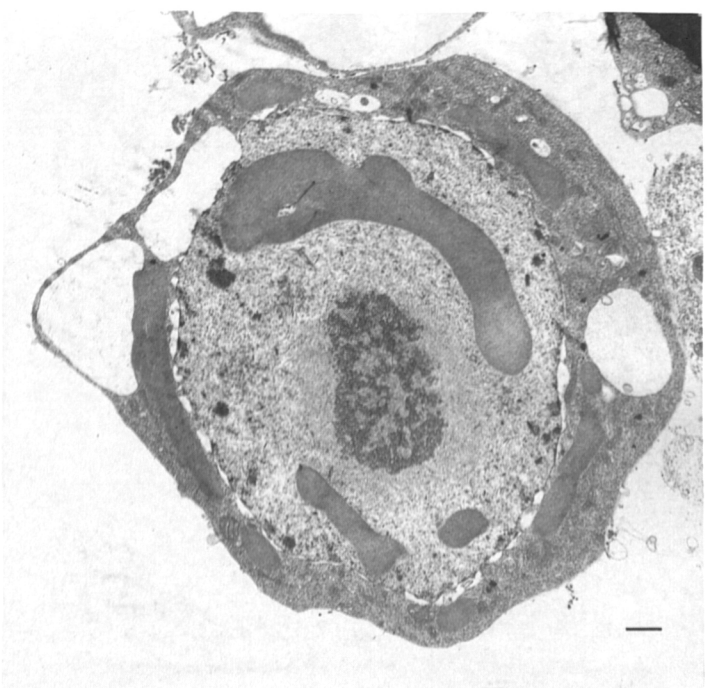

Fig. 6
Morphology of insect cell of baculovirus expression system containing *c-myb* proto-oncogene insert 48 hrs after infection
Bar = 1 μm.

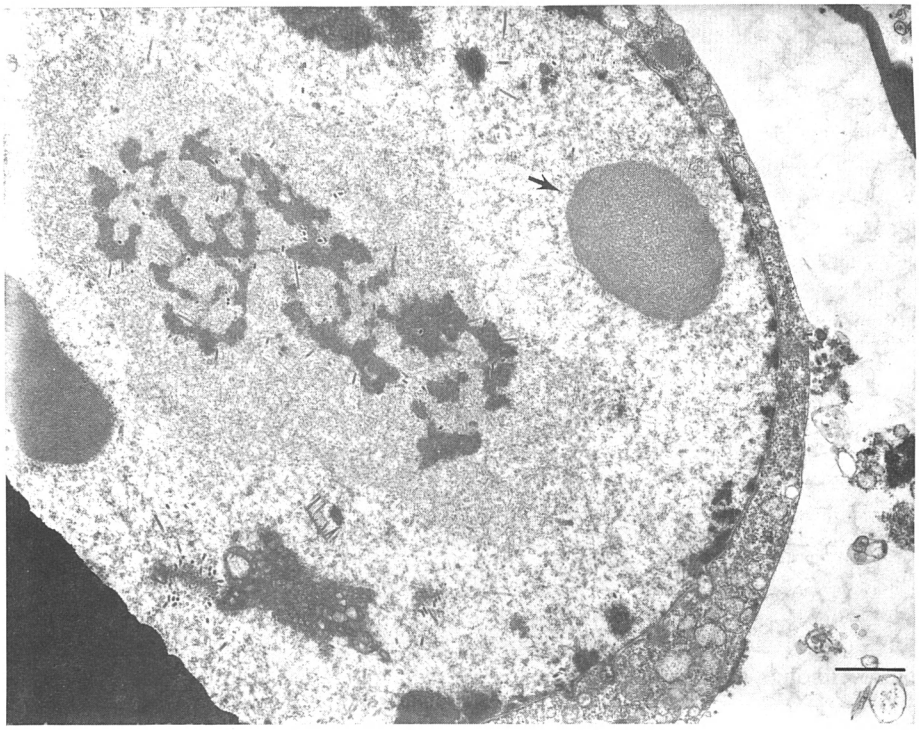

Fig. 7
The same as in Fig. 6 in later stages after infection
Large fibrillar structure is indicated by arrow (bar = 1 μm).

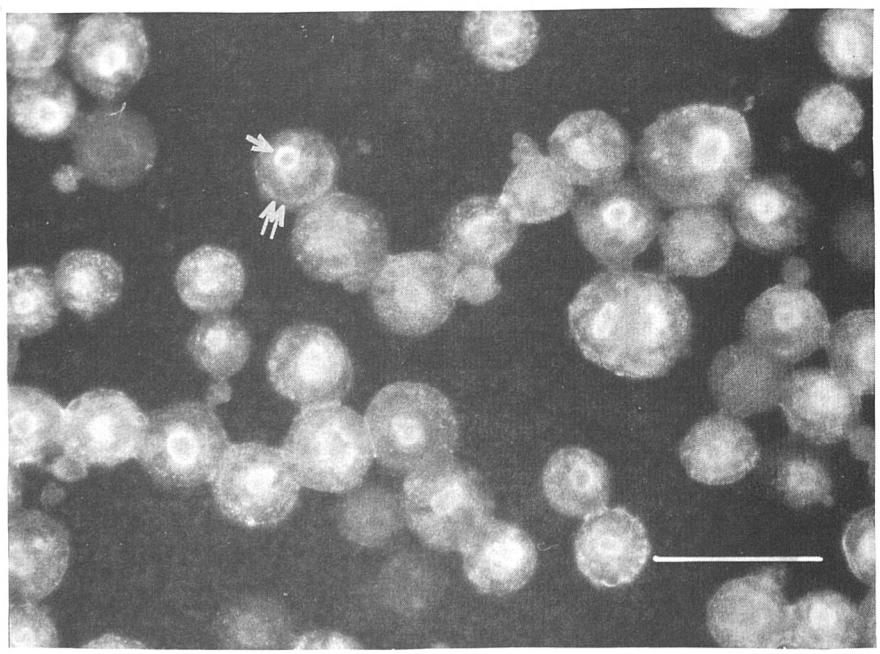

Fig. 8
Immunofluorescence localization of *c-myb* gene product in insect cells
Arrow indicates the marker decorating ring shaped area, double arrow the marker in proximity of nuclear membrane (bar = 10 μm).

tified using anti-fibrillarin antibody (Ochs *et al.*, 1985) and the organization of chromatin was studied using anti-DNA antibody.

In insect cells, which had been infected with baculovirus containing *c-myb* proto-oncogene insert, c-Myb protein was found to be localized predominantly in the nucleus, similarly as was described before in other cell types (Klempnauer *et al.*, 1984; Boyle *et al.*, 1985). The fluorescence marker decorated distinctly the ring-shaped area of the enlarged nucleolus in most of the cells (Fig. 8). Less intensive fluorescence was seen inside the nucleolus. Poor fluorescence was also observed in the proximity of nuclear membrane, suggesting probably some weak interaction of c-Myb protein with lamina.

The localization of v-Myb protein product after *v-myb* gene expression was different in some aspects when compared with c-Myb protein distribution. v-Myb protein was found to be also predominantly localized in the nuclear area. Most of fluorescence was observed in the nucleolus, but in contrast to c-Myb protein a fluorescence marker revealed a significantly more compact and homogeneous distribution in the nucleolus. Although most of fluorescence was detected in the nucleolar area, small but numerous distinct domains were observed also outside the nucleolus in the nuclear compartment (Fig. 9) usually localized in the perinuclear membrane area. These domains corresponded to the nucleolar structures found in the perinuclear area when toluidine blue staining in McIlvain buffer pH 5.2 was used (not shown). This staining revealed markedly enlarged nuclear content which occupied most of the cell area. Results of immunoelectron microscopy confirmed the fluorescence data. The gold marker decorated usually the nucleolar structures (Fig. 10), where dense fibrillar components were predominantly labelled (Fig. 10, insert). No marker in the interstices was observed.

Our results on nuclear localization of *myb* gene products in insect cells are in good agreement with published data for other cell types. Both *v-myb* and *c-myb* gene products have been described to be associated with cell nuclei (Boyle *et al.*, 1984; Klempnauer *et al.*, 1984). However, the data of their interactions with chromatin and nuclear matrix differ substantially (Shen-Ong, 1990). It is expected that a large fraction of the Myb protein is probably associated with the nuclear matrix under physiological conditions (Klempnauer *et al.*, 1984), indicating that it may be involved in DNA replication and/or transcription.

Surprisingly, the obtained data indicate also preferential interaction of Myb proteins with nucleolar structures of insect cells. The fluorescence marker decorated especially large ring-shaped nucleolus which is typical of cancer cells (Busch and Smetana, 1970), whereas its intensity was smaller in the dense nucleolus of normal appearance as well as in small nucleolar structures in the nuclear periphery. Based on electron microscopic data these products seem to

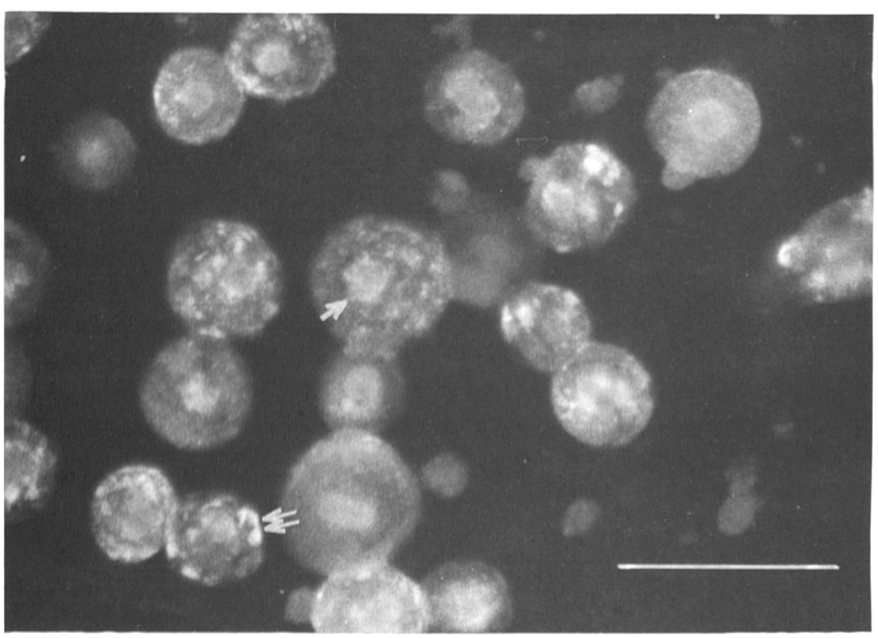

Fig. 9
Immunofluorescence localization of *v-myb* gene product in insect cells
Arrow indicates homogeneous distribution of marker, double arrow the marker outside the nucleolus (bar = 10 μm).

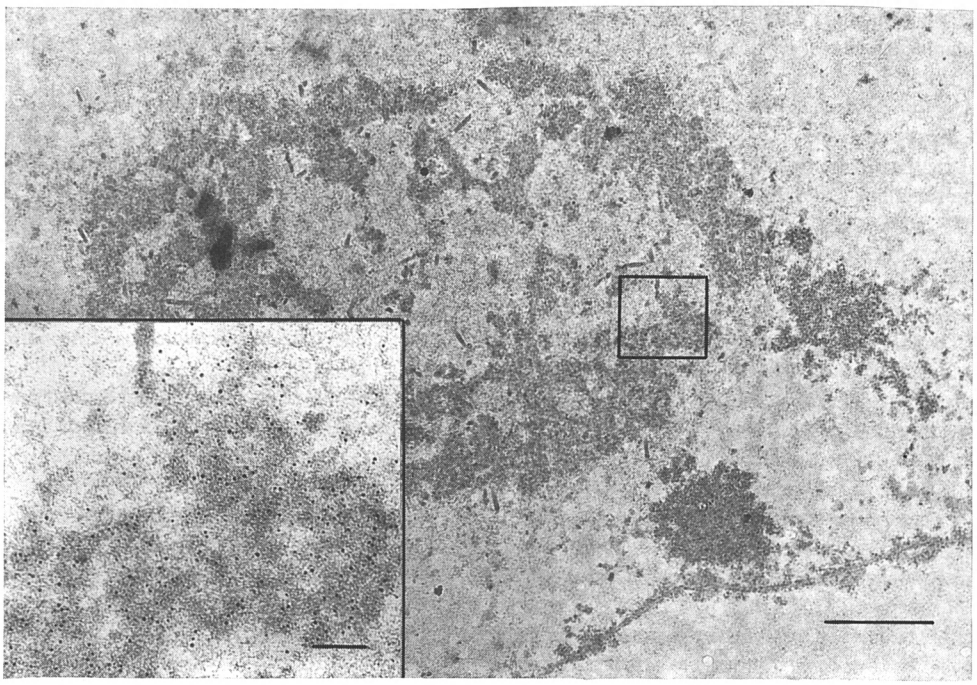

Fig. 10
Immunoelectron microscopic localization of v-Myb protein in insect cells
Bar = 1 μm. Insert: selected area visualized at high magnification (bar = 100 nm).

have affinity for the dense fibrillar components of the nucleolus. This was also in agreement with the results of fluorescence microscopy, where most of the c-Myb and v-Myb protein label revealed the same localization as fibrillarin marker. This 34 K protein is known to be a typical protein of fibrillar centers and dense fibrillar components (Raška *et al.*, 1989). However, some fluorescence of Myb proteins also did not correspond to fibrillarin marker, suggesting their possible additional localization site in the nucleolus.

Dense fibrillar components contain huge amounts of proteins, some of which are involved also in transcription of ribosomal genes (Raška *et al.*, 1989; Raška and Dundr, 1992). The localization of Myb proteins in the nucleolus and small nucleolar structures in the nuclear periphery suggest that they might participate in the processes in which the nucleolus plays an important role. Detailed studies of the mutual interactions of Myb proteins with nuclear matrix and nucleolar proteins are in progress.

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