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Transcriptional Regulation of the BOMBYX MORI A3 Cytoplasmic Actin Promoter in Insect and Mammalian Cells

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TRANSCRIPTIONAL REGULATION OF THE *BOMBYX MORI* A3 CYTOPLASMIC ACTIN PROMOTER IN INSECT AND MAMMALIAN CELLS

by

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Each person whose signature appears below certifies that this dissertation in their opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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LIST OF ABBREVIATIONS

ABP: Actin binding protein
ADP: Adenosine 5'-diphosphate
ATP: Adenosine 5'-triphosphate
ATPase: Adenosine 5'-triphosphate phosphatase
bp: basepair
cDNA: Complementary deoxyribonucleic acid
CHO: Chinese hamster ovary
Ci: Curie
dCTP: Deoxy cytidine 5'-triphosphate
DAPI: 4'6-diamidino-2-phenylindol
DEPC: Diethylpyrocarbonate
DMEM: Dulbecco's minimal essential medum
DNA: deoxyribonucleic acid
DNase: Deoxyribonuclease
EDTA: Ethylenediaminetetraacetic acid
EGTA: Ethylene glycol-*bis*(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
EMEM: Eagle's minimal essential medium
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
HSV-TK: Herpes simplex virus thymidine kinase
hyg: hygromycin B phosphotransferase
kb: kilobase
kDa: kilodalton
LMP: Low melting point
Mb: Megabase
mRNA: messenger ribonucleic acid
MOPS: 3-(*N*-morpholino) propane sulfonic acid
OD: Optical density
ONPG: O-nitrophenyl-β-D-galactopyranoside
pac: Puromycin *N*-acetyl transferase
PBS: Phophate buffered saline
PCR: Polymerase chain reaction
PMSF: Phenylmethylsulfonyl fluoride
RACE: Rapid amplification of cDNA ends
RNase: Ribonuclease
rpm: revolution per minute
RT-PCR: Reverse transcription polymerase chain reaction
SDS: Sodium dodecyl sulfate
SRE: Serum response element
SRF: Serum response factor

SV: Simian virus
TCF: Ternary complex factor
TdT: Terminal transferase
TEMED: *N,N,N′,N′*-tetramethylethylenediamine
Tris: Tris(hydroxymethyl)amino-methane
UTR: Untranslated region
X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside

ABSTRACT

TRANSCRIPTIONAL REGULATION OF THE *BOMBYX MORI* A3 CYTOPLASMIC ACTIN PROMOTER IN INSECT AND MAMMALIAN CELLS

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In this study we have analyzed the activity of the silkworm A3 cytoplasmic actin promoter in homologous insect and heterologous mammalian expression systems. To study the expression of the A3 actin promoter in insect cells we have isolated and characterized a stably transformed *Bombyx mori* cell line containing a novel selectable marker gene, puromycin *N*-acetyl transferase under control of transcriptional regulatory signals from the A3 cytoplasmic actin gene. Integration of the transfected plasmids into the host genome was demonstrated by Southern and *in situ* hybridizations. By using this cell line we have identified alternative transcriptional initiation sites for the A3 cytoplasmic actin gene. One of these start sites is located ~35 bp upstream from the earlier determined transcription initiation site. The two mRNA start sites are utilized with a similar efficiency in the BmN cell line. In addition we detected transcripts that initiated within the first intron of the A3 actin gene. These transcripts may be synthesized under control of an alternative promoter. The mRNA isoforms generated by the use of the

alternative start sites may exhibit different characteristics (stability etc.), therefore the alternative promoter and initiation site usage may be an important factor in regulating the cyclic changes of the A3 actin mRNA level in the silk gland during larval morphogenesis.

Here we also report that A3 cytoplasmic actin based vectors can express foreign genes in various vertebrate cells. Using an A3 actin based expression cassette we have isolated stably transformed mammalian cell lines. Isolation of transformed cells was based on expression of the selected marker gene. Stable integration of A3 actin plasmid construct into the host genome was demonstrated. The integrated plasmid molecules frequently exhibited amplification which may indicate the presence of amplification promoting elements in the 5' or 3' UTR of the A3 actin gene. This is the first reported case for the use of an insect promoter driven selection cassette to establish transformed mammalian cell lines.

The regulatory elements of the A3 actin 5' UTR that are necessary for the expression in mammalian cells were studied by transient transfection assay. We demonstrated that the earlier identified promoter elements of the A3 actin gene are not required for transcriptional activity in mammalian cells. On the contrary, the A3 actin first intron contains a strong promoter that exhibits a transcriptional activity in mouse cells that is comparable to the activity of the strong SV40 early promoter. We suggest that the identified intronic promoter is the same promoter region that regulates the synthesis of the rare A3 actin mRNA isoform observed in *Bombyx mori* cells.

I. INTRODUCTION

Actin is one of the most abundant proteins in eukaryotic cells. In 1942, Straub isolated it as a water soluble component of muscle acetone powder (Straub 1942). Actin can exist either as a monomeric molecule, G-actin, or as a filamentous polymer, F-actin. At increased ionic strength, G-actin molecules can polymerize into F-actin (Szent-Gyorgyi 1951). F-actin is the main component of the thin filaments in sarcomeres of muscle cells. Muscle sarcomeres use ATP hydrolysis to produce mechanical force by sliding the thin actin filament past thick myosin filaments (Huxley and Niedergerke 1954, Huxley and Hanson 1954). In mammals, muscle actin has four isoforms, two in striated muscle (skeletal and cardiac actins) and two in smooth muscle (enteric and vascular actins).

Cytoplasmic actins, which can be found in all cell types are the major components of the cytoskeleton. In vertebrates there are two closely related cytoplasmic actin isoforms, β– and γ–actins. β–actin is one of the most highly expressed proteins in all mammalian non-muscle cells and, together with the γ–actin, is the principal component of microfilaments (Lazarides and Revel 1979). Cytoplasmic actins play an essential role in many cellular processes such as phagocytosis, endo- and exocytosis, intracellular transport processes, cell division and cell motility. They are also implicated in processes which affect the cytoskeletal morphology (Kabsch and Vandekerckhove 1992; Alberts et al. 1994).

Comparative sequence analysis revealed that actin is a highly conserved protein. It is encoded by a multigene family in animals, protozoa, and plants examined so far. The exceptions are some unicellular and filamentous fungi and the ciliate *Tetrahymena pyriformis,* which have a single actin gene (Mounier et al. 1992). The amino acid sequence deduced from cloned genes from a wide range of organisms shows that the protein is extremely conserved. Nucleotide substitutions that result in an amino acid replacement in actins accumulate at a very low rate in comparison to other multigene families such as globins (Hightower and Meagher 1986). Such a high conservation suggests that functional constraints on actin have been extremely severe during evolution. Interestingly, it was found that actin isoforms were more closely related within the tissue in which they expressed than between species (Vandekerckhove and Weber 1978).

A. Biochemical properties of actin

Actin consists of a single polypeptide chain of about 375 residues whose amino acid sequence and biochemical properties are highly conserved throughout evolution. Mammalian actins fall into three classes, depending on their isoelectric point. Alpha actins are found in various types of muscle, whereas β and γ actins are the principal constituents of non-muscle cells (Garrels and Gibson 1976).

Actin binds one molecule of ATP or ADP and has a single high affinity and several low affinity binding sites for divalent cations, believed to be Mg^{2+} in the cell. The

Mg^{2+} can be replaced by Ca^{2+}, which somewhat affects the kinetic properties of actin. The tightly bound cation is directly associated with the phosphates of the nucleotide (Valentin-Ranc and Carlier 1989). In the presence of Ca^{2+}, nucleotide exchange is relatively slow, and monomeric actin is a slow ATPase. Replacement of Ca^{2+} by Mg^{2+} and EGTA leads to a much faster dissociation of the bound nucleotide (Valentin-Ranc and Carlier 1991, Carlier et al. 1994).

Judging from the assembly properties of isolated actin, most of the monomeric actin in cells must exist as a complex with associated proteins that reversibly inhibit its polymerization. This role was attributed to profilin, one of the first actin-binding proteins to be discovered (Carlsson et al. 1977). More recently, thymosin β_4, a 43 amino acid-long polypeptide present in several tissues at high concentrations was recognized as an important actin regulatory polypeptide (Safer et al. 1991).

The assembly of ATP-actin into filaments is initiated by an unfavorable nucleation step and propagated by the addition of subunits to the two ends of the growing filament at different rates. The ends of the filament are referred as "barbed" and "pointed" according to the polarity of the arrowhead-like structure generated on binding myosin subfragment 1(S1) or heavy meromyosin. ATP-actin binds faster to the barbed (plus) end than to the pointed (minus) end. After incorporation of an actin molecule into a filament, bound ATP is hydrolyzed to ADP. This behavior leads to the phenomenon known as treadmilling (Selve and Wegner 1986). Formation of F-actin stimulates the actin ATPase. However, the release of phosphate proceeds more slowly than the formation of the

filament so that the growing filament has a cap of ATP-actin at its barbed end, while monomers containing ADP and phosphate transiently accumulate in the rest of the filament. The mature filament, however, contains only ADP-actin. ADP actin may also be induced to form polymers *in vitro* by raising the salt concentration (Carlier 1991, Carlier and Pantaloni 1997).

Actin interacts with a variety of proteins both *in vitro* and *in vivo* (actin binding proteins: ABPs). These proteins are generally classified according to their effect on actin organization: ABPs that bind monomeric actin and hinder polymerization (e.g. profilins); ABPs able to cap one of the ends of the filament (e.g. cap Z36); ABPs that sever filaments and bind to the barbed ends (e.g. gelsolin); ABPs that bind laterally along the filaments (e.g. tropomyosin); ABPs that move along the filaments (e.g. myosins); and ABPs that connect the filaments with each other and form a network or bundles (e.g. α-actinin, synapsin, villin) (Hartwig and Kwiatkowski 1991, Pollard and Cooper 1986, Stossel et al. 1985, Vanderkerckhove 1990).

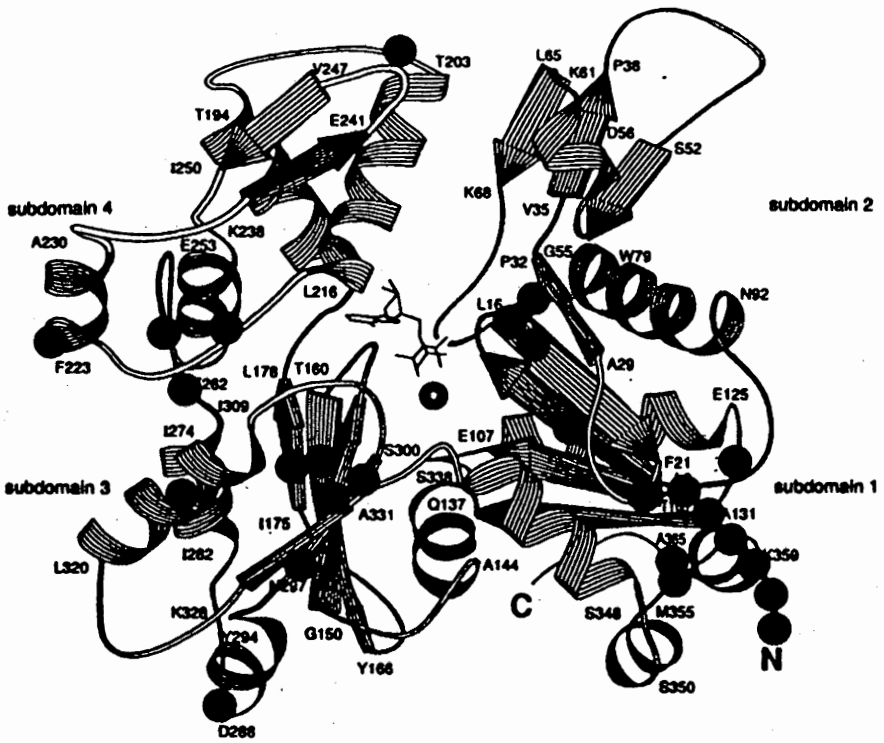
Drugs that stabilize or destabilize actin filaments provide important tools to study their dynamic behavior in cells. The cytochalasins are fungal products that prevent actin from polymerizing by binding to the barbed end of actin filaments. The phalloidins are toxins that bind tightly along the side of actin filaments and stabilize them against depolymerization. Both of these drugs cause dramatic changes in the actin cytoskeleton.

B. Structure of actin

The strong tendency of G-actin to polymerize interferes with the formation of crystals. So far, crystals suitable for X-ray diffraction studies have been obtained as complexes with actin binding proteins that keep actin in its monomeric form. At present, the only structure known at atomic detail has been obtained from the analysis of crystals of rabbit skeletal muscle actin in complex with bovine pancreatic DNase (Mannherz et al. 1977) - in both the ATP and ADP forms - at resolutions of 2.8 Å and 3 Å, respectively (Kabsch et al. 1990).

In agreement with earlier results at low resolution (Kabsch et al. 1985, Suck 1981), actin consist of a small and a large domain with one molecule of ATP or ADP residing in the cleft between the two domains (Figure 1). The overall dimensions of the molecule are approximately 55×55×35 Å. It is now known that the two domains are not very different in size. The small domain contains both the amino- and carboxy-terminus of actin and is built from residues 1-144 and 338-375. The large domain is made up of residues 145-337. The atomic structure revealed that each domain can be further subdivided into two subdomains. The small domain consists of subdomain 1 and subdomain 2. The large domain comprises subdomain 3 and subdomain 4. The course of the polypeptide chain is very similar in subdomains 1 and 3. It is suggested that subdomains 1 and 3 may have evolved by gene duplication, and subdomains 2 and 4 may have been subsequently inserted into subdomains 1 and 3, respectively.

Figure 1. Structure of actin. Solid circles indicate the position of amino acid differences between mammalian cytoplasmic actins and rabbit skeletal muscle actin. The first residue is absent in the cytoplasmic actins (Kabsch and Vandekerckhove 1992, Vandekerckhove and Weber 1978).



	1	2	3	4	5	6	10	16	17	76	103	129	153	162	176	201	225	260	267	272	279	287	297	299	358	365
α	D	E	D	E	T	T	C	L	V	I	T	V	L	N	M	V	N	T	I	A	Y	I	N	M	T	A
β	-	D	D	D	I	A	V	M	C	V	V	T	M	T	L	T	Q	A	L	C	F	V	T	L	S	S
γ	-	E	E	E			I																			

F-actin is a long, flexible filament whose diameter varies between 70 Å and 95 Å. At higher magnifications, the globular subunits of the filament can be recognized, so that the filaments appear as twisted strings of beads. The actin filament can be described as a left handed helix, with a rise per monomer of 27.5 Å and a rotation angle of -166.2° around the filament axis. Each subunit is surrounded by four other subunits, one above and one below, and two to one side (Holmes 1990).

C. Functions of actin

Actin has no important enzymatic activity, although it is a slow ATPase. However, it does inhibit DNase I (Lazarides and Lindberg 1974, Lacks 1981) and activate the myosin ATPase (Lymn and Taylor 1971). Various other enzymes interact with actin (Stossel et al 1985).

Actin has an important structural and dynamic role as a major component of the cytoskeleton in the cell. Both properties depend on its capacity to assemble and disassemble a variety of filamentous structures. Together with the other components of the cytoskeleton, it may help to organize cellular processes in space as well as in time. The organization of the actin filaments and all motility processes involving actin are controlled by the interaction in the G- or the F-form with various actin-binding proteins (ABPs) (Hartwig and Kwiatkowski 1991, Vandekerckhove 1990).

Actin is present in cells in a variety of supramolecular structures. In striated muscle, it forms the stably organized thin filaments of the sarcomere. In non-muscle cells however, organization of actin can be transiently modified in response to extra- or intracellular stimuli. These structures are variations on the theme of filament organization: networks of individual filaments in the cortical cytoplasm; bundled filaments, called stress fibers in interphase fibroblasts; short actin bundles in microvilli and stereocilia; filament bundles ending in focal contacts; filaments running parallel with the membrane at the membrane-cytoskeletal interface. In animal cells one of the most important contractile, belt-like bundle of actin and myosin filaments is the contractile ring (Schroeder 1975). This ring appears beneath the plasma membrane during the M phase of the cell division cycle. Forces generated by it pull inward on the plasma membrane and thereby constrict the middle of the cell, leading the eventual separation of the two daughter cells by a process known as cytokinesis.

In addition to its structural role, actin is also an important element in cellular motility processes. Actin filaments activate the myosin-Mg^{2+}-ATPase activity, and movement of myosin along actin filaments produces force for muscle contraction and other less specialized movements (Cooke 1990). Moreover, amoeboid movements that are responsible for the motility of or in individual cells, such as locomotion and cell ruffling, are important in biological processes such as embryonic development, wound healing and tumor invasion (Theriot and Mitchison 1991).

D. Actin isoforms

In two dimensional gels of total cellular extracts, actin migrates as slightly different spots with constant molecular weights (42 kDa) and, depending on the tissue and/or the species, with very similar pI values (around 5.4) (Garrels and Gibson 1976). Amino acid sequence analysis of skeletal muscle actin (Elzinga et al. 1973) and other isoforms of actin (Vandekerckhove and Weber 1979) confirmed the conserved length (374-375 residues) and explained the pI differences in terms of the number and nature of the acidic residues present in the extreme N termini (Vandekerckhove and Weber 1978). Warm-blooded vertebrates all contain six isoforms of actin expressed solely in a tissue-specific way independent of the species: two striated muscle actins (cardiac and skeletal muscle), two smooth-muscle actins (vascular and visceral), and two non-muscle actins (β and γ) in all non-muscle cells. The muscle actins are typically found in chordates. Early subphyla have only one type. Later, because of consecutive gene duplication (one from Chondrichthyes to Amphibia and one from Amphibia to Reptilia), the ancestral actin branched into four types: two striated- and two smooth-muscle actins (Vandekerckhove and Weber 1984). The cytoplasmic actins are evolutionary older and found in all cells of lower eukaryotes, invertebrates, and in non-muscle cells of vertebrates.

The position of the amino acid exchanges that distinguish skeletal muscle from non-muscle vertebrate actins are mapped in the atomic structure (Figure 1). Most exchanges occur in three-dimensional clusters. The most important cluster is found in the

right-hand lower corner of subdomain 1 and contains positions 1, 2, 3, 4, 5, 6, 103, 129, 357 and 364. Another cluster is present in the central β-sheet of subdomain 3 with substitutions at residues 153, 162, 176 and 298. The third cluster is less pronounced and is located at the interdomain region of subdomains 3 and 4 comprising residues 225, 259, 266 and 271. Interestingly, subdomains 2 and 4 are conserved.

Most of the substitutions located in subdomain 1 coincide with the presumed binding sites of a variety of actin binding proteins. Variations in this region may therefore be considered as structural adaptations to match the contacts with the different associated proteins. This observation suggests that the differences between the isoforms of actin serve to accommodate interactions with associated proteins rather than to vary polymerization properties.

E. Invertebrate actin genes

In invertebrates the discrimination between muscle and cytoplasmic actin isoforms is less evident than in vertebrates. Invertebrate actins resemble vertebrate cytoplasmic actins more than vertebrate muscle actins. Invertebrate actins are thus considered to be cytoplasmic-like actins. Analysis of gene expression has however, indicated that muscle actin genes can be distinguished from cytoplasmic actin genes in a few invertebrate species. In the dipteran *Drosophila melanogaster,* all members of the actin gene family have been cloned. Expression studies of the six genes have determined

that two, 5C and 42A, code for cytoplasmic actins, whereas the other four, 57A, 87E, 88F and 79B, code for muscle actins (Fryberg et al. 1983; Sanchez et al. 1983).

1. Actin genes of the silkworm (*Bombyx mori*)

The lepidopteran *Bombyx mori* has four actin genes (Mounier and Prudhomme 1986; Mange et al. 1996). Two out of the four genes have no intron in their 1131 bp long coding sequences (A1 and A2 actins). The analysis of their expression patterns indicated that they expressed in larval and adult muscle tissues therefore they can be considered as muscle actins (Mounier and Prudhomme 1991). The A1 actin gene is highly expressed in all muscle containing tissues (both visceral and skeletal muscles) during development. On the other hand the A2 gene encodes an actin present only in some adult muscles which form in the late pupa. These latter muscles are skeletal rather than visceral muscles. The A1 actin gene differs from the A2 actin gene by 124 nucleotide substitutions among which 109 are silent, and by 8 amino acid replacements. The structure of the 3' ends of the A1 and A2 actin mRNAs have been analyzed (Mounier and Prudhomme 1991). The A1 actin gene has four different polyadenylation sites, two are selected in pupal muscles and the four are equally used in larval head and bodywall muscles, and in adult thoracic muscles. In contrast, the adult muscle actin gene (A2 actin) has a unique polyadenylation site.

The silkworm genome contains two additional actin genes (A3 and A4 actins). These genes similar to the A1 and A2 genes, code for actin molecules consisting of 376 amino acid residues. Unlike however the A1 and A2 genes the coding region of the A3 and A4 actins is interrupted by an intron at exactly the same position (within codon 116) (Mange et al. 1996). The introns of A3 and A4 are both 92 bp long and show considerable similarity (more than 90% identity). The coding regions of the two actin genes also exhibit an extremely high degree of sequence homology both at the protein (more than 99% identity, 2 amino acid replacements) and the DNA level (98% identity). The high similarity of the A3 and A4 genes suggests that they arose from a recent duplication of an ancestral intron containing actin gene. Both A3 and A4 genes encode typical cytoplasmic actins.

Gene expression studies revealed that both cytoplasmic actin genes expressed in all tissues of *Bombyx mori* during all developmental stages (Mounier et al. 1991; Mange et al. 1996). The accumulation of transcripts of the A3 actin gene has been studied in relation to the silk production cycle in the two secretory regions (posterior and middle) of the silkgland of *Bombyx mori*. This accumulation was roughly similar in the two regions. The amount of A3 mRNA decreased during the first period of the fourth moult, increased on and after the second period of this moult to reach a maximum at day 5 of the fifth instar, then finally decreased. Actin biosynthesis almost completely ceased in the first period of the fourth moult and recovered in the second period of this moult. The accumulation of actin A3 mRNA parallels the development, in the silkgland cells, of the

apical network of microfilaments which are implicated in silk secretion and which are disrupted at the beginning of the moult period and reorganized during the intermoult period. The accumulation of transcripts from actin A3 gene was compared to that of transcripts from silk protein genes and they found to be asynchronous during the secretory cycle, indicating that mechanisms regulating expression of these two types of genes are different in the silkgland.

Analysis of the expression of the A4 actin gene revealed that the A4 and A3 genes are subjected to a similar global temporal regulation during moult (Mange et al. 1996).

F. Transcriptional regulation of the actin genes

Transcriptional regulation of numerous actin genes have been studied in detail. In the actin genes analyzed so far regulation of expression is very complex and involves several positive and negative *cis*-acting sequences. The use of multiple promoters and transcriptional start sites can also been observed, especially in invertebrate cytoplasmic actin genes.

1. Transcriptional regulation of the mammalian β–actin gene

In mammals, the promoter of the β–actin gene consists of at least four *cis*-acting elements: three CCArGG boxes and one CCAAT box (Ng et al. 1989; Frederickson

1989; Kawamoto et al. 1988). Two of the CCArGG boxes are located within the 5' flanking sequences, one distal and one proximal to the mRNA start site, while the third is located within the first intron.

The consensus sequence of the CCArGG box is: $CC(A/T)_6GG$. This sequence motif is the core component of the serum response element (SRE), a DNA element that is required for the transient transcriptional response of many nonmuscle-specific immediate-early response genes, such as β–actin and the cellular proto-oncogene c-*fos,* upon serum or growth factor stimulation (Treisman 1990, 1992). The CCArGG box can be found in all known mammalian actin genes (Minty and Kedes 1986; Muscat and Kedes 1987). Additionally, this conserved DNA element has also been implicated in the regulation of the myosin light chain (Papadopoulos and Crow 1993), muscle creatine kinase (Kharbanda et al. 1993) and dystrophin (Gilgenkrantz et al. 1992) genes.

It has been demonstrated that the CCArGG box interacts specifically with serum response factor ($p67^{SRF}$). $p67^{SRF}$ is a sequence specific DNA binding protein first identified as the factor that binds to the SRE of the c-*fos* promoter (Treisman 1987) and is believed that mediate serum inducibility of that gene (Treisman 1986). Further studies have shown that $p67^{SRF}$ can also negatively regulate transcription from the c-*fos* promoter (Leung and Miyamoto 1989; Shaw et al. 1989a; Konig et al. 1989). Another protein, $p62^{TCF}$ (TCF: Ternary Complex Factor) is also required for serum inducibility of the c-*fos* promoter (Shaw et al. 1989b; Schroter et al. 1990). $p62^{TCF}$ binds to the SRE through interaction with $p67^{SRF}$. It has been suggested that the functional differences between the

CCArGG boxes arise through formation of multi-protein complexes, comprised of CCArGG box-bound $p67^{SRF}$ and various combinations of $p67^{SRF}$-interactive proteins (for example: $p62^{TCF}$) (Danilition et al. 1991).

Transient transfection experiments indicated that β–actin promoter activity in human HeLa cells requires $p67^{SRF}$ binding to the proximal CCArGG box of the promoter (Danilition et al. 1991). The proximal CCAAT box and a heterodimeric transcription factor NF-Y (also known as CP1 or CBF factor) that binds to this element, are also essential for efficient expression. Additionally, it has been demonstrated that the proper alignment of the CCAAT and CCArGG boxes is crucial for the β–actin promoter activity *in vivo*.

2. Transcriptional regulation of the Drosophila 5C cytoplasmic actin gene

The major cytoskeletal actin gene of *Drosophila melanogaster,* the actin 5C gene, has two promoters (Bond-Matthews and Davidson 1988). Alternative promoters and transcriptional start sites are widely used in both prokaryotic and eukaryotic organisms to increase the flexibility of the expression of their genes in response to changing environmental conditions and various developmental stages (Schibler and Sierra 1987; Ayoubi and Van de Ven 1996). Sometimes alternative promoter usage results in protein isoforms which differ from each other in their N-terminal ends. If there is no translational start site in the alternative leader exon the different mRNA forms will encode the same

protein. In such cases the mRNA isoforms may exhibit different stability and/or translation efficiency. Sometimes however there is no plausible explanation for the need of multiple transcriptional start sites. It is possible that in its simplest form the alternative promoter usage makes the gene less susceptible to mutations in the promoter region. This "safety measure" can be especially advantageous in essential genes, like actins.

In the *Drosophila* 5C actin gene the proximal promoter controls constitutive synthesis of actin in all growing tissues (Chung and Keller 1990a). The proximal promoter comprises six positive *cis*-acting regulatory elements, five of which are GAGA sequences. The sixth element is specific to the 5C proximal promoter. The deletion of this element lowers expression to one-third of the wild-type level. The promoter also shows sequences homologous to the alcohol dehydrogenase factor-1 binding site and to the vertebrate CCArGG element, but mutations of these sites did not affect promoter activity in transient transfection assays. This promoter has no classical TATA element although it contains an essential region about 35 bp upstream from the cap site that could be a TATA surrogate.

The TATA-box containing distal promoter of the 5C actin gene regulates synthesis of actin in a tissue- and developmental stage-specific manner (Chung and Keller 1990b). Transient transfection assays identified two negative and six positive *cis*-acting regulatory elements. In addition to these regulatory regions, sequence motifs homologous to binding sites of known *Drosophila* transcription factors and a CCArGG element could also be detected in the nucleotide sequence of the distal promoter. Although removal or

mutation of many of these elements did not affect the promoter activity in transient transfection assays in cultured *Drosophila* S2 cells, it is possible that some of these sequence motifs may regulate the expression of the distal promoter in different tissues at different stages of development.

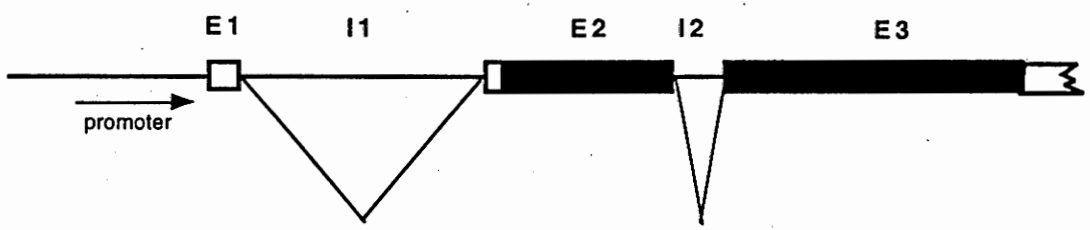
3. Transcriptional regulation of the cytoplasmic actin genes of Bombyx mori

The 5' untranslated region of the A4 cytoplasmic actin gene of *Bombyx mori* shows a similar organization to the *Drosophila* 5C cytoplasmic actin gene (Mange et al. 1996). The A4 gene has two untranslated leader exons transcribed by the use of alternative promoters. The relative abundance of the two types of transcripts is approximately one in many tissues. The transcriptional regulation of the A4 actin gene has not been studied in detail. Sequence analysis indicated that sequence motifs resembling to the vertebrate CCArGG element can be found in both the proximal and distal promoters.

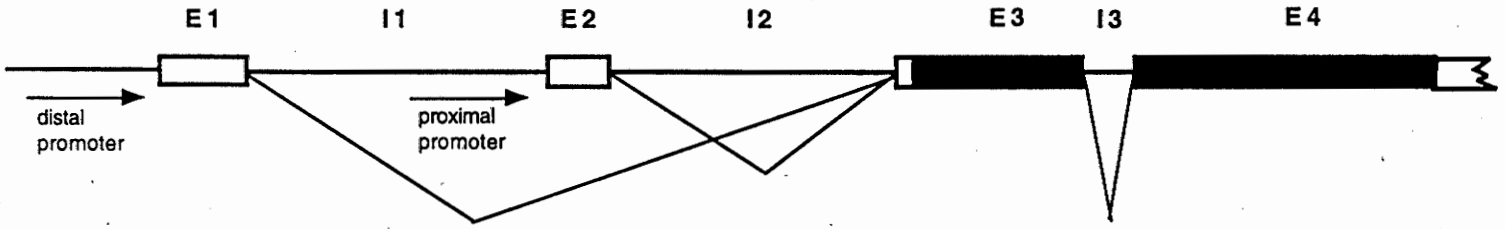
Despite the high degree of similarity between the coding regions of A4 and A3 actin genes the latter exhibits a different organization in its 5' untranslated sequence (Figure 2). Earlier studies indicated that the A3 gene has one promoter region and a short untranslated leader exon followed by a 460 bp long intron (Mange et al. 1997). Transcriptional regulation of the A3 gene has been studied by transient transfection assay in cultured *Spodoptera frugiperda* cells (Sf9).

Figure 2. Organization of the A3 and A4 cytoplasmic actin genes of the silkworm (*Bombyx mori)*. Earlier studies indicated that the A3 gene has one promoter region. The A4 gene has two untranslated leader exons transcribed by the use of alternative promoters. The filled boxes represent the coding sequence.

A3 actin:

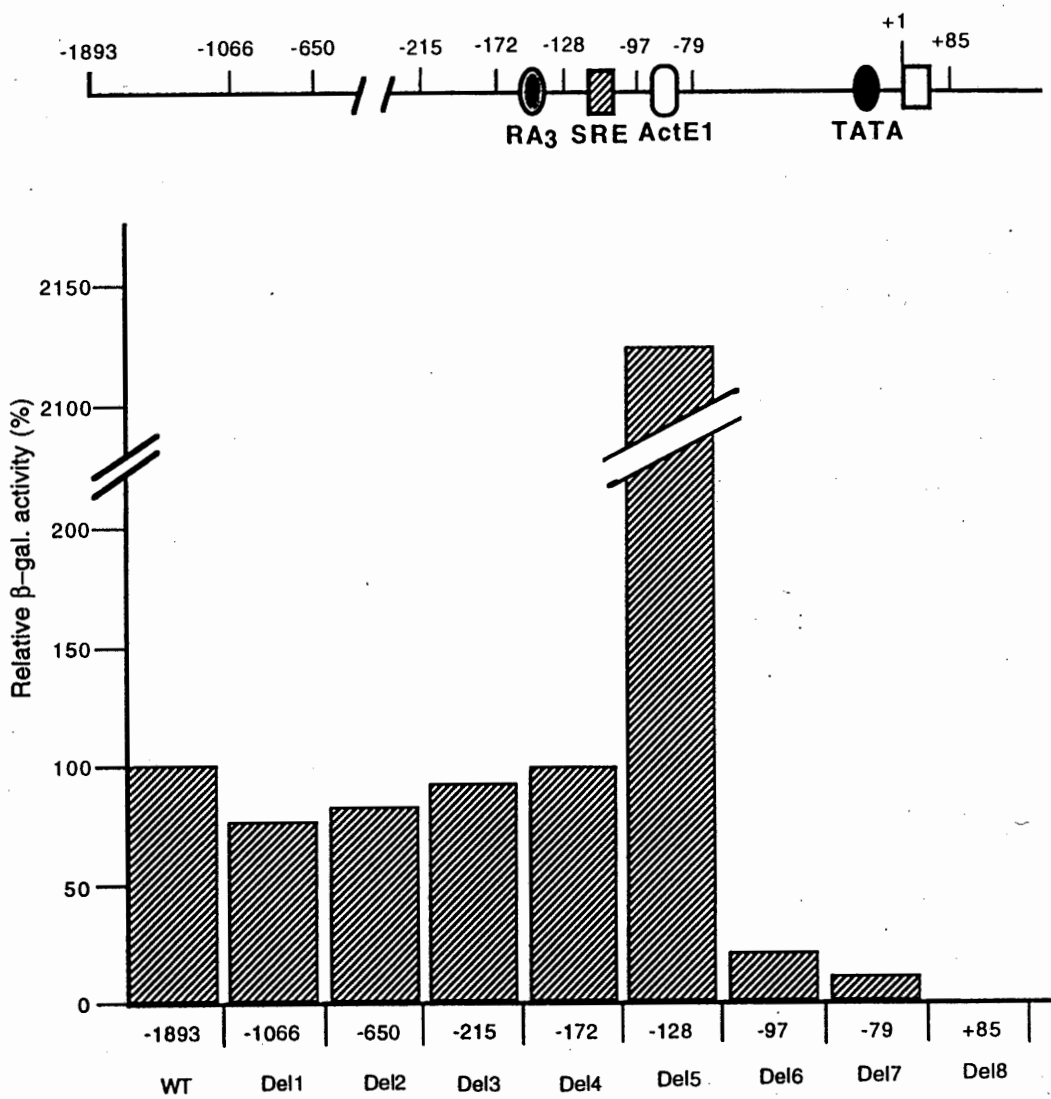


A4 actin:



The analysis revealed the presence of two positive and one negative regulatory elements in the promoter region (Figure 3). Recurrent deletions of the A3 5' untranslated region from -1893 to -172 did not decrease the expression level of the reporter gene. Further trimming the DNA down to -128 resulted in a ~20-fold stimulation of promoter activity indicating that the underlying sequence carries a strong silencer region. Detailed analysis of the silencer domain detected a 10 bp long DNA segment (called RA3 element) capable of interaction with a factor present in silkgland nuclear extract. Deletion down to -97 resulted in a 100-fold drop of promoter activity. This positive domain contains a SRE sequence that is able to bind a $p67^{SRF}$-like protein present in silkgland extract. Further removal of 18 bp 3' of -97 resulted in a twofold decrease of activity, suggesting that this short sequence (named ActE1) also activates A3 expression. No promoter activity was detected in the construct devoid of sequence upstream from +85.

Figure 3. Analysis of the transcriptional regulatory elements of the A3 cytoplasmic actin gene by transient transfection assay in cultured *Spodoptera frugiperda* (Sf9) cells (Mange et al. 1997). The analysis revealed the presence of two positive (SRE and ActE1) and one negative (RA_3) regulatory elements


-1893
-1066
-650
-215
-172
-128
-97
-79
+1
+85
RA3 SRE ActE1
TATA
Relative β-gal. activity (%)
2150
2100
200
150
100
50
0
-1893
-1066
-650
-215
-172
-128
-97
-79
+85
WT
Del1
Del2
Del3
Del4
Del5
Del6
Del7
Del8

II. GOALS AND OBJECTIVES OF THE PROJECT

The transcriptional regulation of the *Bombyx mori* A3 cytoplasmic actin gene has been extensively studied by transient transfection assay in a heterologous lepidopteran cell line (Mange et al. 1997). Although, the use of heterologous expression systems can provide valuable information concerning transcriptional regulation of a gene, one must be careful in the interpretation of the experimental data produced by such systems.

In the A3 gene the first and the second exons are separated by a 460 bp intron that is longer than the average length of introns found in insect genes (Mount et al. 1992). Long introns have been related to the presence of functional elements other than those needed for splicing. These elements include enhancers, silencers, alternative transcriptional initiation sites and promoters (Gillies et al. 1983; Queen and Baltimore 1983). Both in the *Drosophila* 5C and in the *Bombyx* A4 cytoplasmic actin genes the long introns contain alternative promoters associated with multiple *cis*-acting regulatory elements. Earlier studies using the heterologous Sf9 expression system did not address the role of the first intron in the transcriptional regulation of the A3 gene. Here we examined the effect of the first intron on the expression of the A3 promoter both in homologous and heterologous systems.

The CCArGG box containing serum response element plays an important role in the transcriptional regulation of vertebrate actin genes (Mohun et al. 1987; Mohun et al. 1989). SREs are also found in the promoter region of several insect cytoplasmic actin

genes (Chung and Keller 1990a-b; Mange et al. 1996; Mange et al. 1997; Rourke and East 1997). In contrast to the *Drosophila* 5C actin where the mutation of the SRE motif has no effect on transcriptional activity, in the *Bombyx* A3 actin gene the deletion of this element dramatically decrease the promoter strength. The A3 gene is the first example of a SRE-regulated invertebrate actin gene, therefore we decided to examine whether the A3 promoter also functions in vertebrate cells.

III. MATERIALS AND METHODS

A. Cell lines, isolation of stably transformed cell lines

CHO-K20 chinese hamster and LMTK$^-$ mouse cells were grown in F12 medium (Mediatech, Herndon, VA). Human 293 cells were cultured in EMEM (Quality Biological Inc., Gaithersburg, MD) containing 2 mM L-glutamine. COS african green monkey cells were grown in DMEM (Sigma, St. Louis, MO) medium. DT-40 chicken cells were cultured in DMEM with 1×Tryptose phosphate broth (GibcoBRL, Grand Island, NY) and 1% chicken serum (GibcoBRL) (Dieken et al. 1996). BmN silkworm cell line was maintained in IPL-41 (Sigma) medium supplemented with 1×Tryptose phosphate broth. All media used were supplemented with 10% FBS (Gemini Bio-Products Inc., Calabasas, CA). 5×10^6 LMTK$^-$ cells were transfected by the calcium phosphate-DNA transfection method (Chen and Okayama 1987) using 2 μg circular pBmA/HmB plasmid DNA. Transformed cells were selected in 400 μg/ml hygromycin B (Calbiochem, San Diego, CA) containing medium.

Bombyx mori cells (~3×10^6) were also transfected by the calcium phosphate-DNA transfection method using 2 μg circular pBmA/Pur and 5 μg pTK-β plasmids. Three days after transfection, cells were collected in 50% conditioned medium and the cell suspension was dispensed into a 96 well microtiter plate. The next day the medium was changed to 50% conditioned medium containing 5 μg/ml puromycin (Sigma). After four

weeks selection the resistant cell line was transferred to non-selective medium and cultured under the same conditions until the cell population was large enough to transfer into a 60 mm petri dish. From that time on the transformed cell line was maintained in medium containing 5 μg/ml puromycin.

B. DNA techniques, plasmid constructs

All DNA manipulations were performed using conventional techniques (Sambrook et al. 1989). Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA) Boehringer Mannheim (Indianapolis, IN) and Promega (Madison, WI). All enzymes were used at the recommended temperatures in buffers supplied by the manufacturers. The list of plasmids created during this study and the construction routes are given below:

1. pBmA/Pur

Plasmid pBmA/Pur was constructed from pBmA/HmB by replacing the hygromycin B phosphotransferase gene (*hyg*) with the 660 bp ClaI-HindIII fragment of pBabe-Puro (Morgenstern and Land 1990) containing the *Streptomyces alboginer* puromycin *N*-acetyl transferase gene (*pac*). pBmA/HmB was a kind gift from Dr. K. Iatrou. pBmA/HmB is a derivative of the pBmA expression vector (Johnson et al. 1992) containing the *Streptomyces hygroscopicus* hygromycin B phosphotransferase gene inserted into the polylinker region of the plasmid.

2. pBL

Plasmid pBL was constructed from pBmA/HmB by replacing the hygromycin B phosphotransferase gene containing HindIII-BamHI fragment with the 3.7 kb HindIII-BamHI fragment of pCH110 (Pharmacia, Piscataway, NJ) containing the bacterial β-galactosidase gene and the SV40 early polyadenylation signal. Finally, to remove the SV40 polyadenylation signal from the plasmid the 2.5 kb EcoRV-NotI fragment was replaced by the 2.3 kb EcoRV-NotI fragment of plasmid pTK-β (Clontech, Palo Alto, CA) that contains the 3' end of the β-galactosidase gene without polyadenylation signal.

3. pBSL

The 0.4 kb HindIII-BamHI fragment of pBabe-Puro (Morgenstern and Land 1990) containing the SV40 early promoter was inserted into the HindIII site of pBL.

4. pSBL

The 1.5 kb SalI-KpnI fragment containing the 5' UTR of the A3 actin gene was removed from pBSL to produce pSBL.

5. pΔP-BmpA/LacZ

The promoter-less pΔP-BmpA/LacZ was constructed from pSBL with the removal of the 0.4 kb SalI-HindIII fragment containing the SV40 early promoter.

6. pBmA-Δ443/LacZ

The 1 kb EcoRV-HindIII fragment of pBmA/Pur containing the 443 bp long promoter segment and the first intron of the A3 actin gene was cloned into the pUC18 plasmid vector (pHE3). Next, the insert was isolated from pHE3 as a KpnI-HindIII

restriction fragment and then it was inserted into pSBL by replacing the 0.4 kb SalI-HindIII fragment containing the SV40 early promoter.

7. pBmA-ΔI/LacZ

The 1.5 kb KpnI-HindIII fragment of pBmA/Pur containing the 1 kb promoter region and the first intron of the A3 actin gene was cloned into pUC18 vector (pKH1). In the next step, the intron was removed from the pKH1 plasmid by replacing the 550 bp intron containing HindIII-SnaBI fragment with the equivalent intronless 90 bp long HindIII-SnaBI RT-PCR fragment resulting in the ΔI5 plasmid (This RT-PCR segment was obtained by the following procedure: 1. an RT-PCR reaction was carried out on poly-A^+ RNA template prepared from the stably transformed BmN cell line containing the pBmA/Pur plasmid by using an intron preceding forward primer (BMAF) and a *pac* gene specific reverse primer (PUR2). 2. The 700 bp long intronless RT-PCR product was digested by HindIII-SnaBI and the resulting 90 bp DNA fragment was isolated.). The correct structure of the ΔI5 plasmid was confirmed by sequencing. Following that, the 1 kb KpnI-HindIII fragment of ΔI5 was used to replace the 0.4 kb HindIII-SalI fragment of pSBL resulting in the pBmA-ΔI/LacZ plasmid construct.

8. pBmA-Δ5'XI/LacZ

Plasmid pHE3 (see above) was digested by HindIII-SnaBI restriction enzymes and the 3.15 kb SnaBI-HindIII fragment was isolated. Next, this fragment was ligated to the 0.5 kb HindIII-DraI fragment of the same plasmid producing pHE3-Δ5'XI plasmid that lacks the 43 bp SnaBI-DraI fragment containing the 5' splice site of the first intron of

the A3 actin gene. Finally, the 1 kb SacI-HindIII fragment of pHE3-Δ5'XI was used to replace the 0.4 kb HindIII-SalI fragment of pSBL producing pBmA-Δ5'XI/LacZ. The structure of the pBmA-Δ5'XI/LacZ plasmid was confirmed by sequencing.

9. pBmA-Δ176/LacZ, pBmA-Δ125/LacZ, pBmA-Δ110/LacZ, pBmA-Δ73/LacZ, pBmA-Δ27/LacZ, pBmA-Δ29(-)/LacZ

These deletion constructs were generated by a PCR based method which consisted of the following steps: 1. PCR reactions were performed on pBmA/Pur (see above) plasmid template by using nested forward primers (URAE, USRE, DSRE, ACED, SSP, BMAF) and a common reverse primer (BMA3R). 2. The PCR products were digested by SnaBI or SpeI restriction enzymes and the deleted promoter fragments were ligated to the 3.2 kb SnaBI-KpnI or the 3 kb SpeI-KpnI fragment of pHE3 plasmid resulting in pBmpr176, pBmpr125, pBmpr110, pBmpr73, pBmpr27 and pBmpr29(-) plasmid constructs. The structures of these plasmids were checked by sequencing. 3. The plasmids exhibiting the expected structures were digested by SacI-HindIII restriction endonucleases and the DNA fragments containing the 5' deleted promoter regions were ligated to the 7.6 kb SalI-HindIII fragment of pSBL producing pBmA-Δ176/LacZ, pBmA-Δ125/LacZ, pBmA-Δ110/LacZ, pBmA-Δ73/LacZ, pBmA-Δ27/LacZ, and pBmA-Δ29(-)/LacZ plasmid constructs.

10. pBmA-Δ137(-)/LacZ

The pBmA-Δ176/LacZ plasmid was digested by BstBI-SalI. End repair and recircularization of the 8.1 kb DNA fragment resulted in the pBmA-Δ137(-)/LacZ construct.

11. pBmA-Δ244(-)/LacZ

The pBmA-Δ244(-)/LacZ was constructed analogous to pBmA-Δ137(-)/LacZ but instead of BstBI-SalI double digestion the pBmA-Δ176/LacZ plasmid was digested by SpeI and SalI restriction endonucleases. Following end repair and recircularization pBmA-Δ244(-)/LacZ was obtained that lacked an additional 107 bp fragment of the A3 first intron.

12. pBL-ΔTATA

pBL-ΔTATA plasmid was created through several intermediary constructs. A 900 bp segment of the A3 actin 5' UTR, starting immediately upstream of the TATA-box was generated by PCR (template: pKH1; primers: TBR, Reverse 2) The PCR product was subsequently cloned into the SmaI site of pUC18 plasmid resulting in pBmpr900/u. The structure of pBmpr900/u was confirmed by sequencing. The insert was isolated from the plasmid as a BamHI-KpnI fragment. Following T4 polymerase treatment the fragment was inserted into T4 polymerase treated SacI-EcoRI sites of pBmpr27 plasmid (see above). The structure of the resulting pKH-ΔTATA was checked by sequencing. Finally, the 0.9 kb PacI-HindIII fragment of pBL was replaced by the equivalent restriction

fragment of pKH-ΔTATA (that contains the mutated TATA-box) producing the pBL-ΔTATA plasmid construct.

13. pBmA-5'I/LacZ

pIP7 plasmid was created by inserting the 1 kb HindIII-KpnI fragment of ΔI5 into the HindIII-ClaI digested pBmpr29(-) plasmid. Next, the 340 bp SpeI-HindIII fragment of pBmA-Δ29(-)/LacZ was replaced by the 1.4 kb SpeI-HindIII restriction fragment of pIP7 resulting in pBmA-5'I/LacZ.

14. pBmA-3'I/LacZ

pBmA-3'I/LacZ was obtained by inserting the 0.55 kb SnaBI-HindIII fragment of pBmA-Δ73/LacZ containing the first intron of the A3 actin gene into the SacII site of pBmA-ΔI/LacZ.

15. pSBL-5'$(I)_4$, pSBL-5'I

The 0.55 kb SnaBI-HindIII fragment of pBmA-Δ73/LacZ that contains the first intron of the A3 actin gene was treated with Klenow polymerase. Following the ligation of this fragment to SalI linearized and Klenow polymerase treated pSBL plasmid the pSBL-5'$(I)_4$ plasmid construct was obtained. This plasmid contains 4 tandemly arranged intron containing DNA fragments inserted immediately upstream of the SV40 early promoter. pSBL-5'I was created from pSBL-5'$(I)_4$. Following SpeI digestion the 8.6 kb SpeI fragment was circularized producing the pSBL-5'I plasmid.

16. pSBL-3'I

The 0.4 kb SalI-HindIII fragment of the pSBL plasmid that contains the SV40 early promoter was ligated to the 8.3 kb SalI-HindIII fragment of pBmA-3'I/LacZ to obtain the pSBL-3'I construct.

C. β-galactosidase cytochemical staining and enzyme assay

1. β-galactosidase cytochemical staining

Cells at ~50% confluency (in 60mm petri dish) were transfected with 3.75 μg of pBL or pCH110 plasmids by a synthetic, cationic lipopolyamine (Transfectam, Promega) with the exception of the LMTK$^-$ and BmN cell lines which were transfected by the calcium phosphate-DNA transfection method. β-galactosidase expression was monitored by cytochemical staining 60 hours (BmN cell line) or 36 hours (mammalian cell lines) after transfection using the following procedure (Bondi et al. 1982): Cells were rinsed with cold PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; pH~7.3). The PBS solution was replaced with ice cold fixative solution (2% formaldehyde, 0.2% glutaraldehyde in PBS) and it was left on the cells for 5 minutes. The cells were rinsed with PBS again and the staining solution (1 mM X-gal; 5 mM $K_3Fe(CN)_6$; 5 mM $K_4Fe(CN)_6$; 2 mM $MgCl_2$ in PBS) was added to the petri dishes (~3 ml/60 mm dish). To allow the enzyme reaction to take place the dishes were incubated at 37 °C for 4-12 hours. After incubation the cells were rinsed with PBS and the

blue cells were counted by microscope using visible light. The samples can be stored in 50% ethanol at 4 °C.

2. β–galactosidase enzyme assay

An equal number of LMTK$^-$ cells (~1×10^7) were plated into 100 mm petri dishes. The cells were allowed to grow 12-16 hours before they were transfected with plasmid DNA (10 μg plasmid DNA/100 mm petri dish). Forty eight hours following transfection the cells were collected by trypsinization and β-galactosidase activities of the cytoplasmic extracts were determined. For quantitative β-galactosidase activity measurement two methods were used:

a. ONPG method (MacGregor et al. 1991)

Cells were washed with cold PBS. After centrifugation (2,000 rpm, 10 minutes, 4 °C) the pelleted cells were resuspended in 450 μl PM-2 buffer (23 mM NaH_2PO_4; 77 mM Na_2HPO_4; 0.1 mM $MnCl_2$; 2 mM $MgSO_4$; 40 mM β–mercaptoethanol). The cells were lysed by 0.1% Triton-X100 in PM-2 buffer and centrifuged for 15 minutes at 13500 g. Protein concentrations of the supernatants were determined by the Bradford method (Bradford 1976). Aliquots of supernatants containing 0.5-1 mg protein were used to measure β-galactosidase activity. The volumes of the cytoplasmic extracts were adjusted to 800 μl with PM-2 buffer and they were transferred into 1 ml plastic cuvets. The enzymatic reactions were initiated by the ddition of 200 μl of ONPG solution (O-nitrophenyl-β-D-galactopyranoside dissolved at 4 mg/ml in PM-2 buffer) to each cuvet. The samples were incubated at 37 °C until a yellow color became apparent. The

reactions were stopped by the addition of 500 µl of 1 M $NaCO_3$. Absorbance of each sample was read at 420 nm.

b. Chemiluminescent β–galactosidase assay

To determine the β–galactosidase activities of the cytoplasmic extracts prepared from the transfected LMTK$^-$ cells a chemiluminescent detection system (Boehringer Mannheim) was also applied. Cells were collected by trypsinization and washed twice in cold PBS. The cytoplasmic extract preparation and the β–galactosidase activity determination were carried out according to the manufacturers instructions. The chemiluminescent measurements were performed on a luminometer by manual initiation using 5 second integration time without delay.

To determine the transfection efficiency 1/10-th volume of the transfected cell suspensions were embedded in 0.5% LMP agarose. Following proteinase-K deproteinization, the agarose embedded DNAs were digested with HindIII restriction endonuclease, separated on 0.8% agarose gel and transferred onto Hybond-N membrane (Amersham, Arlington Heights, IL). Membranes were hybridized with ^{32}P-labeled β-galactosidase gene specific PCR probe and washed under high stringency conditions (see below). Hybridizing spots were cut out from the membrane and counted on a scintillation counter. The measured β-galactosidase activities were corrected for plasmid size and transfection efficiency. The β–galactosidase activities were determined at least twice in separate experiments for each plasmid construct.

D. Southern hybridization

The presence of the plasmid vectors in the isolated cell lines was demonstrated by Southern blot hybridization. High molecular weight agarose embedded genomic DNA was prepared from the mammalian cell lines as described (Fatyol et al. 1994). Cells ($1\text{-}3\times10^7$)were collected from the culture dishes by trypsinization. The cells were washed with PBS. After centrifugation (2,000 rpm, 10 minutes, 4 °C) the pelleted cells were resuspended in 80 µl lukewarm 0.5% LMP agarose solution (0.5 g LMP agarose boiled in 100 ml PBS until the agarose completely dissolved then the solution cooled down to ~50 °C). The cell suspension was poured into a plastic mold and stored at 4 °C until the agarose plug solidified (~20 minutes). The solid agarose plugs were transferred into LET buffer (1% Na-lauroyl-sarcosine, 100 µg/ml Proteinase-K in 0.5 M EDTA; pH 8) and incubated for 12-16 hours at 50 °C. The agarose plugs were stored in 50 mM EDTA solution at 4 °C. Prior to restriction enzyme digestion the agarose plugs were incubated in 1 mM PMSF containing TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8) for 2 hours at 37 °C to inactivate the residual protease activity. Subsequently, the plugs were rinsed in TE buffer and then equilibrated in the recommended restriction buffer for 2 hours at 37 °C. The restriction buffer was then replaced with a fresh aliquot of the same buffer containing ~10 U restriction endonuclease/1 of µg DNA. Restriction digestions were performed at the recommended temperature for 12-16 hours.

Genomic DNA was isolated from the insect cell lines by lysing the cells in TE buffer containing 1% SDS. Proteinase-K was added to a concentration of 100 μg/ml and incubated at 50 °C for 12-16 hours. The lysate was extracted with equal volumes of phenol, phenol/chloroform (1:1) and chloroform and precipitated with 2.5 volumes of ethanol in the presence of 2.5 M NH_4-acetate. The DNA was dissolved in TE buffer containing 10 μg/ml RNase A. Restriction endonuclease digestions of the genomic DNA were carried out under conditions recommended by the manufacturers.

Restriction enzyme digested genomic DNAs were separated on 0.8% agarose gel in 1×TAE electrophoresis buffer (40 mM Tris-acetate; 2 mM EDTA). The gel was incubated in 0.25 N HCl for 10 minutes to improve the subsequent DNA transfer. The DNA was denatured in situ by incubating the gel in a denaturation solution (0.5 M NaOH; 1.5 M NaCl) for 40 minutes. Prior to the DNA transfer the gel was neutralized with a neutralization solution (0.5 M Tris-HCl; 1.5 M NaCl; pH 7) for 40 minutes. In both cases after the first 20 minutes the respective solution was replaced with a fresh aliquot of the same buffer. Following the neutralization the DNA was blotted onto Hybond-N nylon membrane (Amersham) by the capillary transfer method (Sambrook et al. 1989) using 20×SSC (3 M NaCl; 0.3 M Na_3-citrate; pH 7) as a transfer buffer. Membranes were hybridized with ^{32}P labeled DNA fragments. $\alpha^{32}P$-dCTP (Du Pont NEN Products, Boston, MA) labeled DNA probes were prepared by the random priming method using the Megaprime kit from Amersham. After the labeling reaction the non-incorporated nucleotides were separated from the DNA fragments by a SEPHADEX G-

50 column (Pharmacia). Membranes were incubated overnight at 42 °C in a hybridization solution (5×SSPE; 5×Denhardt's solution; 0.5% SDS; 20 μg/ml denatured herring sperm DNA; 50% formamide) (20×SSPE: 3 M NaCl; 200 mM NaH_2PO_4; 20 mM EDTA; pH 7.4) containing the radioactivelly labeled, denatured DNA probe. After hybridization, membranes were washed successively in the following buffers: 2×SSPE, 0.1% SDS at room temperature for 10 minutes (2×); 1×SSPE, 0.1% SDS at 65 °C for 15 minutes; 0.1×SSPE, 0.1% SDS at 65 °C for 10 minutes. Membranes were exposed to X-ray films between intensifying screens at -80 °C.

E. Northern hybridization

For Northern hybridization poly-A^+ RNAs were prepared from both the mammalian and insect cell lines by using the Oligotex Direct mRNA kit (Qiagen, Santa Clarita, CA). 10 μg denatured poly-A^+ RNA samples were run on 1% agarose formaldehyde gel in 1×MOPS buffer (appropriate amount of agarose melted in water, cooled to 60 °C, 5×MOPS buffer and formaldehyde were added to give final concentrations of 1× and 2.2 M respectively. 1×MOPS buffer: 20 mM MOPS, pH 7; 8 mM Na-acetate; 1 mM EDTA, pH 8). Following electrophoresis gels were treated with 0.05 N NaOH for 20 minutes and 20×SSC for 45 minutes. The RNA was blotted onto Hybond-N membrane (Amersham) by the capillary transfer method using 20×SSC as a transfer buffer. Membranes were hybridized with radioactivelly labeled DNA probes as

described above (see Southern hybridization). After hybridization, membranes were washed in the following buffers: 1×SSC, 0.1% SDS at 68 °C for 20 minutes; 0.2×SSC, 0.1% SDS at 68 °C for 20 minutes (3×). Membranes were exposed to X-ray films as described above.

F. In situ hybridization

a. Preparation of metaphase chromosomes from cultured cell lines

Cells were grown in the presence of 5 μg/ml colcemid for 3-5 hours. The mitotic cells were collected by selective detachment from the culture dish and centrifuged for 10 minutes at 1,000 rpm. The pelleted cells were resuspended in 10 ml of 75 mM KCl and incubated at room temperature for 15 minutes. The cells were pelleted again (10 minutes at 1,000 rpm) and then resuspended in 10 ml of fixative solution (methanol/glacial acetic acid, 3:1; precooled to -20 °C). The cell suspension was incubated on ice for 15-20 minutes. The cells were spin down as above and resuspended in 2-3 ml of cold fixative solution. Next, the cell suspension was dropped on a microscope slide (cleaned with ethanol/HCl, 99:1) and the slide was air dried.

b. Preparation of biotin labeled DNA probes

DNA probes were labeled with biotin-16-dUTP by using a nick translation kit (Boehringer Mannheim) according to the manufacturer's instructions. The non-incorporated nucleotides were separated from the labeled DNA fragments by

chromatography through a SEPHADEX G-50 column (Pharmacia). The labeled DNA probe (0.5-1 μg) was mixed with 50 μg of sonicated herring sperm DNA and then precipitated with 2.5 volume of ethanol in the presence of 0.3 M Na-acetate (pH 5.2). The precipitated nucleic acids were dissolved in 50 μl of hybridization solution (50% formamide; 10% dextran-sulfate; 2×SSC).

c. Pretreatment of chromosome preparations

Pretreatment of chromosome preparations, hybridization and probe detection were carried out according to the published protocol with minor modifications (Wiegant et al. 1991).

RNase A treatment: chromosome preparations were incubated in 2×SSC containing 100 μg/ml DNase free RNase A at 37 °C for 1 hour. Slides were subsequently washed with 2×SSC for 5 minutes at room temperature, three times.

Pepsin treatment: slides were incubated in 0.01 N HCl containing 0.005% pepsin for 5 minutes at 37 °C. Slides were washed twice with PBS for 5 minutes at room temperature then once with PBS/$MgCl_2$ (50 mM) for 5 minutes at room temperature.

Postfixation: slides were incubated in PBS/$MgCl_2$ containing 1% formaldehyde for 10 minutes at room temperature then washed in PBS (5 minutes, room temperature). Finally, the chromosome preparations were dehydrated through an ethanol series (70%, 90%, 100% ethanol, 3 minutes each) and vacuum dried.

d. Hybridization

The biotinylated DNA probe and the chromosome preparations were denatured in one step. Ten microliter of probe (dissolved in hybridization buffer, see above) was added to a slide. The slide was covered with a coverslip and then sealed with rubber cement. Subsequently the slide was placed on a glass plate, preheated to 80 °C in an oven and denatured for 4 minutes. After denaturation the slide was transferred into a moist chamber and incubated for 12-16 hours at 37 °C.

e. Fluorescence probe detection

After hybridization the coverslips were removed from the slides and then they were washed in the following buffers:

- 2×SSC/50% formamide; 3×5 minutes; 45 °C,
- 2×SSC; 5×2 minutes; room temperature,
- TNT (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% Tween-20); 5 minutes; room temperature.

To block the non-specific binding sites the slides were incubated in TNB buffer (100 mM Tris-HCl, pH 7.5; 150 mM NaCl; 3% BSA). Next, the slides were incubated with FITC-avidin for 30 minutes at 37 °C (Vector Laboratories, Burlingame, CA; 500×dilution in TNB buffer) and then they were washed three times with TNT buffer at room temperature (5 minutes each). The slides were then incubated with biotinylated anti-avidin (Vector Laboratories, 200×dilution in TNB buffer) for 30 minutes at 37 °C

and washed as above. Finally, the slides were incubated with FITC-avidin again and then they were washed in TNT buffer three times (5 minutes each; room temperature).

f. DNA counterstaining

The chromosomes were counterstained with DAPI (50 ng/μl in PBS) or propidium iodide (3 μg/μl in PBS) for 5-10 minutes. The slide were rinsed briefly in PBS. Finally, 100 μl of antifade solution (90% glycerol, 1 mg/ml paraphenylene-diamine) was applied to the slides, covered with coverslips and sealed with nail polish. The specimens were examined by fluorescence microscope.

G. Primer extension

For primer, extension total cellular RNA was prepared from the cultured cell lines. The medium was removed from the culture dish and the monolayer was washed twice with 7 ml of ice-cold PBS. The cells were lysed with 2 ml of 10 mM EDTA (pH 8), 0.5% SDS in the culture dish. The cell lysate was scraped into a polypropylene tube. The plate was rinsed with 2 ml of 0.1 M Na-acetate (pH 5.2), 10 mM EDTA (pH 8). This solution was combined with the cell lysate. The cell lysate was extracted with 4 ml of phenol (equilibrated with water) and precipitated with 2 volumes of ice-cold ethanol in the presence of 0.1 M Tris-HCl (pH 8) and 0.2 M NaCl. The RNA was collected by centrifugation and dissolved in 200 μl of TE buffer. The RNA was precipitated again with 2.5 volumes of ethanol in the presence of 0.1 M NaCl, and finally dissolved in

100 µl of DEPC treated water (solutions were treated with 0.1% DEPC for ~12 hours at 37 °C and then they were autoclaved). The concentration of the RNA solution was determined by the measurement of OD_{260}.

Prior to primer extension, the 5' ends of the non-phosphorylated oligonucleotides were radioactivelly labeled by using T4 polynucleotide kinase. Ten pmol oligonucleotide was labeled in each reaction. The labeling reactions were carried out in a 10 µl volume using 250 µCi of γ^{32}P-ATP (Du Pont NEN Products) as phosphate donor and 10 U of T4 polynucleotide kinase (Promega) as catalyst. The reaction buffer used was supplied by the manufacturer. The reactions were incubated for 10 minutes at 37 °C. The enzyme was inactivated by boiling the samples for 2 minutes. The samples were precipitated twice with ethanol to remove the non-incorporated nucleotides. Finally, the precipitated oligonucleotides were dissolved in 50 µl water. The efficiency of the labeling reaction was determined by measuring a 1 µl aliquot on a scintillation counter.

Primer extensions were performed using 30 µg (insect cells) or 100 µg (mammalian cells) of total RNA. The RNA was mixed with 10^5-10^6 cpm of the labeled oligonucleotide primer. The mixture was precipitated with 2.5 volumes of ethanol in the presence of 0.3 M Na-acetate (pH 5.2). The RNA was collected by centrifugation, washed with 70% ethanol and dried at room temperature. The precipitated nucleic acids were dissolved in 30 µl of hybridization buffer (40 mM PIPES, pH 6.4; 1 mM EDTA, pH 8; 0.4 M NaCl; 80% formamide). The hybridization mixture was incubated at 85 °C for 10 minutes to denature the nucleic acids and then transferred immediately to a 30 °C water

bath and incubated for 8-12 hours. After hybridization, the volume of the mixture was adjusted to 200 µl with water and the nucleic acids were precipitated with 2 volumes of ethanol. The nucleic acids were collected by centrifugation, washed with 70% ethanol and dried at room temperature. The pellet was dissolved in 19.2 µl of reverse transcriptase buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 5 mM $MgCl_2$; 1 mM dNTP-mix; 5 U/µl RNase inhibitor). After addition of 20 U (0.8 µl) of AMV reverse transcriptase the reaction mixture was incubated at 42 °C for 1 hour. The reaction was stopped by the addition of 1 µl of 0.5 M EDTA (pH 8) and 0.5 µl of DNase free RNase A (10 µg/ml). The mixture was incubated at 37 °C for 30 minutes. After incubation, 150 µl of TE buffer containing 0.1 M NaCl was added to the sample and then extracted with 200 µl of phenol/chloroform (1:1). The nucleic acids were precipitated with 2.5 volumes of ethanol and collected by centrifugation. The pellet was washed with 70% ethanol, dried at room temperature and dissolved in 10 µl TE buffer. Prior to gel electrophoresis 10 µl of formamide loading buffer (Promega) was added to the sample. The mixture was denatured for 5 minutes by boiling and then transferred immediately to an ice bath. The samples were analyzed on a 6% denaturing polyacrylamide gel (7 M urea; 6% acrylamide/bis-acrylamide; 0.05% ammonium-persulfate; 0.005% TEMED) in 1×TBE buffer (89 mM Tris-borate; 2 mM EDTA, pH 8). Following electrophoresis the gel was transferred onto a Whatmann 3MM filter paper and it was exposed to X-ray film between intensifying screens at -80 °C.

H. Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were performed by the "Expand PCR System" (Boehringer Mannheim) on a GeneAmp PCR System 9600 thermocycler (Perkin Elmer, Norwalk, CT). In the PCR reactions 10 ng of plasmid DNA or 100-500 ng of genomic DNA was used as template. The PCR reactions were carried out in 50 µl volume. The primer concentrations were 0.4 pmol/µl. The most frequently used amplification conditions were the following: 1. denaturation at 94 °C, first time for 2 minutes and then 5 seconds in all the subsequent cycles; 2. annealing at 62 °C for 30 seconds; 3. extension at 67 °C for 2 minutes. After 10 cycles the extension time was increased by 20 seconds after each cycles. Twenty additional cycles were performed with gradually increasing extension time.

I. RT-PCR, 3' and 5' RACE

1. RT-PCR

Poly-A^+ RNAs were prepared from both the mammalian and insect cell lines by using the Oligotex Direct mRNA kit (Qiagen). Prior to cDNA synthesis the poly-A^+ RNA was treated with RNase free DNase I (Boehringer Mannheim): 1 µg of RNA was incubated with 25-30 U of DNase I for 30 minutes at 37 °C. Following DNase treatment, the reaction mixture was extracted with 1 volume of chloroform. The RNA was

precipitated with 2.5 volumes of ethanol in the presence of 0.3 M Na-acetate (pH 5.2). Single stranded cDNA was synthesized by a 1st Strand cDNA Synthesis kit (Boehringer Mannheim) in a 20 µl volume using 100-300 ng of DNase I treated poly- A^+ RNA as template. For PCR reactions usually 10 µl of cDNA synthesis reaction mixture was used as a template. PCRs were performed as described above.

2. 3' RACE

cDNA synthesis was carried out using the ANCHT oligonucleotide as a primer. Following the cDNA synthesis two sets of PCR amplification were performed. All of the following amplifications were carried out under the same conditions as the RT-PCRs unless otherwise indicated:

a. 3' RACE on cDNA template prepared from the insect cell line

In the first set of amplification a *pac* gene specific forward primer (PUR1) and a reverse primer, specific to the 5' end of the ANCHT oligonucleotide were used (ANCH1). In the second PCR 1 µl from the first PCR reaction was reamplified using the same gene specific forward primer (PUR1) and a nested reverse primer (ANCH2). The PCR reactions contained 25 pmol of each primer.

b. 3' RACE on cDNA template prepared from the mammalian cell line

The PCR amplifications were carried out as described above except that in the first set of amplifications *hyg* gene specific forward primers were used (HygroF or HygroRF). In the second set of PCR reactions the HygroF or the HygroF2 *hyg* gene

specific forward primers were used. The reverse primers were ANCH1 and ANCH2 respectively.

3. 5' RACE

cDNA was synthesized using oligo $(dT)_{17}$ as a primer on poly-A^+ template prepared from the insect cell line. Following cDNA synthesis the excess primer was removed by a QIAquick PCR purification column (Qiagen). Poly-A tail was synthesized by terminal transferase (TdT, Boehringer Mannheim). After TdT treatment the sample was purified again by a QIAquick PCR purification column. In the next step the TdT treated single stranded cDNA was used as a template in PCR. In the first PCR reaction 2 pmol ANCHT, 25 pmol ANCH1 and 25 pmol BMPR (BMPR is a reverse primer, specific to the 3' UTR of the A3 actin gene) primers were used. The PCR was performed under the same conditions as the RT-PCR except that before the first cycle an additional segment was inserted into the program to allow the synthesis of the second cDNA strand. This segment included the following steps: 1. denaturation for 5 minutes. at 94 °C; 2. the annealing consisted of two steps: a, 75 °C for 30 seconds (the enzyme was added to the reaction at this step), b, 35 °C for 5 minutes; 3. 2nd strand cDNA synthesis at 67 °C for 40 minutes. The reamplification reaction contained a *pac* gene specific reverse primer (PUR2) and ANCH2 primer (25 pmol each).

The PCR products were sequenced directly or first they were cloned into pUC18 plasmid vector.

J. DNA sequence analysis, oligonucleotide synthesis

DNA sequence analysis and oligonucleotide synthesis were performed by the DNA analysis core facility of the Center for Molecular Biology and Gene Therapy.

K. Nucleotide sequences of the primers used

ACED: 5' AAA CTG GAG CCG ATA CTG TGT AAA T 3'

ANCH1: 5' CCA GTG AGC AGA GTG ACG AGG ACT C 3'

ANCH2: 5' AGG ACT CGA GTC GAC CTC AAG CTT T 3'

ANCHT: 5' CCA GTG AGC AGA GTG ACG AGG ACT CGA GTC GAC CTC AAG CTT TTT TTT TTT TTT TT 3'

BMAR: 5' AAA TAA GTG ACA AGT ACG TAG ATG CGA ATG 3'

BMA3F: 5' GGA ATG AGA ATG TAG TTT GAA TT 3'

BMIF: 5' GGT GTG CTC GAA CAG TGC GCA TTC G 3'

BMPF: 5' GGC TCA AAT TAC GCT TGT GAT CTT G 3'

BMPF2: 5' TTA TTT ATT AAA GTT ACT AAC CCC 3'

DSRE: 5' GAC AAA AAC TGA GTC AGC CCG CGA T 3'

HSVPR: 5' TAT TTC TTC CGG GGA CAC CGC CAG C 3'

HygroF: 5' GAA TCT CGT GCT TTC AGC TTC GAT 3'

HygroF2: 5' CGG GCA ACT TGT TTA TTG CAG CTT A 3'

HygroR2: 5' TGC CGT CAA CCA AGC TCT GAT AGA G 3'

HygroRF: 5' GAA GTA CTC GCC GAT AGT GGA AAC 3'

IS: 5' CAG AAA TTG GGA ACA GTG AAT TAT G 3'

LacZF: 5' GCG CTG TAT CGC TGG ATC AAA TCT G 3'

LacZR: 5' TTC GGT TGC ACT ACG CGT ACT GTG A 3'

Primer A (BMAF): 5' CAT TCG CAT CTA CGT ACT TGT CAC T 3'

Primer B (BIF): 5' GTA TGG AAT GAG AAT GTA GTT TGA A 3'

Primer C (BMA3R): 5' CCA ACG CGG CAA CTT CTT CGT CGC A 3'

Primer D_1 (PUR2): 5' TCA GGC ACC GGG CTT GCG GGT CAT 3'

Primer D_2 (BMPR): 5' GGC AAC AAC AAC ATT CCG TTC GTT T 3'

Primer D_3 (HygroR): 5' GTT TCC ACT ATC GGC GAG TAC TTC 3'

PUR1: 5' AAT GAC CGA GTA CAA GCC CAC GGT G 3'

PUR1R: 5' GCA CCG TGG GCT TGT ACT CGG TCA T 3'

SSP: 5' TAG TTT ATC CTC ACG AGT CGG TTC T 3'

TBR: 5' CCG TTA TCA CAA TTT ACA CAG TAT C 3'

URAE: 5' TCA GTT ATC AAG GCA TCG CCT TAT C 3'

USRE: 5' GTT ACC ATA TAT GGT GAC AAA AAC T 3'

IV. RESULTS

A. Molecular characterization of a stably transformed *Bombyx mori* cell line: identification of alternative transcriptional initiation sites of the A3 cytoplasmic actin gene

1. Isolation and characterization of a stably transformed *B. mori* cell line

The coding regions of the two silkworm cytoplasmic actin genes show an extremely high degree of sequence homology both at the protein (99%) and the DNA level (98%) (Mange et al. 1996). The high similarity of the two genes makes it difficult to analyze the transcriptional regulation of the A3 and A4 genes independently. One possible solution to this problem is the insertion of a heterologous sequence between the regulatory regions of the actin gene to be studied. After re-introduction of this construct into the host cells the heterologous sequence can serve as a specific probe to follow the transcripts synthesized under the control of the regulatory elements of interest. For the re-introduction of the modified gene into the host cells, usually the transient transfection is the method of choice. However, if the host cells can not be transfected efficiently, isolation of stably transfected cell lines may become necessary.

To study the transcriptional regulatory signals of the A3 actin gene we constructed a selectable plasmid vector which contained the *Streptomyces alboginer* puromycin *N*-

acetyl transferase (*pac*) gene (Vara et al. 1985; Vara et al. 1986) under the control of the A3 actin promoter and polyadenylation signal (pBmA/Pur, Figure 4). Established cell lines of *B. mori* can not be transfected efficiently with plasmid molecules, therefore the analyses of the regulatory signals of the A3 gene would have been difficult in the transient transfection assay. To overcome this problem we decided to isolate a stably transfected cell line containing the pBmA/Pur expression vector. The pBmA/Pur and pTK-β (Clontech) plasmids were co-transfected into BmN cells. After four weeks selection in 5 μg/ml puromycin containing medium, one resistant clone was obtained and analyzed further. To confirm the presence of the selected *pac* gene, genomic DNA from the isolated cell line was subjected to Southern analysis (Figure 5). Restriction enzymes that linearize the pBmA/Pur plasmid (BamHI, HindIII, XbaI) produced strong bands at 6.4 kb, suggesting that the selected plasmid is significantly amplified resulting in head-to-tail arranged repeats. The intense 3.2 kb SacI, 1.4 kb and 5 kb SacII fragments corresponding to the similar sized fragments of pBmA/Pur, indicated the presence of unrearranged plasmid copies. The presence of the non-selected pTK-β plasmid sequences in the isolated cell line was also demonstrated by Southern hybridization. The pTK-β plasmid had been amplified similar to pBmA/Pur and a significant portion of the plasmid molecules also showed tandem organization (Figure 6).

In situ hybridization was carried out on metaphase chromosomes of the isolated cell line to examine whether the foreign DNA integrated into the host genome.

Figure 4. Structure of the pBmA/Pur A3 cytoplasmic actin based plasmid vector.

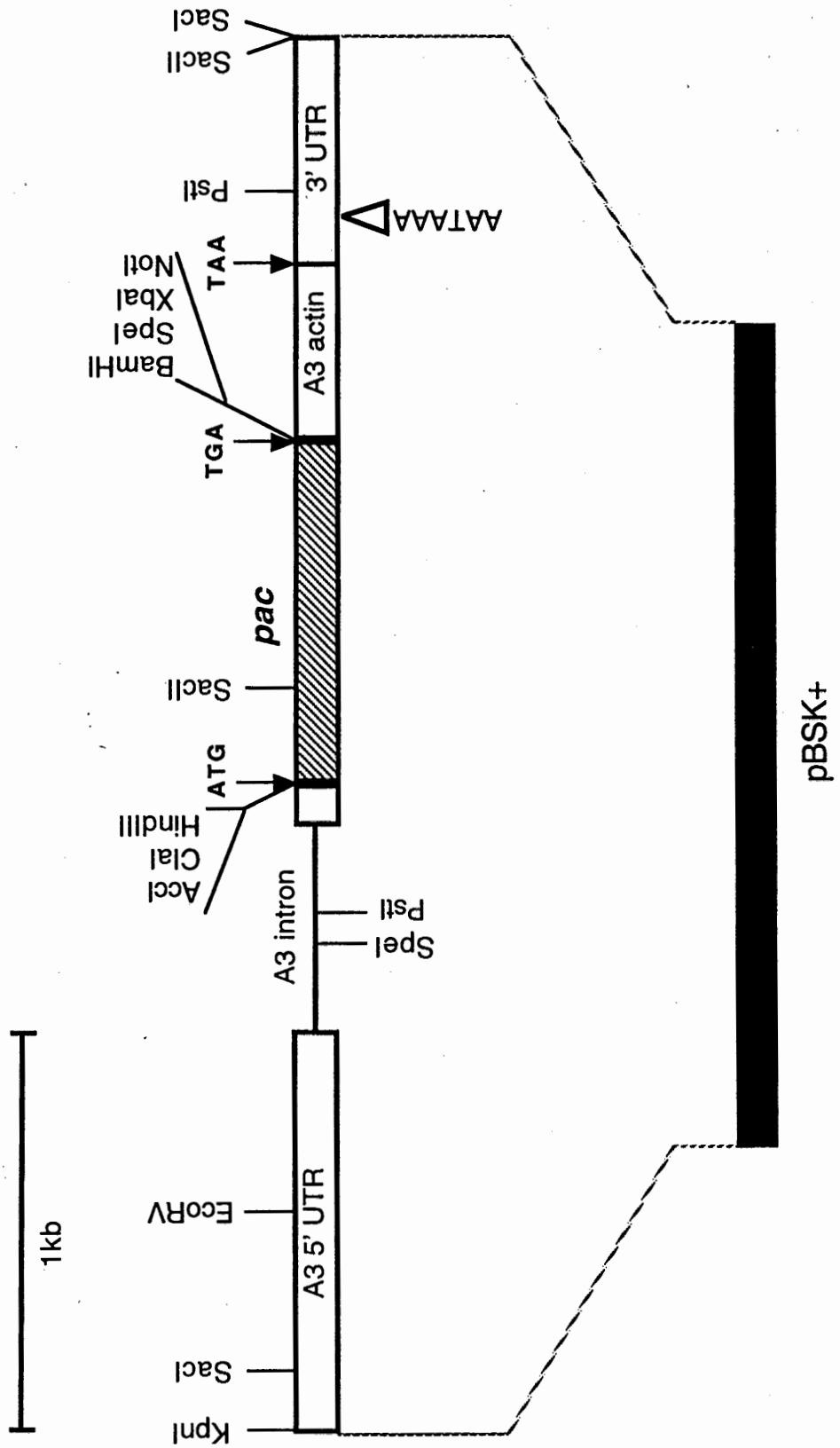

SacI
SacII
PstI
AATAAA
3' UTR
TAA
NotI
XbaI
SpeI
BamHI
A3 actin
TGA
pac
SacII
ATG
HindIII
ClaI
AccI
A3 intron
PstI
SpeI
pBSK+
1kb
EcoRV
A3 5' UTR
SacI
KpnI

Figure 5. Southern analysis of the puromycin resistant *Bombyx mori* cell line (BAT). Genomic DNA of transformed cell line was digested with the indicated restriction endonucleases and probed with ^{32}P labeled *pac* gene specific PCR fragment. Genomic DNA prepared from non-transformed BmN cells was used as a negative control. pBmA/Pur plasmid DNA digested with the indicated enzymes served as a positive control.

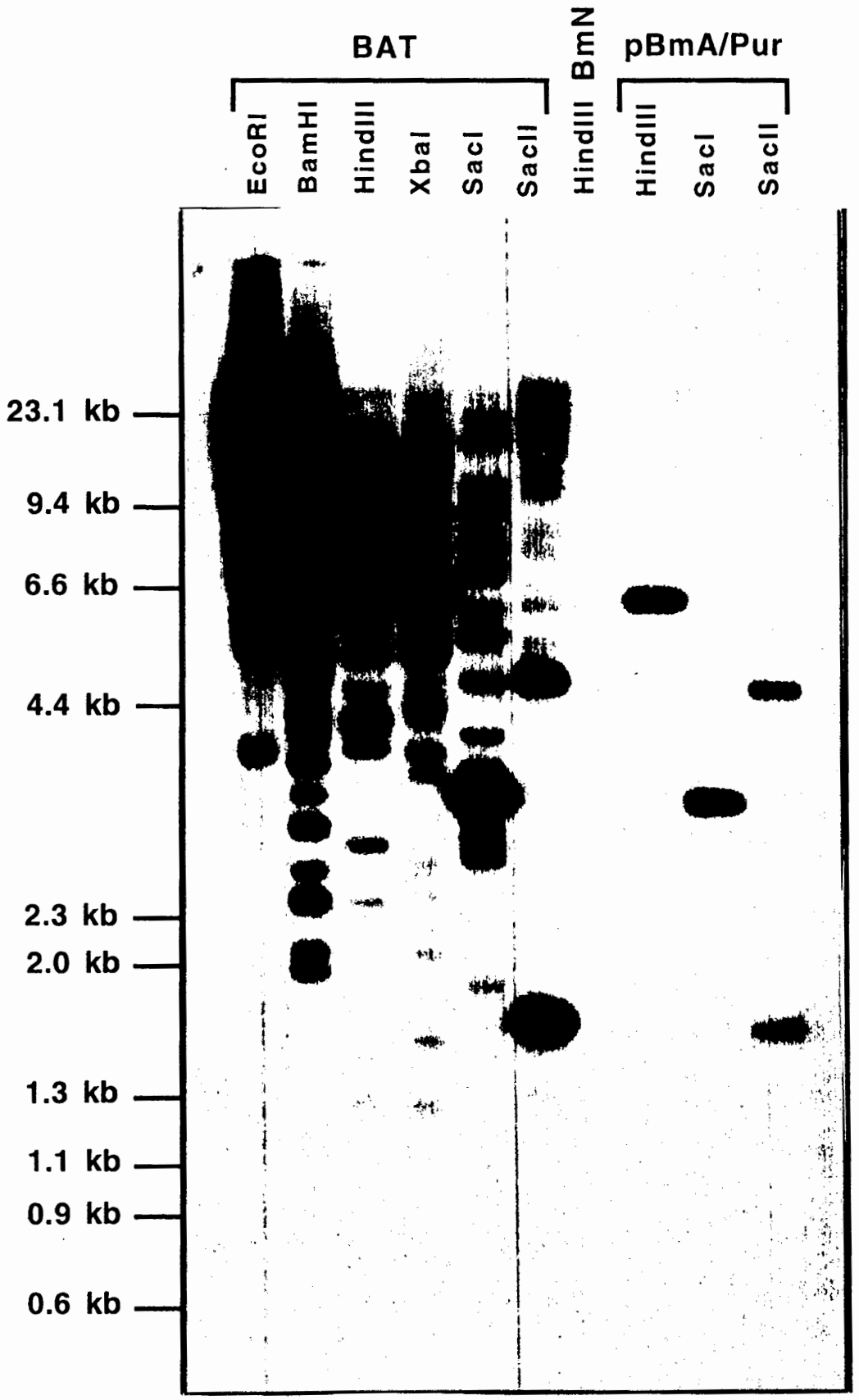
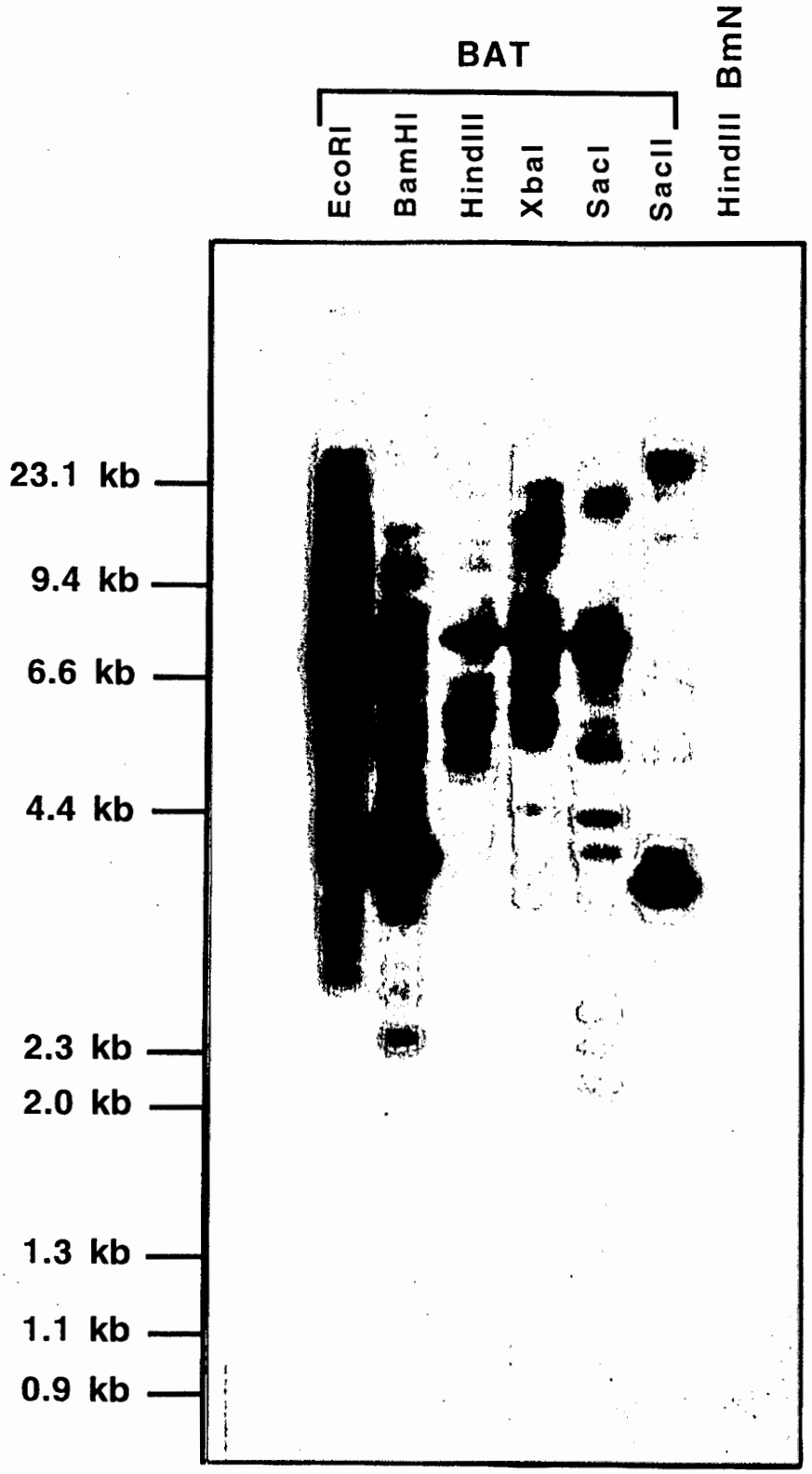

BAT
HindIII BmN
pBmA/Pur
EcoRI
BamHI
HindIII
XbaI
SacI
SacII
HindIII
SacI
SacII
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
1.3 kb
1.1 kb
0.9 kb
0.6 kb

Figure 6. Detection of the presence of the non-selected pTK-β plasmid in the stably transformed *Bombyx mori* cell line by Southern hybridization. ^{32}P labeled β–galactosidase gene specific PCR fragment was used as a probe.


BAT
EcoRI
BamHI
HindIII
XbaI
SacI
SacII
HindIII BmN
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
1.3 kb
1.1 kb
0.9 kb

By using a biotin-labeled *pac* gene probe, a strong hybridization signal was detected on one chromosome in the majority of the cells examined (Figure 7). Occasionally, spreads with two or more chromosomes exhibiting strong hybridization signals were also observed. The overall higher chromosome number however, indicated that the cells represented by these chromosome spreads are polyploids, spontaneously generated from the original transformed cell line. In summary, our data confirm that at least a portion of the transfected DNA resides within the host chromosomes.

2. Expression of the pac gene in the stably transformed insect cell line

To demonstrate the expression of the selected *pac* gene, poly-A^+ RNA purified from the transformed insect cell line was used in Northern analysis. The radiolabeled *pac* gene probe detected an abundant 1.8 kb transcript (Figure 8). This transcript corresponds to the full-sized *pac* mRNA. 3' RACE indicated that the poly-A tail was attached to this transcript at the earlier identified polyadenylation site of the A3 actin gene (241 bp downstream from the TAA codon) (Mounier and Prudhomme 1991). The structures of the minor RNA species detected at lower molecular weight were also analyzed by 3' RACE. By using this PCR technique we could recover two cDNA molecules which corresponded to rearranged *pac* transcripts (Figure 9).

Figure 7. *In situ* hybridization on metaphase chromosomes of the stably transformed *Bombyx mori* cell line. Biotinylated *pac* gene specific PCR fragment was used as a probe. In the majority of the cells a strong hybridization signal could be detected on a medium sized chromosome.

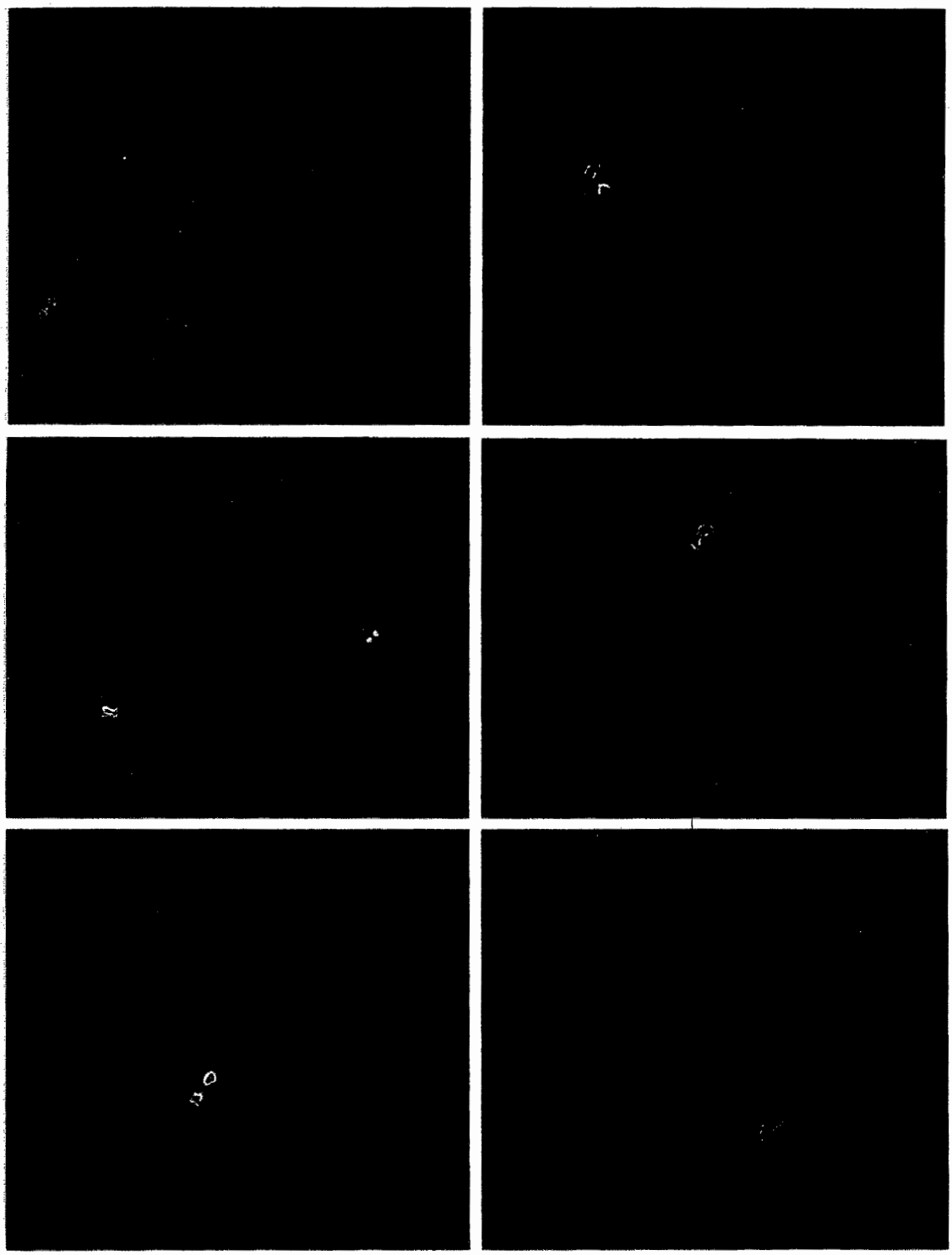


Figure 8. Analysis of the expression of the *pac* gene in the stably transformed *Bombyx mori* cell line by Northern hybridization. Poly-A^+ RNA prepared from the transformed cell line was separated on a 1% agarose formaldehyde gel and probed with ^{32}P labeled *pac* gene specific PCR fragment.

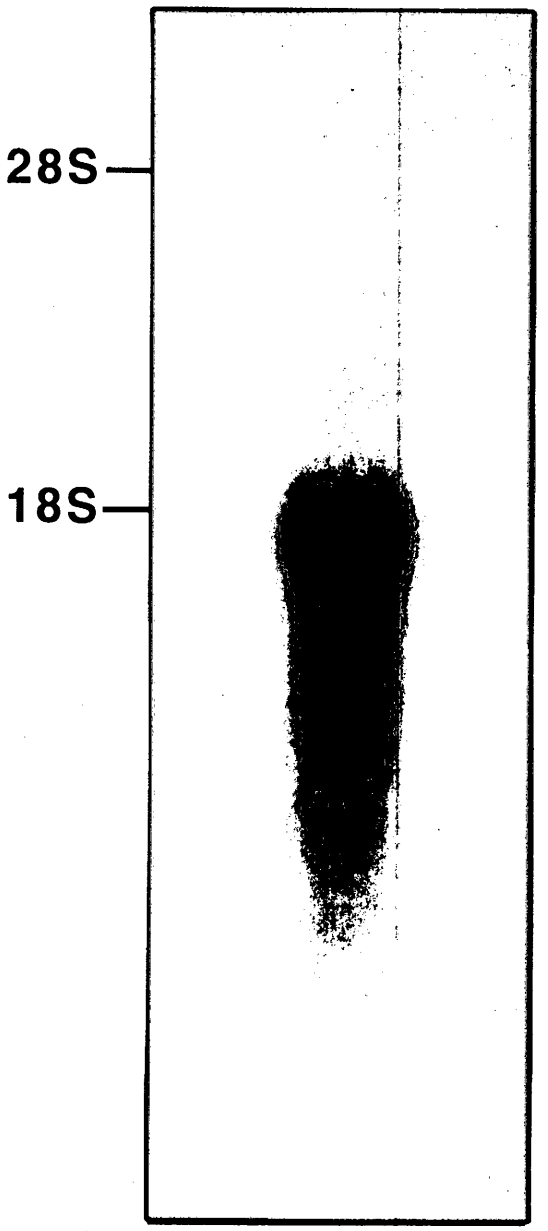
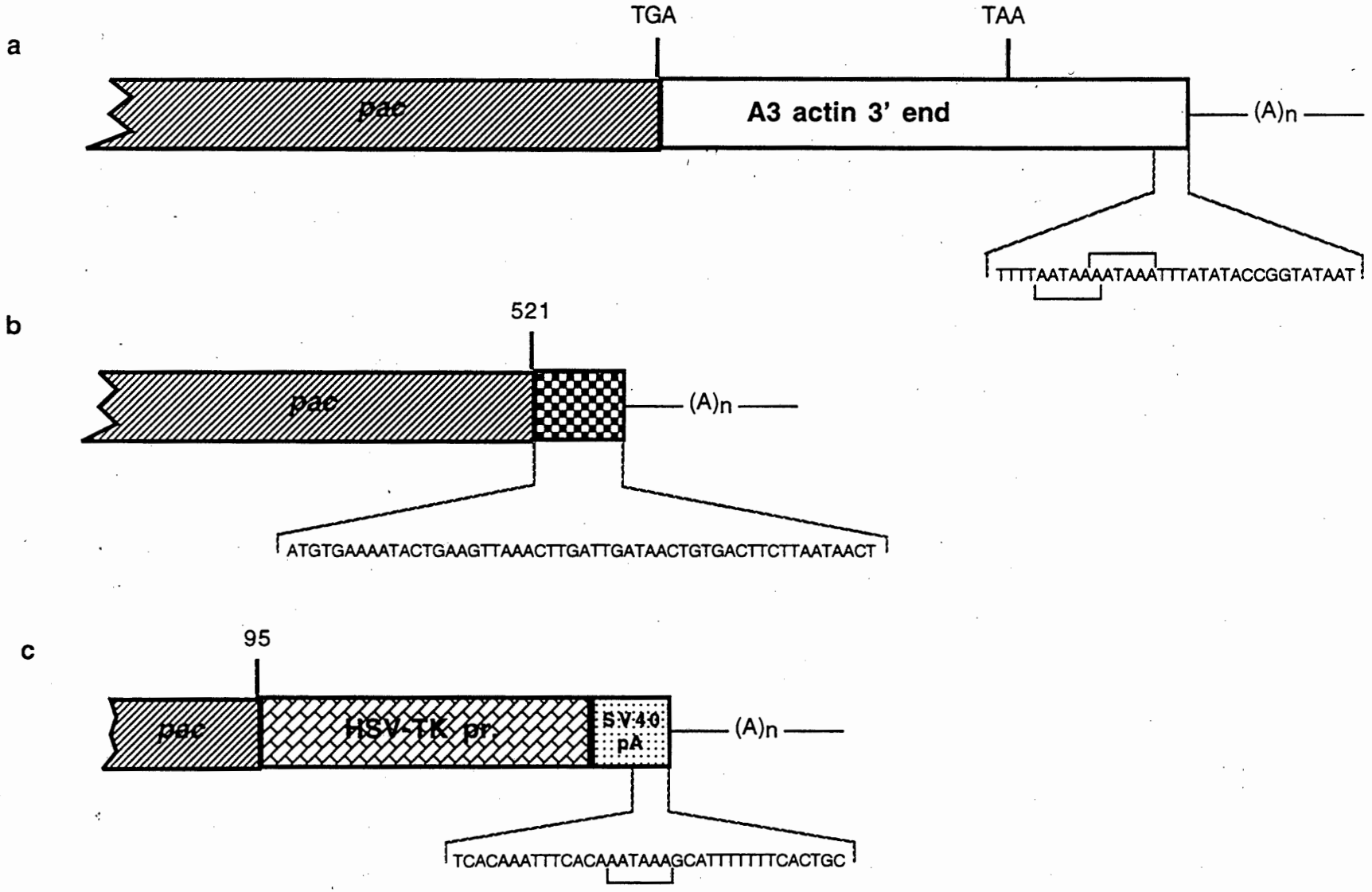

28S
18S

Figure 9. Analysis of the 3' end of the *pac* gene specific mRNA molecules by 3'RACE. (**a**) Structure of the major mRNA variant expressed in the stably transformed *Bombyx mori* cell line. In this mRNA species the polyadenylation occurs at the A3 actin specific polyadenylation signal. (**b, c**) Structures of the 3' ends of the minor *pac* mRNA species detected in the transformed insect cell line. In one of the rearranged transcripts no consensus polyadenylation signal was observed while in the other the poly-A tail attachment took place at the SV40 polyadenylation signal. The consensus polyadenylation signals are bracketed.

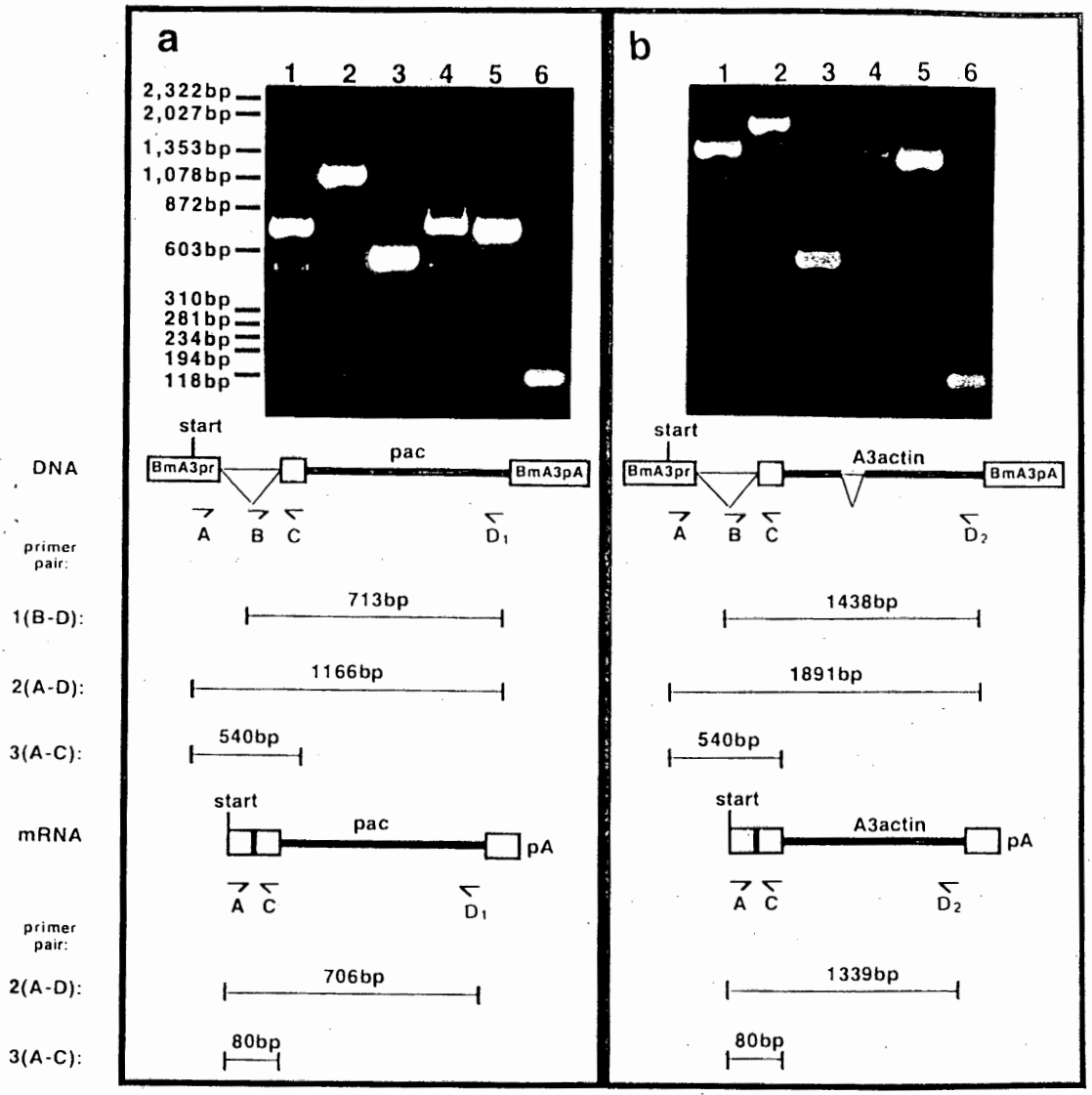

a
TGA
TAA
pac
A3 actin 3' end
(A)n
TTTTAATAAAATAAATTTATATACCGGTATAAT
b
521
pac
(A)n
ATGTGAAAATACTGAAGTTAAACTTGATTGATAACTGTGACTTCTTAATAACT
c
95
pac
HSV-TK pr.
SV40 pA
(A)n
TCACAAATTTCACAAATAAAGCATTTTTTTCACTGC

In one of these cDNAs, the last 79 bp of the *pac* coding sequence and the 3' end of the A3 actin gene (~690 bp) were replaced by a 52 bp long DNA segment. Although the RNA molecule represented by this cDNA was apparently polyadenylated, consensus polyadenylation signal was not found at the 3' end of this transcript. The RNA represented by the second recovered cDNA molecule is transcribed from a transcription unit which is a result of a complex rearrangement between the transfected pBmA/Pur and pTK-β plasmids. In this transcript, the site of polyadenylation was determined by the SV40 polyadenylation signal. This observation is in agreement with previous data that the SV40 polyadenylation signal can be utilized by insect cells (Angelichio et al. 1991).

3. Transcription initiation within the first intron of the A3 actin gene

The pBmA/Pur plasmid contains the first, 460 bp long intron of the A3 actin gene. We studied the excision of this intron from the *pac* mRNA in the transformed *B. mori* cell line by RT-PCR. Poly-A^+ RNA purified from the transformed cell line served as a template for reverse transcription. Three sets of primers were used to study the structure of the *pac* mRNA (Figure 10a). First we used an intron specific forward primer (primer B) in combination with a *pac* gene specific reverse primer (primer D_1). In the PCR reaction, using genomic DNA as a template, this primer pair is predicted to produce a DNA fragment that starts within the intron and ends within the *pac* coding region.

Figure 10. Analysis of the structure of the *pac* (**a**) and the A3 actin (**b**) mRNAs by RT-PCR. See details in the text.


a
b
1 2 3 4 5 6
2,322bp
2,027bp
1,353bp
1,078bp
872bp
603bp
310bp
281bp
234bp
194bp
118bp
DNA
start
BmA3pr
pac
BmA3pA
A3actin
primer pair:
1(B-D):
2(A-D):
3(A-C):
713bp
1166bp
540bp
1438bp
1891bp
mRNA
pA
706bp
80bp
1339bp

With the use of this primer pair, on the cDNA template there should be no product, if the splicing is complete. By using genomic DNA as a template the expected fragment was amplified (Figure 10a, lane 1). Unexpectedly however, in RT-PCR reaction the same DNA fragment was also detected (Figure 10a, lane 4). This result might mean that the removal of the intron from the mRNA is incomplete, or it could also indicate that there is an alternative trancriptional start site in the intron itself. To decide between these two possibilities, we replaced the intron-specific forward primer with a primer which precedes the 5' end of the intron (primer A). If the splicing is incomplete, this primer, in combination with the *pac* gene-specific reverse primer used above, should produce two products, an intron containing and an intron-less DNA fragment in RT-PCR. On the other hand if the intron removal is complete, there should be only one intron-less RT-PCR product. The results of this experiment showed one, intron lacking DNA fragment (Figure 10a, lane 5). The correct splicing of the intron was confirmed by the sequence analyses of the RT-PCR product. To further confirm complete removal of the first A3 actin intron from the *pac* mRNA, a third RT-PCR reaction was carried out using two intron-flanking primers. In agreement with the previous results, only an 80 bp long intron-less RT-PCR fragment was detected (Figure 10a, lane 6).

In a next set of experiments the *pac* gene-specific reverse primer was replaced with an A3 actin gene-specific primer and the equivalent PCR and RT-PCR reactions were carried out using templates prepared from non-transformed BmN cells. These experiments provided similar results to those discussed above (Figure 10b). In summary,

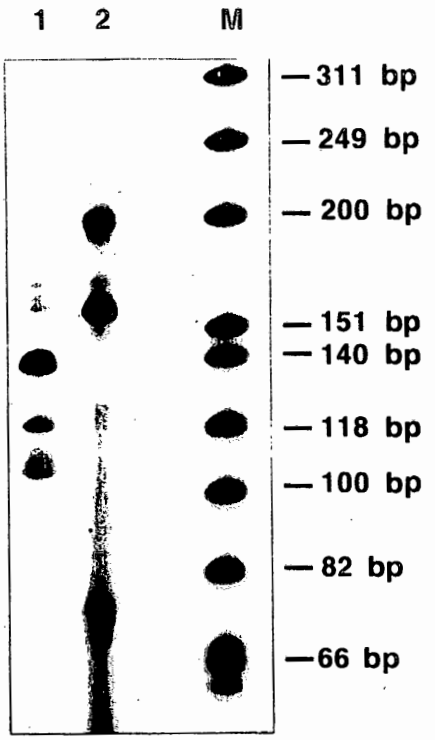
the accumulated data indicate that transcripts initiated in front of the first intron of the A3 actin are properly processed. Additionally, our results also strongly suggest that the RT-PCR product detected by the intron-specific forward primer represents alternative transcripts initiated in the first intron of the A3 actin gene.

4. The A3 actin gene has two major transcription initiation sites in front of the first intron

Transcriptional start sites within the A3 actin promoter were analyzed by two different methods. Primer extension experiments were carried out on total RNA template purified from the stably transfected BmN cell line. By using a primer specific for the cytoplasmic actin genes of *B. mori,* multiple bands were detected (Figure 11a, lane 1). The smallest specific band (~108 bp) represents transcripts initiated around the earlier identified start site of the A3 actin mRNA (69 bp upstream from the first exon-intron junction). The remaining bands were difficult to interpret although most likely many of them represent A4 cytoplasmic actin-specific transcripts. Previously, it has been reported that the A4 actin gene has two alternative promoters (Mange et al. 1996). One of them does not have a TATA-box, therefore transcription initiation can occur at multiple sites at this promoter (Smale 1997). To identify those bands which represent 5' ends of transcripts initiated at the A3 actin promoter, a *pac* gene-specific primer was used for primer extension. By using this primer, three major bands were detected (Figure 11a, lane 2).

Figure 11. Primer extension analysis of the 5' termini of the cytoplasmic actin and *pac* gene transcripts in *B. mori* cells (**a**). The following primers were used: lane 1. cytoplasmic actin specific primer (BMA3R); lane 2. *pac* gene specific primer (PUR1R). Lane M: HinfI digested ΦX174 DNA marker. The positions of the transcriptional start sites were determined by 5' RACE (**b**). The identified start sites are underlined. If the cDNA ended near a T residue the exact position of the start site could not be determined. In such cases the first residue preceding the poly-T tract was underlined. The A residue, suggested earlier as the transcriptional start site is boxed. Alignment of the two major transcriptional start regions of the A3 actin gene (**c**).

a



b

GTGATAACGGCTCTTTTATATAGTTTATCCTCACGAGTCGGTTCTCATTTACTAAGGTGT

c

upstream initiation region: CGG--CTCtTTTA-TAtAG

downstream initiation region: CGGttCTCaTTTAcTA-AG

The smallest one (~75 bp) most likely represents a reverse transcription artifact because no corresponding band was detected by the actin gene-specific primer. The band at ~160 bp is equivalent to the 108 bp fragment detected by the actin primer. The largest band at ~200 bp indicated a second transcriptional start site. The existence of this upstream, earlier undetected initiation site was confirmed by 5' RACE. The end points of the recovered cDNAs clustered in two, well separated regions of the A3 promoter (Figure 11b). The first, downstream initiation region includes the A residue identified earlier as the transcriptional start site. The second initiation region is located ~35 bp upstream from the first one. Interestingly, this DNA segment shows a significant sequence homology to the downstream initiation region (Figure 11c). The use of this upstream initiation site was also demonstrated in non-transformed BmN cells by RT-PCR. A forward primer located between the two initiation sites in combination with an A3 actin gene specific reverse primer produced a strong intron-less DNA fragment. On the other hand when we used a forward primer preceding the upstream start site, no specific product was detected in RT-PCR. (data not shown). Considering the similar intensities of the two primer extension products representing the alternative leader exons, transcription starts with approximately the same frequency at both initiation regions in BmN cells.

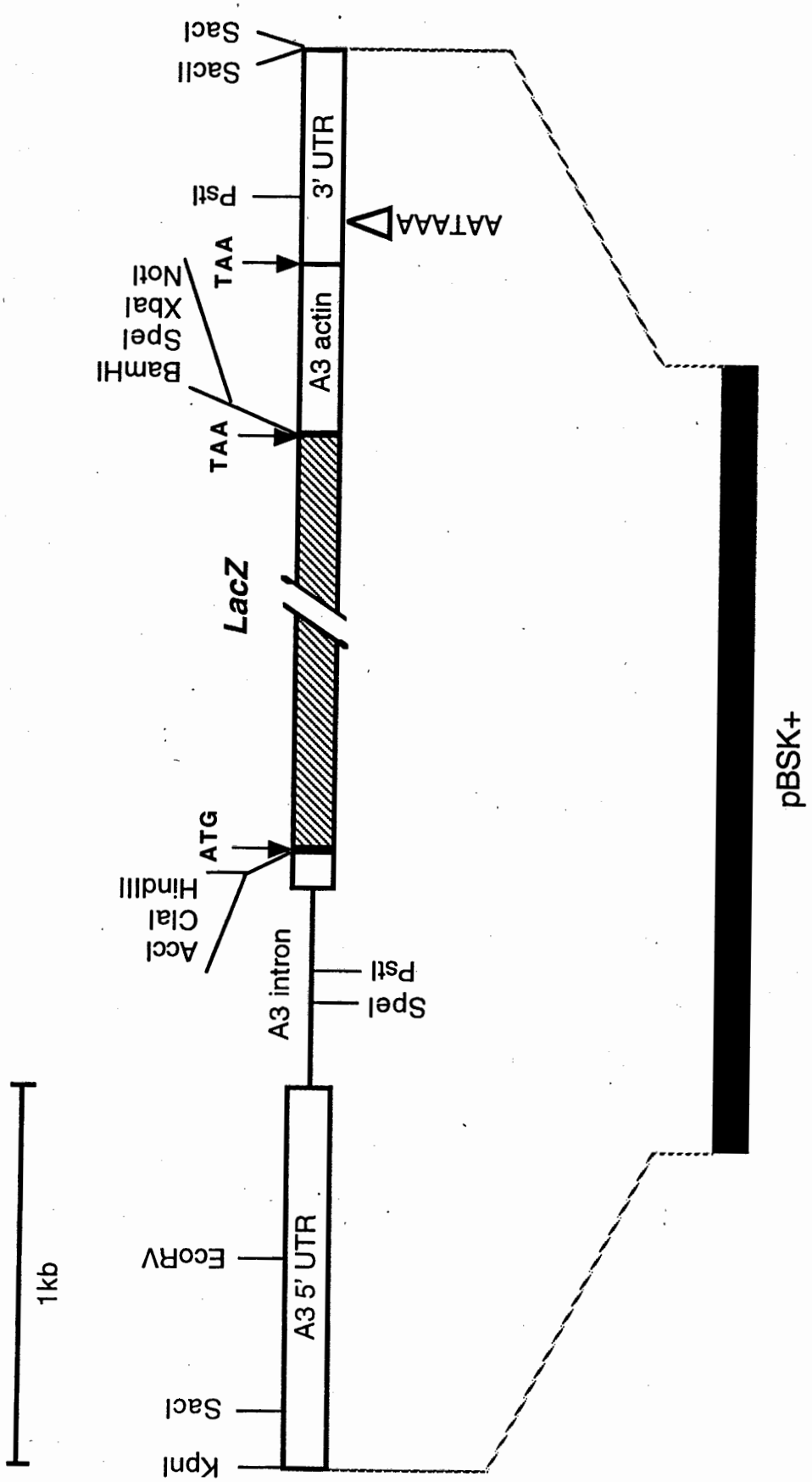
B. The use of a *Bombyx mori* A3 cytoplasmic actin based selection cassette to establish stably transformed mammalian cell lines

1. The 5' and 3' UTRs of the *B. mori* A3 cytoplasmic actin gene are functional in different vertebrate cell lines

The similarities between the *Bombyx* A3 actin promoter and the promoter region of vertebrate cytoplasmic actin genes indicated that transcriptional signals of the A3 actin might function in vertebrate cells. To examine this question, we constructed a reporter plasmid by placing the bacterial β-galactosidase gene under control of the A3 actin 5' UTR and polyadenylation signal (Figure 12). The resulting pBL construct was subsequently introduced into different mammalian (Chinese hamster: CHO-K20, African green monkey: COS, human: 293, mouse: LMTK$^-$), avian (chicken: DT-40) and insect (*B. mori:* BmN) cell lines. In parallel, control tranfections were also carried out using pCH110 plasmid (Pharmacia) which contains the β-galactosidase gene under the transcriptional control of the SV40 early promoter and polyadenylation signal. Transient expression of the β-galactosidase gene was monitored by cytochemical staining 60 hours (*B. mori* cells) or 36 hours (all other cell lines) following transfection.

Transfection efficiencies of the mammalian cell lines were in the range of 5-20% as determined from the number of blue cells detected using the pCH110 plasmid.

Figure 12. Structure of the pBL A3 cytoplasmic actin based reporter plasmid.


SacI
SacII
PstI
3' UTR
AATAAA
TAA
NotI
XbaI
SpeI
BamHI
A3 actin
TAA
LacZ
ATG
HindIII
ClaI
AccI
A3 intron
PstI
SpeI
1kb
EcoRV
A3 5' UTR
SacI
KpnI
pBSK+

After transfection of the pBL construct 0.05-0.2% of blue cells were detected in every mammalian cell line. The activity of both pCH110 and pBL plasmids could also be demonstrated in the avian DT-40 cells (Figure 13). These results indicate that the *B. mori* A3 actin 5' UTR can drive the expression of the β-galactosidase gene in different mammalian and even in avian cells. The apparently lower transfection efficiencies obtained with pBL could be a consequence of the low activity of the *B. mori* A3 actin 5' UTR in vertebrate cells or the decreased stability of β-galactosidase mRNA due to deficient polyadenylation. To address this problem, we constructed a series of plasmid vectors and assayed them for transient β-galactosidase activity in LMTK$^-$ cells (Figure 14). The pBL plasmid showed 34.4% of the β-galactosidase activity observed after transfection of equimolar amount of pCH110. The replacement of the A3 actin 5' UTR with the SV40 early promoter (pSBL) increased the relative β-galactosidase activity to 109.2%, indicating that the insect polyadenylation signal provides approximately the same stability to the β-galactosidase mRNA in mammalian cells as the SV40 early polyadenylation signal does. The removal of the promoter from the pSBL plasmid (pΔP-BmpA/LacZ) decreased the relative β-galactosidase activity to 3% confirming that the pBluescript SK+ vector itself does not contain DNA elements which can function as promoters in mammalian cells. In summary, these results indicate that the transcriptional signals of the silkworm A3 cytoplasmic actin gene can drive the expression of the bacterial β-galactosidase gene in different vertebrate cells in transient transfection assay.

Figure 13. Analysis of the transcriptional activity of the pBL, A3 cytoplasmic actin based reporter plasmid in various vertebrate and *Bombyx mori* cells by β–galactosidase cytochemical staining. The following cell lines were used: (**a**) CHO-K20, Chinese hamster; (**b**) COS, African green monkey; (**c**) 293, human; (**d**) $LMTK^-$, mouse; (**e**) DT-40, chicken; (**f**) BmN, *Bombyx mori*.

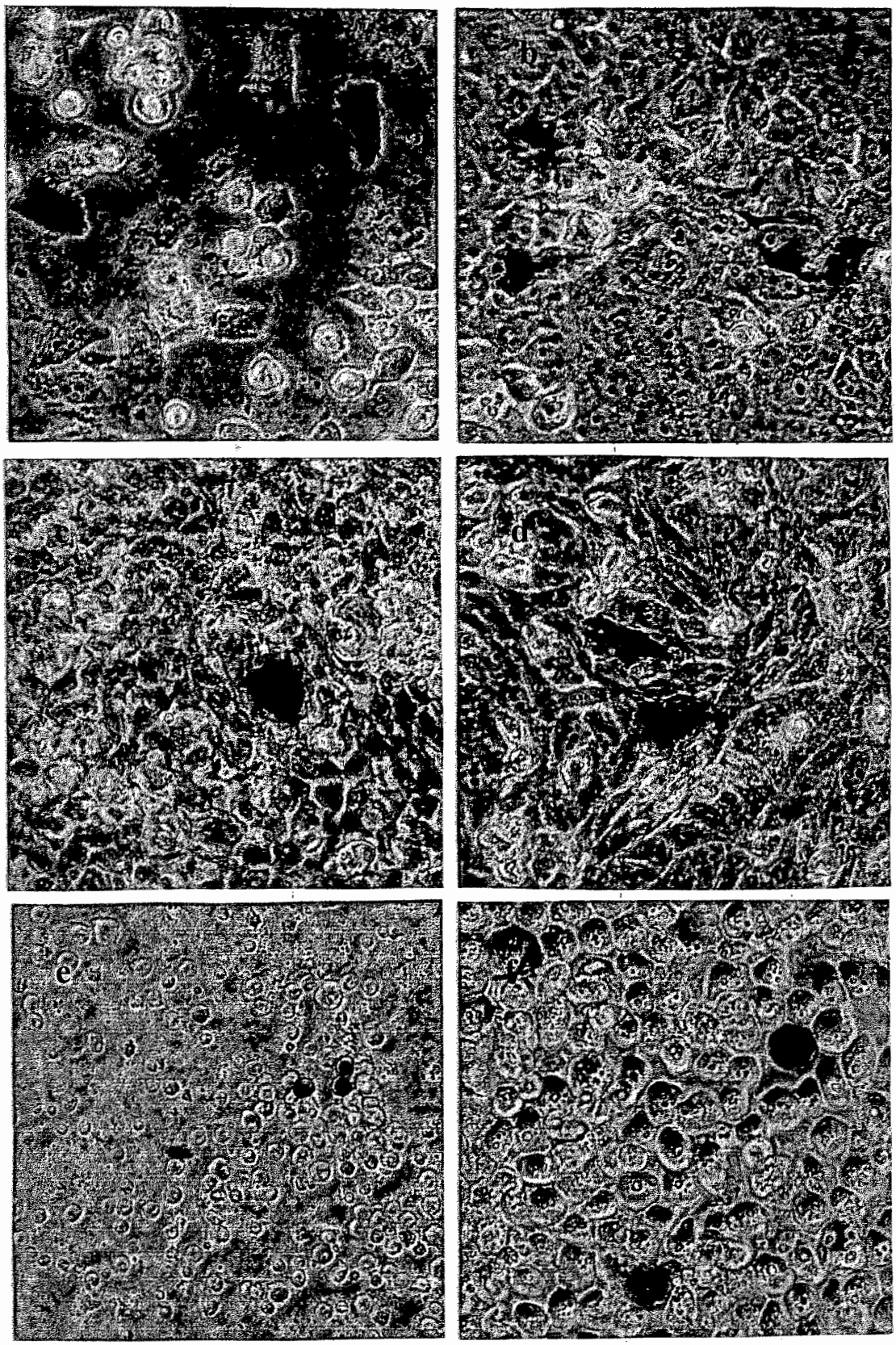
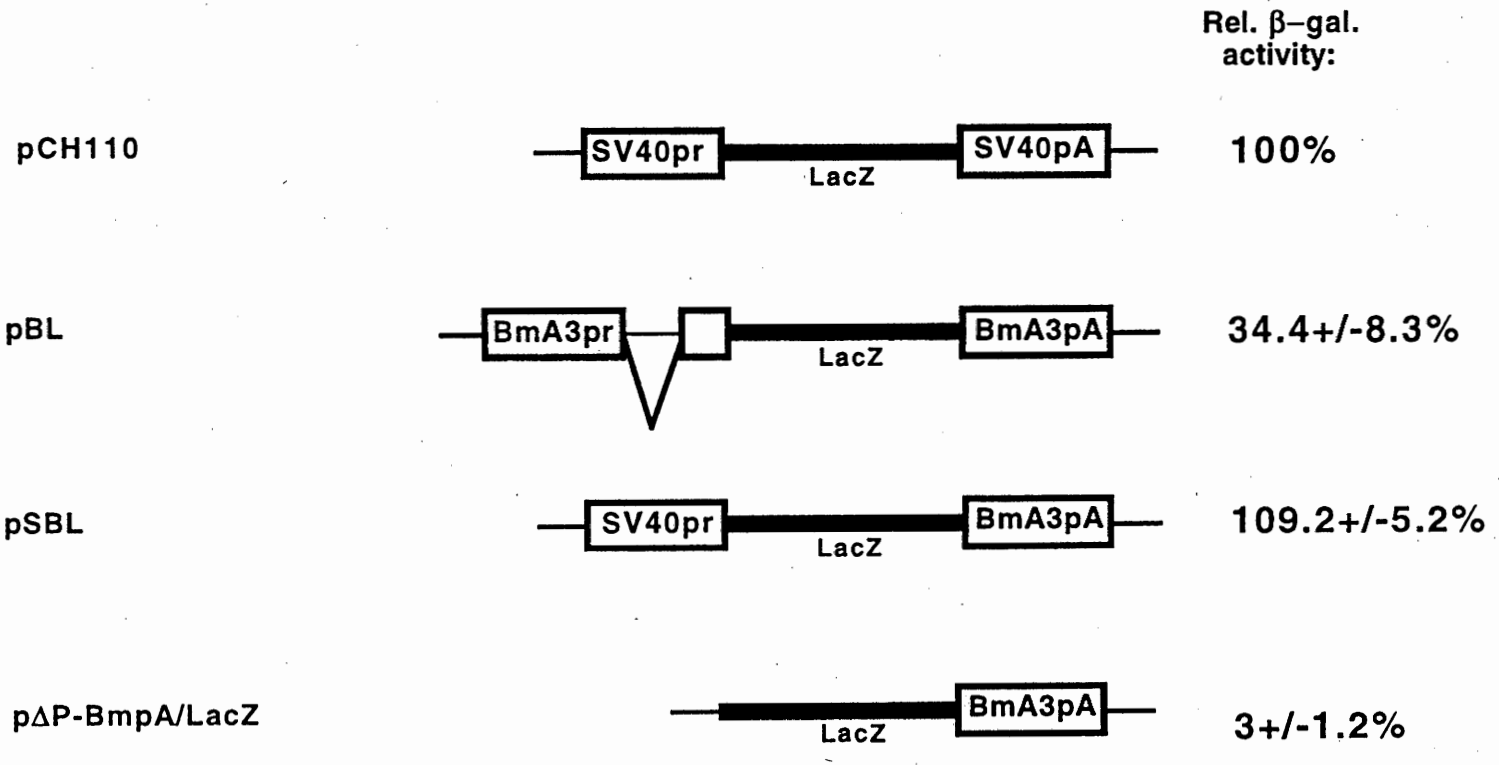

a
b
c
d
e
f

Figure 14. Analysis of the transcriptional activities of different, β–galactosidase gene containing plasmid constructs in LMTK$^-$ cells. β–galactosidase activities are presented relative to the value obtained with pCH110 plasmid construct. The β-galactosidase activities were determined three times for each plasmid construct. Standard deviations from the mean are indicated.


Rel. β–gal. activity:
pCH110
SV40pr
LacZ
SV40pA
100%
pBL
BmA3pr
LacZ
BmA3pA
34.4+/-8.3%
pSBL
SV40pr
LacZ
BmA3pA
109.2+/-5.2%
pΔP-BmpA/LacZ
LacZ
BmA3pA
3+/-1.2%

The transfection efficiency of 1-2% could be achieved in *B. mori* cells by using the pBL construct. After transfection of pCH110 plasmid into *B. mori* cells, activity of the SV40 early promoter could not be detected by β-galactosidase staining. Blue cells could not be seen even after transfection of pSBL, indicating that the lack of detectable β-galactosidase production was not due to inefficient polyadenylation.

2. Isolation of stably transformed mammalian cell lines using an A3 actin based expression vector

To answer the question of whether the transcriptional signals of the silkworm A3 cytoplasmic actin gene can function integrated into mammalian chromosomes, we examined the ability of the *Streptomyces hygroscopicus* hygromycin B phosphotransferase (*hyg*) gene (Gritz and Davies 1983) containing selection cassette of the pBmA/HmB plasmid (Figure 15) to generate stably transformed mammalian cell lines. We introduced the pBmA/HmB plasmid into LMTK$^-$ cells by calcium phosphate-DNA co-transfection. After two weeks selection in 400 μg/ml hyromycin B containing medium, 53 cell colonies were isolated. Twenty-eight randomly selected resistant cell lines were analyzed for the presence of the vector in the mouse genome by Southern blot hybridization. The agarose embedded high molecular weight genomic DNAs were digested with EcoRI restriction endonuclease that linearizes the pBmA/HmB plasmid.

Figure 15. Structure of the pBmA/HmB A3 cytoplasmic actin based plasmid vector.

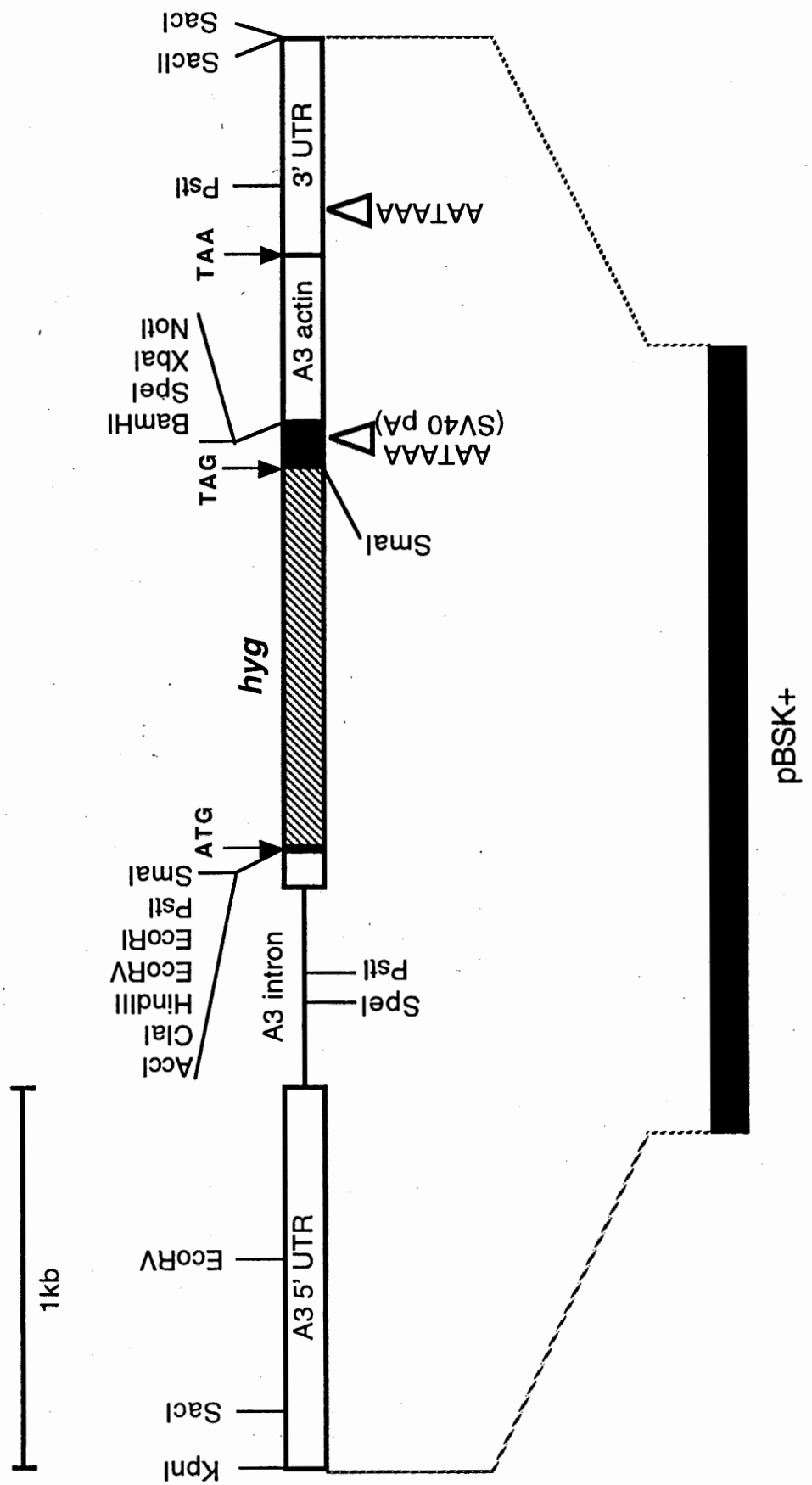
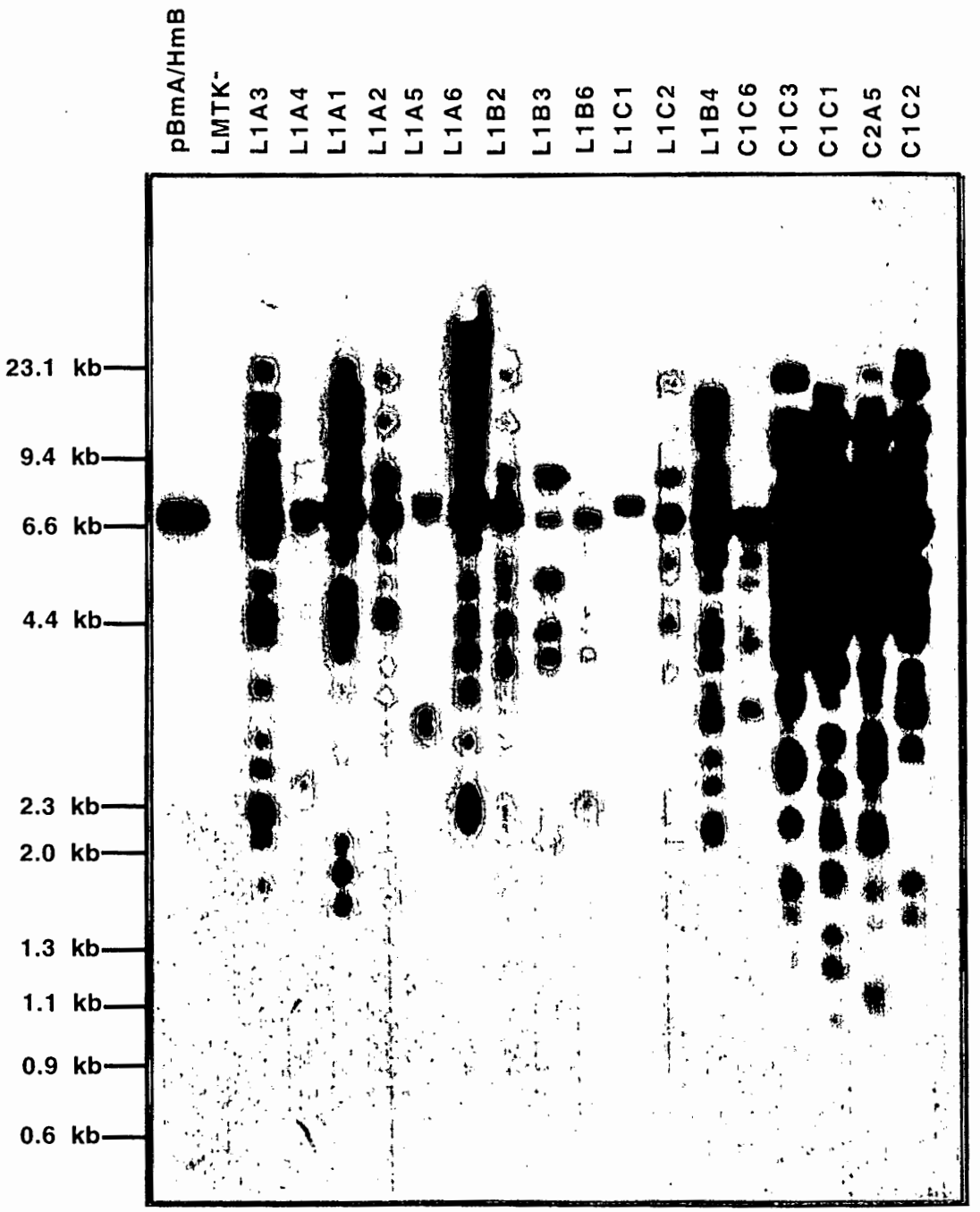

SacI
SacII
PstI
3' UTR
AATAAA
TAA
A3 actin
NotI
XbaI
SpeI
BamHI
(SV40 pA)
AATAAA
TAG
SmaI
hyg
ATG
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
AccI
A3 intron
PstI
SpeI
EcoRV
A3 5' UTR
SacI
KpnI
1kb
pBSK+

Figure 16 shows the autoradiogram of the Southern blot of 17 cell lines, obtained after hybridization with radioactivelly labeled *hyg* gene-specific PCR probe. In approximately 70% of the cell lines, more than ten different hybridizing bands could be detected. Based on the intensity of these bands we concluded that many of them represent more than one copy. A prominent 7 kb fragment co-migrating with the EcoRI linearized plasmid was detected in the majority of the cell lines. This band may correspond to head-to-tail arranged plasmid copies. In more than 30% of the analyzed cell lines the number of hybridizing bands and the intensity of these bands indicated the presence of more than 100 plasmid molecules per cell. The high copy number of the plasmid molecule may provide a selective growth advantage for the cells during hygromycin selection. In the resistant cell lines that contain no more than 5-10 plasmid molecules per cell (see figure 16, lanes 7, 11 and 12) the transfected plasmids might be integrated adjacent to strong cellular promoters or enhancers. The amplification pattern of the foreign DNA was similar to that observed in the stably transformed *B. mori* cell line (see section A.1.). It is interesting to speculate whether the high amplification rate of the introduced plasmid DNA may be due to the presence of a sequence element in the 5' or 3' regulatory regions of the A3 actin gene that can induce amplification both in mouse and *B. mori* cells.

The presence of the intact selection cassette in the transfected cell lines was examined by SacI digestion.

Figure 16. Southern analysis of the hygromycin resistant stably transformed mammalian cell lines. EcoRI digested genomic DNAs were separated on a 0.8% agarose gel and probed with ^{32}P labeled *hyg* gene specific PCR fragment. Genomic DNA isolated from non-transformed LMTK$^-$ mouse cells served as a negative control. EcoRI linearized pBmA/HmB plasmid DNA was used as a positive control. The amount of plasmid DNA used was equivalent with 20 plasmid copies per diploid mouse genome.


pBmA/HmB
LMTK-
L1A3
L1A4
L1A1
L1A2
L1A5
L1A6
L1B2
L1B3
L1B6
L1C1
L1C2
L1B4
C1C6
C1C3
C1C1
C2A5
C1C2
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
1.3 kb
1.1 kb
0.9 kb
0.6 kb

In 14 out of 16 randomly selected cell lines the *hyg* gene specific probe detected a major 3.8 kb SacI fragment that corresponded to the similar sized Sac I fragment of the pBmA/HmB plasmid, containing the intact selection cassette (data not shown).

The physical condition of the plasmid molecules in the transformant cell lines was analyzed by pulsed field gel electrophoresis and *in situ* hybridization. Undigested, agarose embedded, high molecular weight genomic DNAs of 17 randomly selected hygromycin resistant LMTK$^-$ cell lines were separated by pulsed field gel electrophoresis under electrophoretic conditions which allowed separation of DNA molecules up to ~1.5 Mb. Hybridization signals could not be detected with the *hyg* specific probe except at the start positions of the gel, suggesting that no free plasmid exists in the transformed mouse cells (Figure 17). The results of the *in situ* hybridizations were in agreement with these data. In the analyzed cell lines, hybridization signals were detected on different mouse chromosomes, indicating that the foreign DNA integrated into the mouse genome (Figure 18).

To test the stability of the transfected DNA, one of the hygromycin resistant LMTK$^-$ cell lines (L1C2, see Figure 16 lane 13) was cultured in non-selective medium and later on the plating efficiency was determined (relative number of colonies obtained in the presence and in the absence of selection). After 4 months (~30 passages) the cell line still showed high plating efficiency (76.8%), confirming that the hygromycin resistance of the cells was due to plasmid molecules integrated stably into the genome.

Figure 17. Analysis of the hygromycin resistant stably transformed mammalian cell lines by pulsed field gel electrophoresis and Southern hybridization. ^{32}P labeled *hyg* gene specific PCR fragment was used as a probe. The positions of the gel well and the circular plasmid are indicated by arrows.

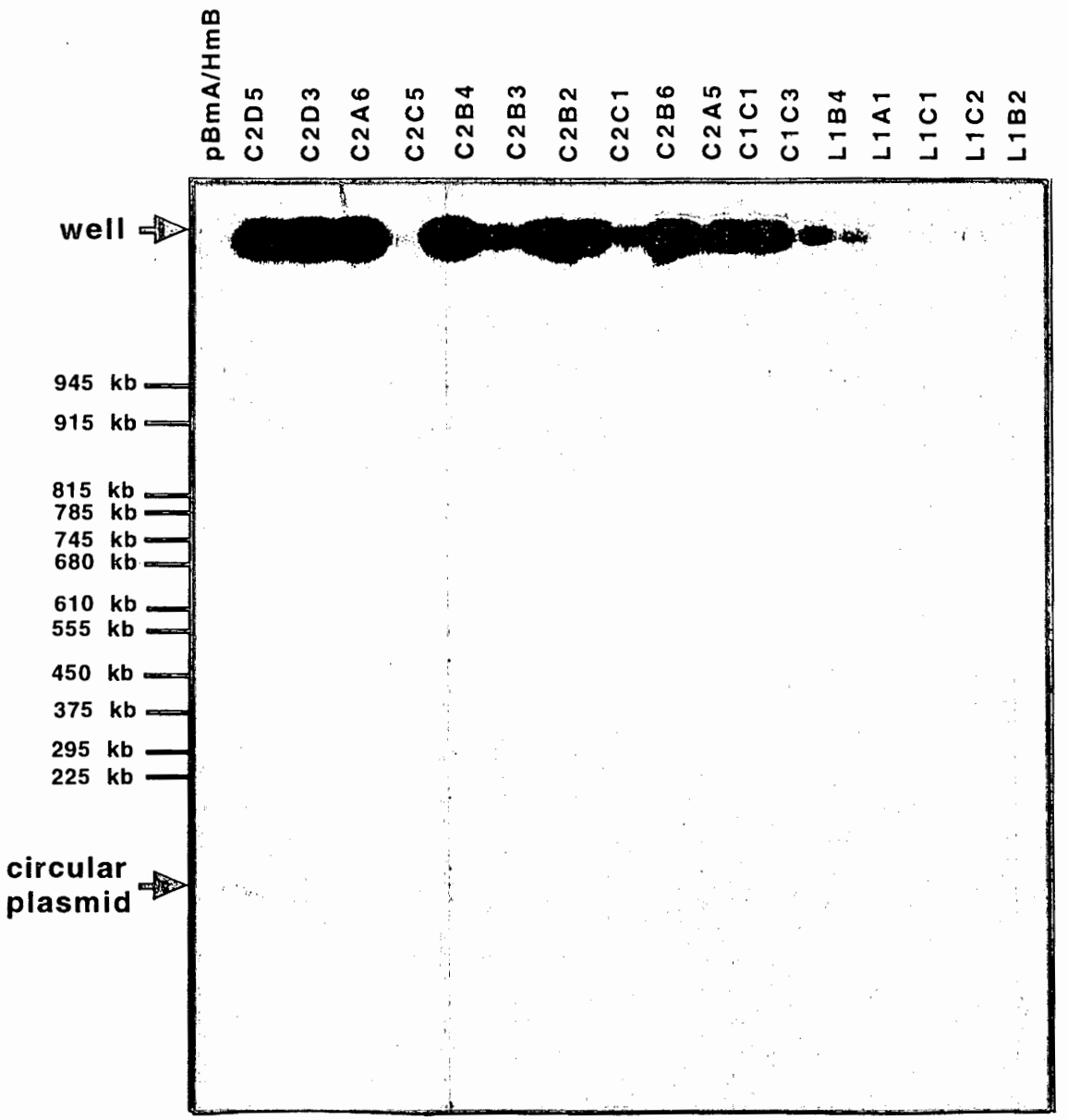
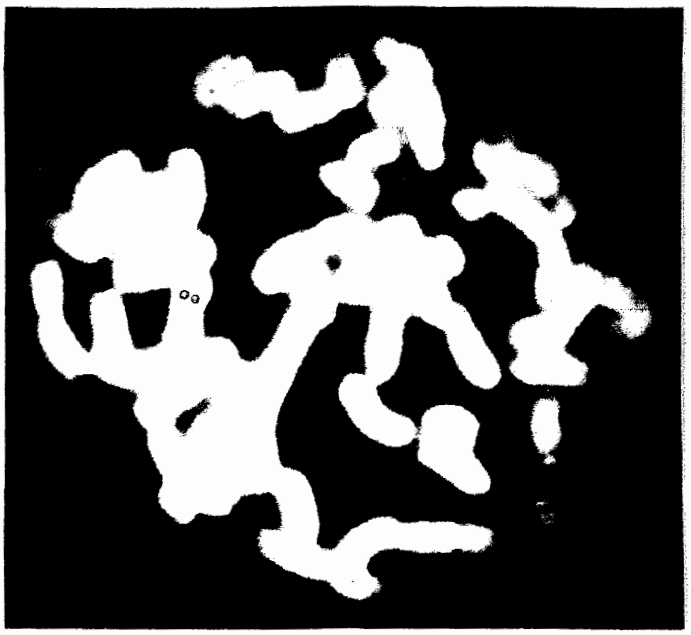

pBmA/HmB
C2D5
C2D3
C2A6
C2C5
C2B4
C2B3
C2B2
C2C1
C2B6
C2A5
C1C1
C1C3
L1B4
L1A1
L1C1
L1C2
L1B2
well
945 kb
915 kb
815 kb
785 kb
745 kb
680 kb
610 kb
555 kb
450 kb
375 kb
295 kb
225 kb
circular plasmid

Figure 18. *In situ* hybridization on the metaphase chromosomes of a stably transformed mouse cell line. Biotinylated pBmA/HmB plasmid was used as a probe. A strong hybridization signal could be detected on a mouse chromosome.



3. Expression of the hyg gene in the stably transformed mammalian cell lines

To demonstrate the expression of the selected *hyg* gene, poly-A^+ RNA purified from the transformant cell lines was used for RT-PCR and Northern analysis. Figure 19 shows the results of the RT-PCR reactions obtained on 5 randomly selected transformant cell lines. In each case the *hyg* gene-specific forward and reverse primers produced the expected 893 bp PCR fragment. In Northern analysis, three transcripts were detected with a *hyg* gene-specific probe (Figure 20). The two longer transcripts (~2.5 kb and ~1.8 kb) represent *hyg* mRNAs generated by the alternative usage of the two polyadenylation signals present in the pBmA/HmB plasmid as confirmed by 3' RACE (Figure 21). The 1.8 kb mRNA was generated by the use of the SV40 early polyadenylation signal located ~300 bp downstream from the stop codon of the *hyg* gene. On the other hand, in the 2.5 kb *hyg* transcript, the poly-A tail was attached to the mRNA ~700 bp further downstream by the use of the A3 polyadenylation signal. Interestingly, mammalian cells utilize the insect polyadenylation signal differently from insect cells. The poly-A tail attachment site is located 5 bp upstream from the position that is observed in insect cells.

The smallest transcript was difficult to interpret. This RNA species could be observed in two independent cell lines, therefore it is unlikely that it originates from truncated or rearranged *hyg* gene. The polyadenylation of many eukaryotic mRNA molecules is determined by a hexanucleotide consensus sequence, AATAAA, located 10-30 bp upstream from the poly-A tail attachment site (Brinstiel et al. 1985).

Figure 19. Demonstration of the expression of the *hyg* gene in 5 randomly selected, stably transformed mouse cell lines by RT-PCR. Various control PCR reactions were used: 1. RNA template (without cDNA synthesis); control for genomic DNA contamination. 2. cDNA template prepared from non-transformed mouse cells; control for non-specific PCR products; 3. pBmA/HmB plasmid DNA template as a positive control.

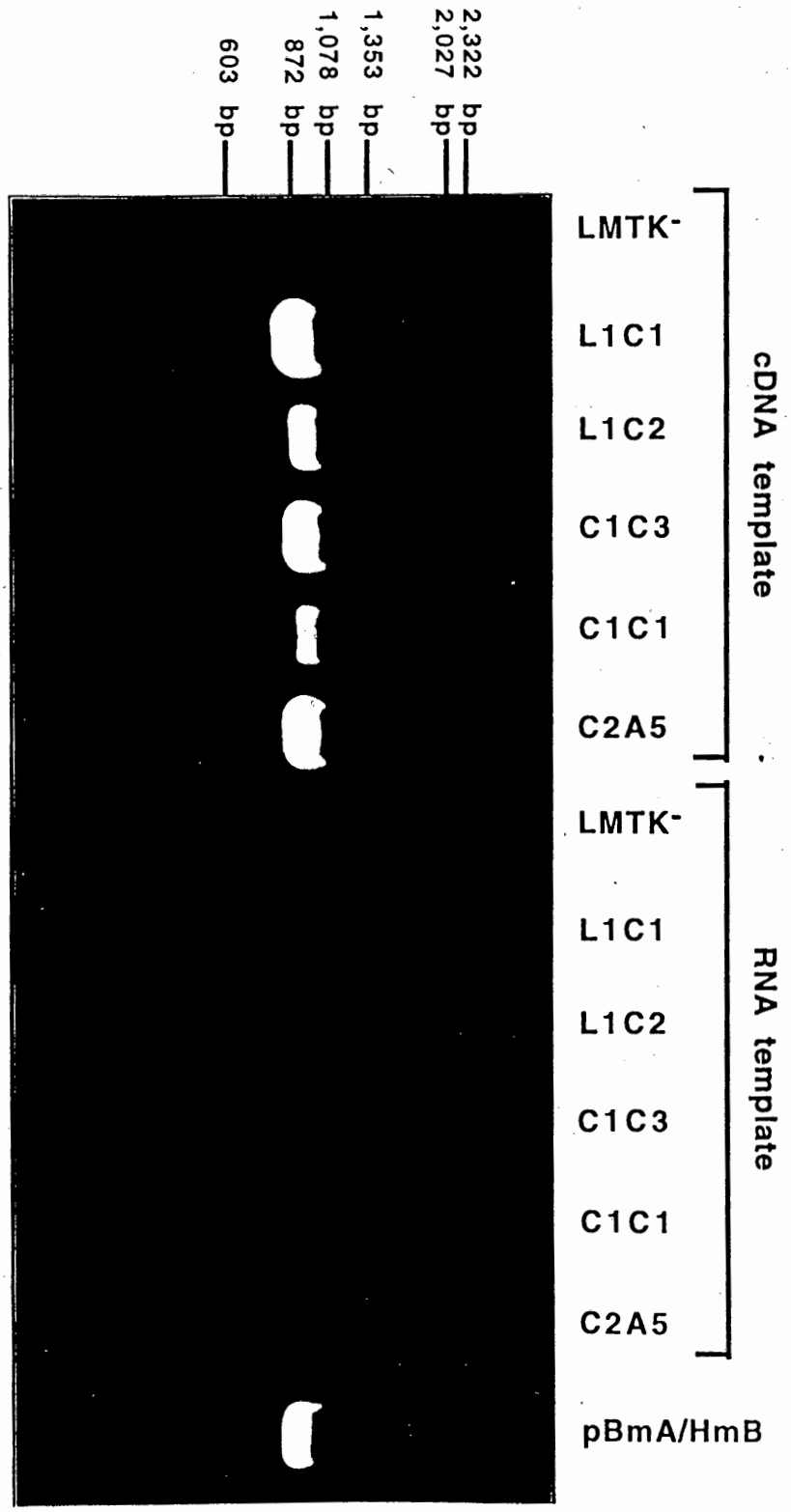

2,322 bp
2,027 bp
1,353 bp
1,078 bp
872 bp
603 bp
LMTK-
L1C1
L1C2
C1C3
C1C1
C2A5
cDNA template
LMTK-
L1C1
L1C2
C1C3
C1C1
C2A5
RNA template
pBmA/HmB

Figure 20. Expression of the *hyg* gene in the stably transformed mouse cell lines was analyzed by Northern hybridization. Poly-A^+ RNA was separated on a 1% agarose formaldehyde gel and probed with ^{32}P labeled *hyg* gene specific PCR fragment.

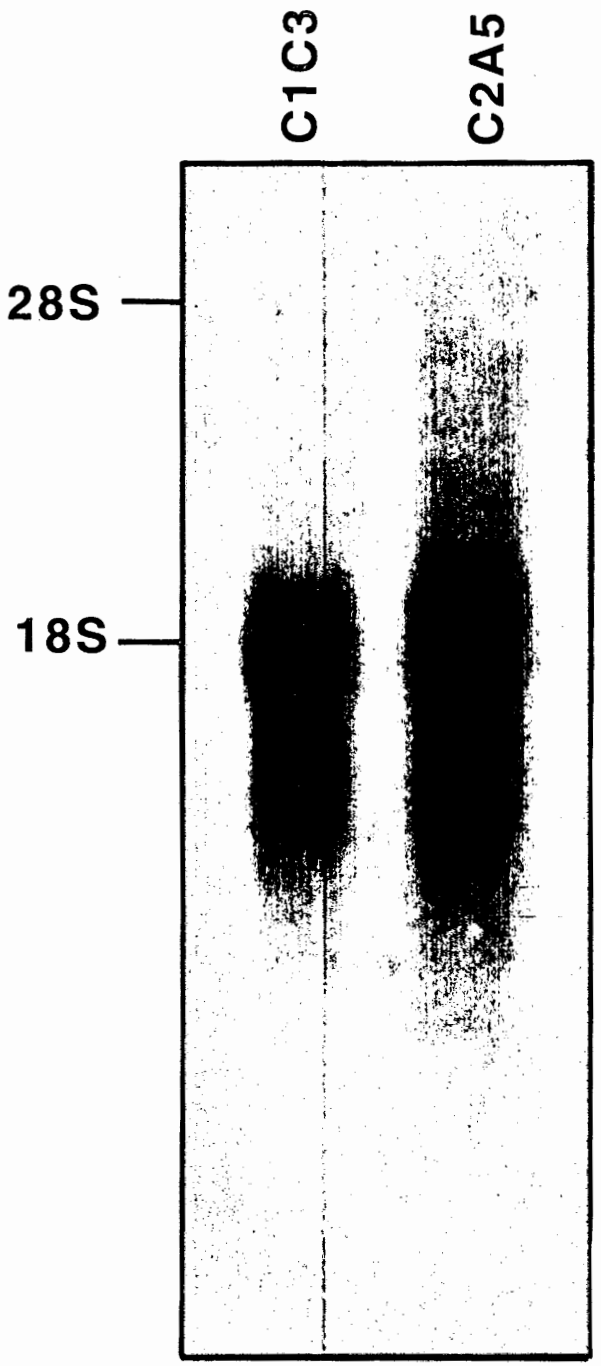
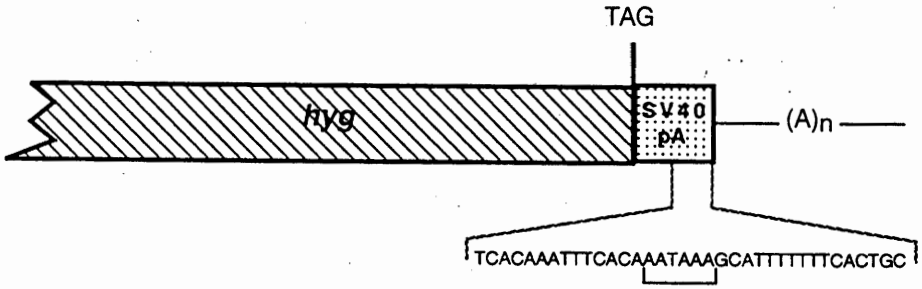
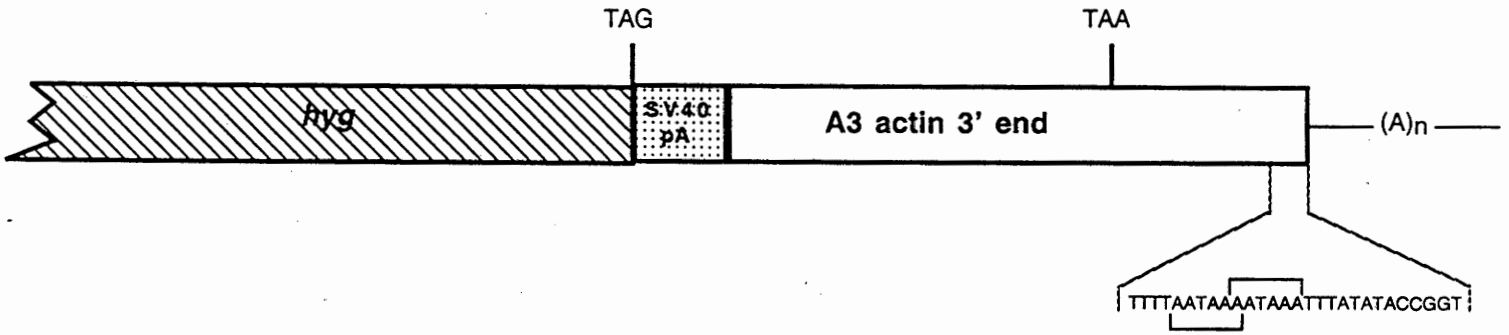

C1C3
C2A5
28S
18S

Figure 21. The structure of the 3' end of the *hyg* gene specific transcripts detected in stably transformed mammalian cells. The mammalian transcription apparatus could utilize both the insect (**b**) and the viral (**a**) polyadenylation signals. Note, that the poly-A tail attachment site at the insect polyadenylation signal is located 5 bp upstream compared to the position that was observed in insect cells (see Figure 9a). The consensus polyadenylation signals are bracketed.

a


TAG
hyg
SV40
pA
(A)n
TCACAAATTTCACAAATAAAGCATTTTTTTCACTGC

b

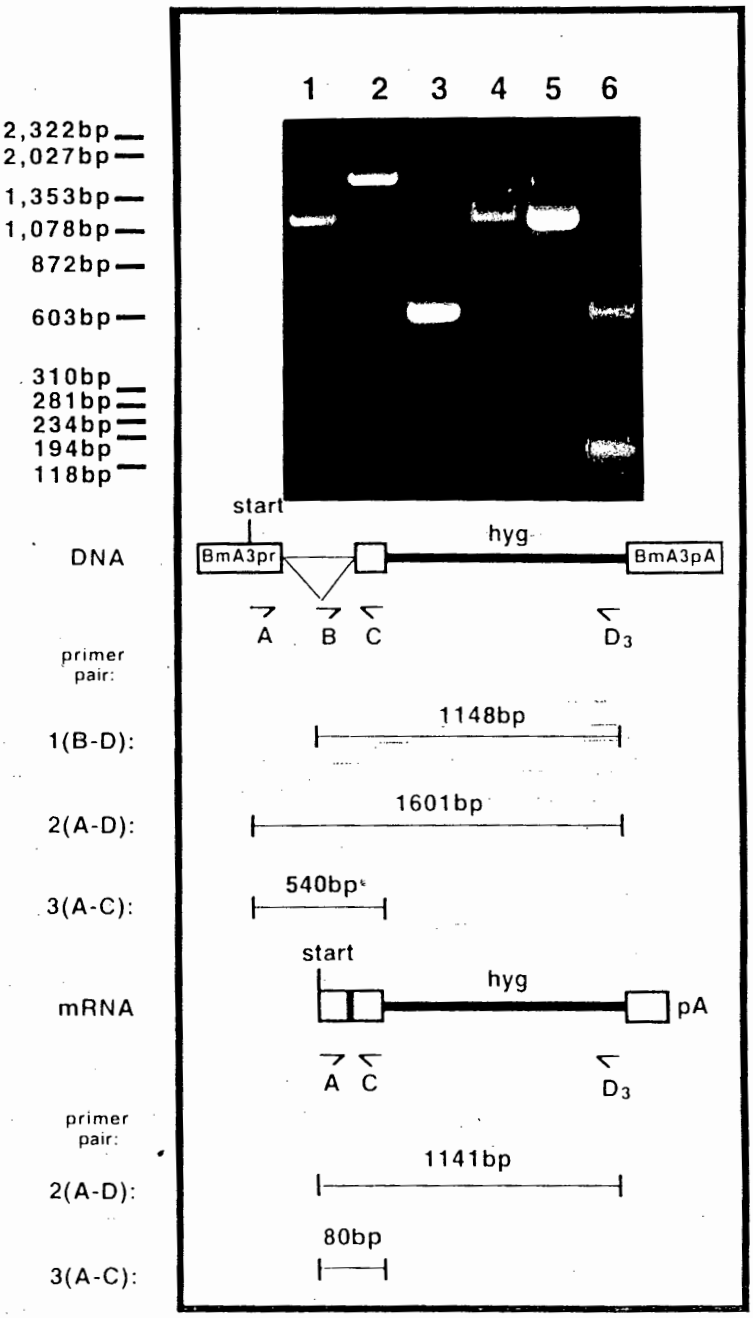

TAG
TAA
hyg
SV40
pA
A3 actin 3' end
(A)n
TTTTAATAAAATAAATTTATATACCGGT

The *hyg* coding sequence does not contain AATAAA sequence motifs that could serve as alternative sites for polyadenylation and would result in an even shorter transcript than the two mRNAs discussed above. Therefore, the smallest band may represent an mRNA species that is synthesized under control of a cryptic promoter residing within the *hyg* coding region and polyadenylated by the use of one of the polyadenylation signals identified. This assumption would also explain the result that we recovered only two RNA species by 3' RACE ended near the SV40 or the A3 polyadenylation signals.

The pBmA/HmB plasmid, similar to pBmA/Pur, contains the first intron of the A3 actin preceding the ATG start codon of the *hyg* gene. We studied the removal of this intron from the *hyg* mRNA in the transformed mammalian cell lines by RT-PCR. We performed the equivalent PCR and RT-PCR reactions that were used for the characterization of the *pac* mRNA expressed by the stably transformed *B. mori* cell line. In these reactions however a *hyg* gene-specific reverse primer was used instead of a *pac* gene-specific primer (Figure 22).

The intron-specific forward primer (primer B), in combination with the *hyg* gene specific reverse primer (primer D_3), produced the same 1148 bp DNA fragment both in PCR and RT-PCR reactions (Figure 22, lane 1, 4). By using the A-D_3 primer pair, an intense RT-PCR fragment corresponding to the intron-less *hyg* mRNA (as confirmed by sequencing) and a minor, intron containing product were observed (Figure 22, lane 5).

Figure 22. RT-PCR analysis of the structure of the *hyg* gene specific transcripts expressed in a stably transformed mammalian cell line. See details in the text.


1 2 3 4 5 6
2,322bp
2,027bp
1,353bp
1,078bp
872bp
603bp
310bp
281bp
234bp
194bp
118bp
start
hyg
DNA
BmA3pr
BmA3pA
A B C
D3
primer pair:
1(B-D):
1148bp
2(A-D):
1601bp
3(A-C):
540bp
start
hyg
mRNA
pA
A C
D3
primer pair:
2(A-D):
1141bp
3(A-C):
80bp

In contrast to the stably transformed insect cell line, in mammalian cells two bands were detected by RT-PCR using the two intron flanking primers (primers A and C, Figure 22, lane 6). The major 80 bp product derived from processed mRNA. The size of the larger fragment was the same as the size of the intron containing PCR fragment obtained by using genomic DNA as a template (Figure 22, lane 3). In summary, these results indicate that the first A3 actin specific intron can be spliced out correctly from the majority of the *hyg* mRNA in mammalian cells. The existence of the minor intron containing transcript can be explained by supposing that the removal of the insect intron in mammalian cells is slower than in insect cells, therefore the intron containing mRNA intermediary can accumulate to a level where it becomes detectable by RT-PCR. However, there could be an alternative explanation for the incomplete removal of the A3 actin intron from the *hyg* mRNA. Southern hybridization indicated that the LMTK$^-$ cell line used in these experiments contains at least one hundred pBmA/HmB plasmid molecules per cell (Figure 16, lane 16). It is possible that in some plasmid copies mutations destroyed the exon-intron junctions which in turn could prevent the removal of the intron from the *hyg* mRNA transcribed from such plasmids.

C. Identification of an alternative promoter within the first intron of the *Bombyx mori* A3 cytoplasmic actin gene

1. Transcriptional regulatory elements of the A3 actin promoter are not required for the activity of the A3 5' UTR in mammalian cells

The successful application of the A3 actin-based expression vector for the isolation of stably transformed mouse cell lines has prompted us to examine the activities of the earlier identified transcriptional regulatory elements of the A3 promoter in mammalian cells. Our previous observations also showed that an A3 actin mRNA isoform initiated within the first intron exists in *B. mori* cells (see section A.3.). RT-PCR data indicated that the intronic start site is used less frequently in BmN cells than the two major initiation sites preceding the intron. Although, the low abundance of this mRNA species hindered us from determining its 5' end, RT-PCR experiments carried out by using nested intron-specific primers narrowed down the initiation region to the last fourth of the intron. It is possible that this mRNA isoform was synthesized under the control of an alternative promoter. In a recent study, Mange and his colleagues identified only one promoter region of the A3 actin gene (Mange et al. 1997). However, we can not rule out the existence of a second promoter. These authors analyzed the regulatory regions of the A3 actin in the heterologous Sf9 cells by transient transfection assays. Therefore, a weak alternative promoter might remain undetected.

To identify the regulatory regions of the A3 actin 5' UTR necessary for expression in mammalian cells, we constructed a series of 5' deletions of the promoter region by a PCR-based strategy. Next, we inserted the deleted DNA fragments into a plasmid vector containing the bacterial β–galactosidase reporter gene and the A3 actin polyadenylation signal. We used the same reference point to name our deletion plasmids as Mange and his coworkers did, the earlier suggested initiation site (+1) which is located 69 bp upstream from the first exon-intron junction (Figure 23). The resulting plasmid molecules were subsequently introduced into LMTK$^-$ cells. Forty-eight hours after transfection, cytoplasmic extracts were prepared from the transfected cells and the β–galactosidase activities were determined (Figure 24).

Deletion mutants to -443 and -176 exhibited approximately the same activity as the plasmid containing the intact promoter region. To our surprise, sequential deletion of the earlier identified regulatory elements (deletions to -125, -110 and -73) did not decrease the promoter activity but on the contrary, an almost 2-fold increase could be observed. Subsequent removal of a 46 bp DNA segment containing the TATA-box and the upstream initiation site resulted in an ~4.5-fold increase compared to the intact promoter. The deletion derivative generated by the further removal of the downstream initiation site still exhibited a ~3.5-times higher transcriptional activity than the original pBL plasmid did.

Figure 23. Structures of the plasmid constructs containing deletion derivatives of the 5' UTR of the A3 cytoplasmic actin gene.

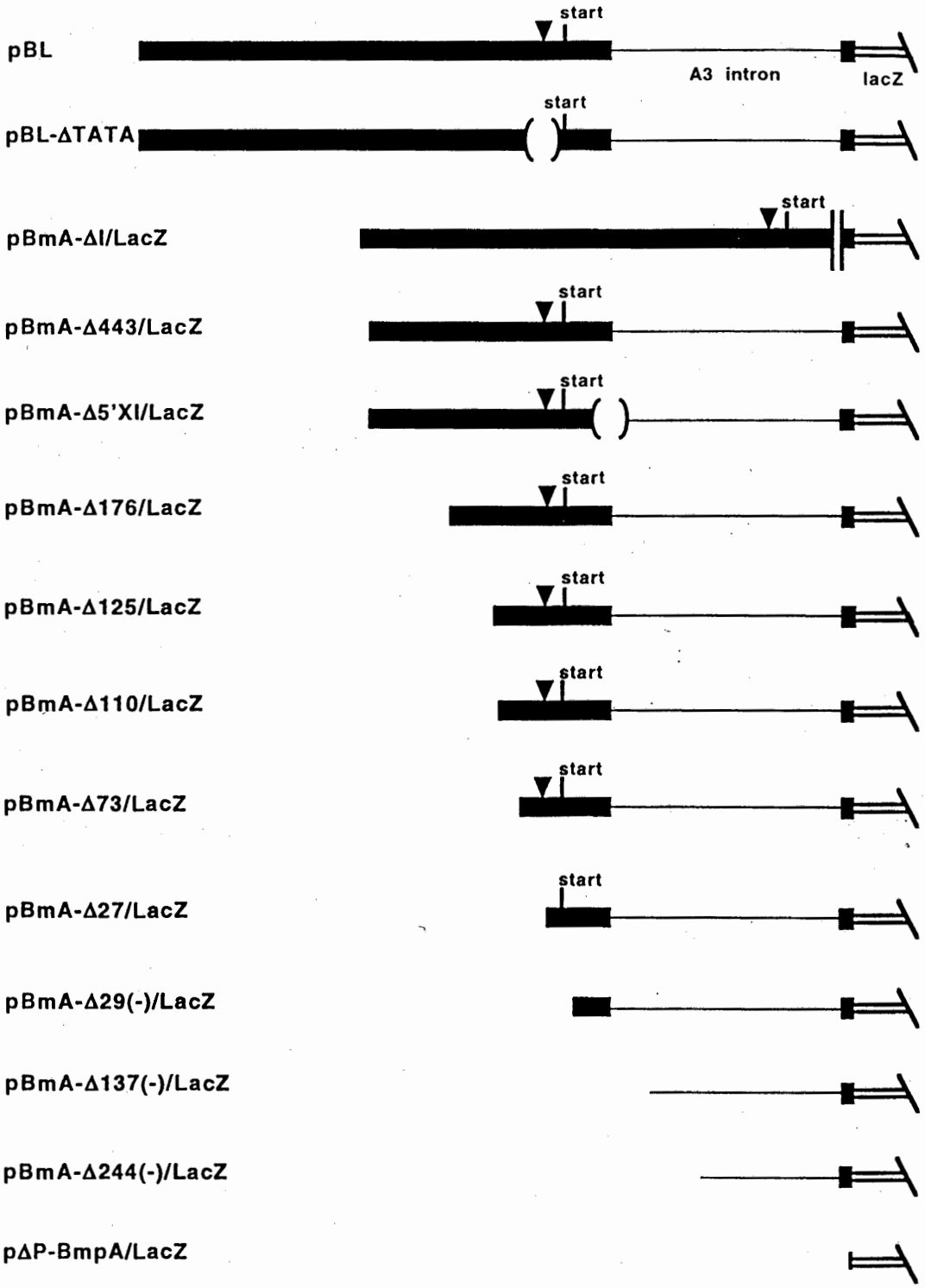
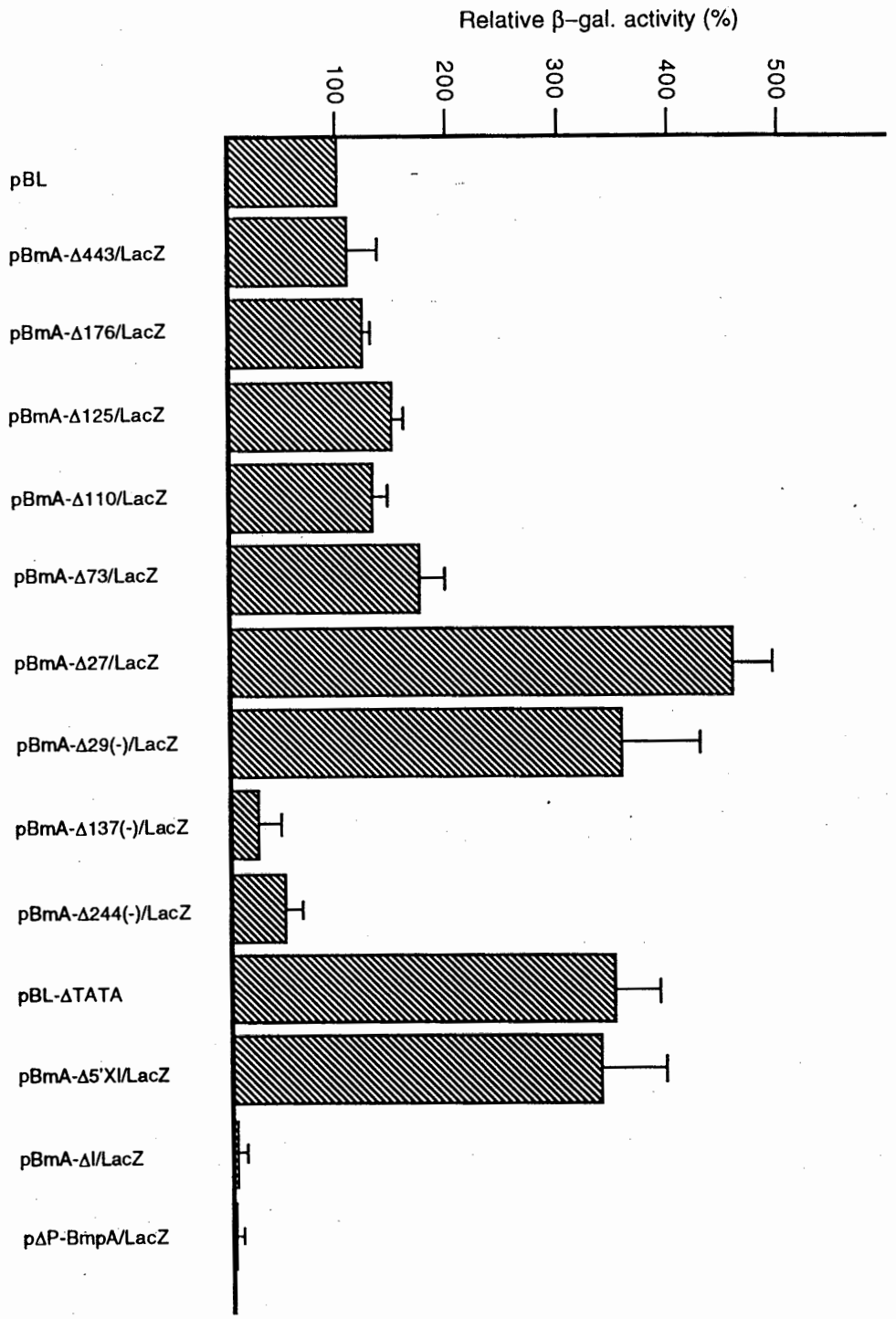

pBL
start
A3 intron
lacZ
pBL-ΔTATA
start
pBmA-ΔI/LacZ
start
pBmA-Δ443/LacZ
start
pBmA-Δ5'XI/LacZ
start
pBmA-Δ176/LacZ
start
pBmA-Δ125/LacZ
start
pBmA-Δ110/LacZ
start
pBmA-Δ73/LacZ
start
pBmA-Δ27/LacZ
start
pBmA-Δ29(-)/LacZ
pBmA-Δ137(-)/LacZ
pBmA-Δ244(-)/LacZ
pΔP-BmpA/LacZ

Figure 24. Analysis of the transcriptional regulation of the 5' UTR of the A3 cytoplasmic actin gene by transient transfection assay in LMTK$^-$ cells. β–galactosidase activities are given relative to the value obtained with pBL plasmid. The β-galactosidase activities were determined three times for each plasmid construct. Error bars indicate standard deviations from the mean.


Relative β–gal. activity (%)
100
200
300
400
500
pBL
pBmA-Δ443/LacZ
pBmA-Δ176/LacZ
pBmA-Δ125/LacZ
pBmA-Δ110/LacZ
pBmA-Δ73/LacZ
pBmA-Δ27/LacZ
pBmA-Δ29(-)/LacZ
pBmA-Δ137(-)/LacZ
pBmA-Δ244(-)/LacZ
pBL-ΔTATA
pBmA-Δ5'XI/LacZ
pBmA-ΔI/LacZ
pΔP-BmpA/LacZ

To assess the effect of the removal of the TATA-box only, we created a mutant plasmid construct in which the 10 bp long DNA segment containing the TATA element was replaced by a 9 bp DNA fragment from the polylinker region of the pUC18 plasmid vector. Although, the rest of the promoter remained unchanged the promoter activity increased by 3.5-fold. In summary, these data indicate that despite the apparent similarities between the *Bombyx* A3 and the vertebrate actin promoters, the regulatory elements of the A3 promoter region, including the SRE motif, are not required for the transcriptional activity of the A3 5' UTR in mammalian cells.

2. The first intron is essential for the transcriptional activity of the A3 actin 5' UTR in mammalian cells

The results presented above suggested that the regulatory elements required for the activity of the A3 5'UTR are located within the 460 bp long first intron. To confirm this presumption we constructed an internal deletion derivative of the pBL plasmid from which only the intron was missing (pBmA-ΔI/LacZ). When we tested this mutant plasmid in transient transfection assay we observed a complete loss of promoter activity. In an earlier study Bunchman and Berg observed that an SV40 expression vector failed to express the rabbit β–globin sequence unless an intron was included in the transcription unit (Buchman and Berg 1988). The size of this effect was unexpectedly large: a difference of several hundred-fold was observed between recombinants containing or

lacking an intervening sequence. Detailed analysis revealed that the efficient expression of the β–globin gene required the integrity of the exon-intron junction sequences that are needed for splicing. The authors suggested that the inhibition of the splicing process decreased the mRNA stability, finally resulting in a low expression level. We tested this possibility by creating a mutant plasmid lacking the 5' exon-intron junction (pBmA-Δ5'XI/LacZ). Interestingly, this mutation did not decrease the promoter activity but on the contrary, a more than 3-fold increase was detected relative to the activity obtained with the equivalent, 5' splice junction-containing plasmid construct (pBmA-Δ443/LacZ).

3. The first intron of the A3 actin gene contains a promoter that is transcriptionally active in mammalian cells

Next, we addressed the question of whether the intron contains the promoter elements that are required for transcription initiation. First we removed 68 bp from the 5' end of the intron. This deletion resulted in a ~17-fold drop in promoter activity (relative to the activity obtained with pBmA-Δ29(-)/LacZ), but this activity was still 22.8% of the value obtained with the pBL plasmid. Further removal of a 108 bp DNA segment increased the β–galactosidase activity by 2-fold. We could draw two conclusions from these experiments:

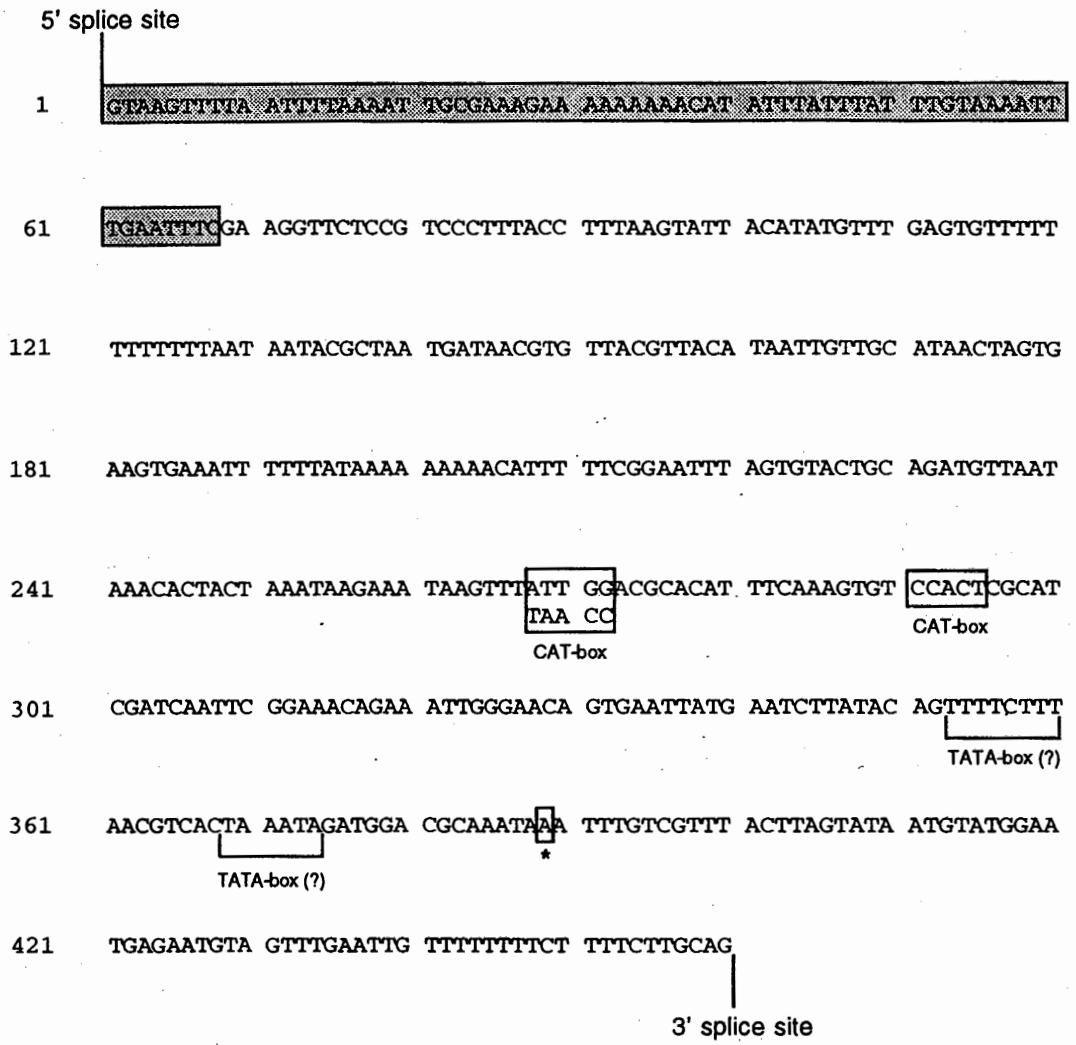
- i., the 5' 68 bp long segment of the intron contains a strong positive, possibly an enhancer-like element,

- ii., the promoter elements required for the basic transcriptional activity of the A3 5' UTR in mammalian cells are localized in the last 284 bp region of the intron.

To further confirm the existence of the intronic promoter we decided to determine the initiation site of the A3 transcripts in mammalian cells. The pBmA/HmB plasmid containing the intact 5' UTR of the A3 actin gene was transfected into LMTK$^-$ cells. Forty-eight hours after transfection total cellular RNA was extracted from the transfected mouse cells and the 5' termini of the A3 transcripts were analyzed by primer extension. After separation of the primer extension reaction on a 6% denaturing polyacrylamide gel, an ~110 bp long extension product was detected (Figure 25) indicating that the transcription initiation site is located ~72 bp upstream from the 3' splice site. Approximately 20-30 bp upstream from the identified transcription initiation site, several A-T rich sequence motifs can be observed. One of these sequence elements may serve as a TATA-box for the intronic promoter. Additionally, two CCAAT-boxes can also be recognized 100-120 bp upstream from the transcriptional start site (Figure 26). In summary, our data strongly suggest that the expression of the A3 actin 5' UTR in mammalian cells is controlled by an efficient promoter region located in the second half of the A3 first intron.

Figure 25. Mapping of the transcriptional initiation site at the intronic promoter of the A3 actin in mouse cells by primer extension. BMA3R actin gene specific primer was used. ^{32}P labeled HinfI digested ΦX174 DNA was used as a molecular weight marker.

Figure 26. Organization of the putative intronic promoter of the A3 cytoplasmic actin gene. The transcriptional start site is indicated by an asterisk. The A-T rich DNA segments that may serve as TATA-boxes are bracketed. The CCAAT-boxes are also indicated. The shaded region indicates the 68 bp DNA segment that has a strong positive effect on transcriptional activity in mouse cells.

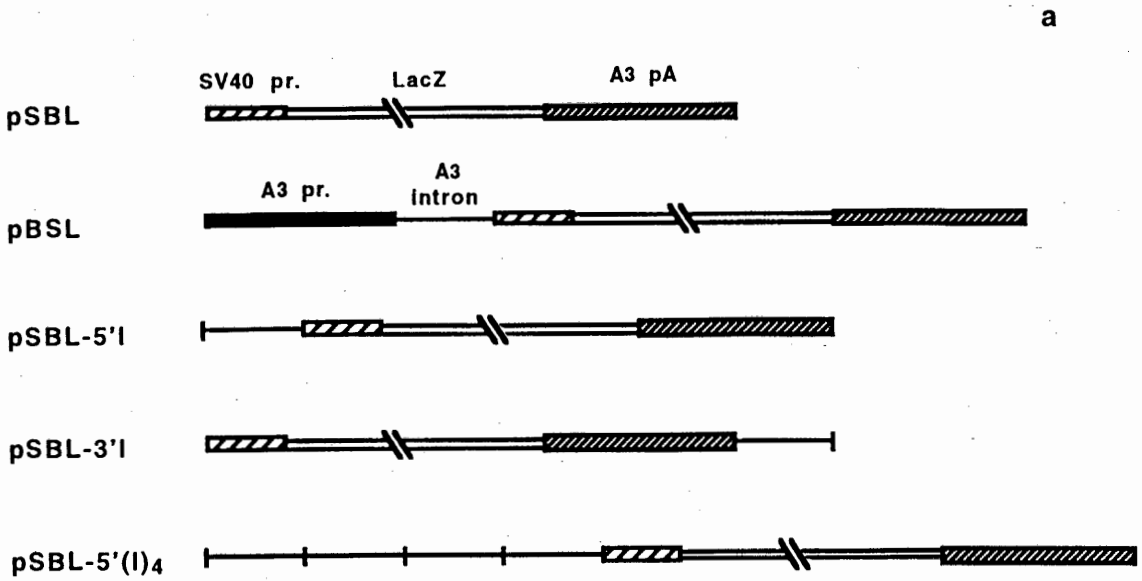

5' splice site
1 GTAAGTTTTA ATTTTAAAAT TGCGAAAGAA AAAAAAACAT ATTTATTTAT TTGTAAAATT
61 TGAATTTCGA AGGTTCTCCG TCCCTTTACC TTTAAGTATT ACATATGTTT GAGTGTTTTT
121 TTTTTTTAAT AATACGCTAA TGATAACGTG TTACGTTACA TAATTGTTGC ATAACTAGTG
181 AAGTGAAATT TTTTATAAAA AAAAACATTT TTCGGAATTT AGTGTACTGC AGATGTTAAT
241 AAACACTACT AAATAAGAAA TAAGTTTATT GGACGCACAT TTCAAAGTGT CCACTCGCAT
TAA CC
CAT-box
CAT-box
301 CGATCAATTC GGAAACAGAA ATTGGGAACA GTGAATTATG AATCTTATAC AGTTTTCTTT
TATA-box (?)
361 AACGTCACTA AATAGATGGA CGCAAATAAA TTTGTCGTTT ACTTAGTATA ATGTATGGAA
TATA-box (?)
*
421 TGAGAATGTA GTTTGAATTG TTTTTTTTCT TTTCTTGCAG
3' splice site

4. The first intron of the A3 actin gene can activate a heterologous promoter

Deletion experiments demonstrated that a 68 bp DNA segment of the A3 first intron located near the 5' splice site substantially augment the activity of the intronic promoter. The size of the effect suggested that it may be due to a strong enhancer-like element. By definition, transcriptional enhancers can increase the activity of promoters from large distances regardless of their orientation and position relative to the transcription unit (Hertel et al. 1997, Sauer and Tjian 1997). Enhancer elements can frequently activate not only their cognate promoters but also other heterologous promoters. To examine whether the A3 intron contains any enhancer-like element, we inserted the intron into the pSBL reporter plasmid both upstream and downstream from the transcription unit (Figure 27). The pSBL plasmid expresses the β–galactosidase enzyme under transcriptional control of the SV40 early promoter and the A3 actin polyadenylation signal. In transient transfection assay, this plasmid provided approximately the same β–galactosidase activity in mouse cells as the pCH110 mammalian expression vector did (see section B.1.). Both intron containing derivatives of the pSBL plasmid exhibited a substantially higher β–galactosidase activity in LMTK$^-$ cells than the original construct. By increasing the number of introns in the pSBL plasmid, the promoter activity could not be augmented further.

We also tested the effect of the A3 intron on the activity of the intron-less A3 promoter (Figure 28).

Figure 27. Analysis of the positive effect of the A3 first intron on the activity of the heterologous SV40 early promoter by transient transfection assay. Structure of the plasmid constructs that were used to analyze the effect of the A3 intron on the transcriptional activity of the SV40 early promoter (**a**). β–galactosidase activities are given relative to the activity obtained with the pSBL plasmid (**b**). The β-galactosidase activities were determined three times for each plasmid construct. Error bars indicate standard deviations from the mean.


a
SV40 pr.
LacZ
A3 pA
pSBL
A3 pr.
A3 intron
pBSL
pSBL-5'I
pSBL-3'I
pSBL-5'(I)4

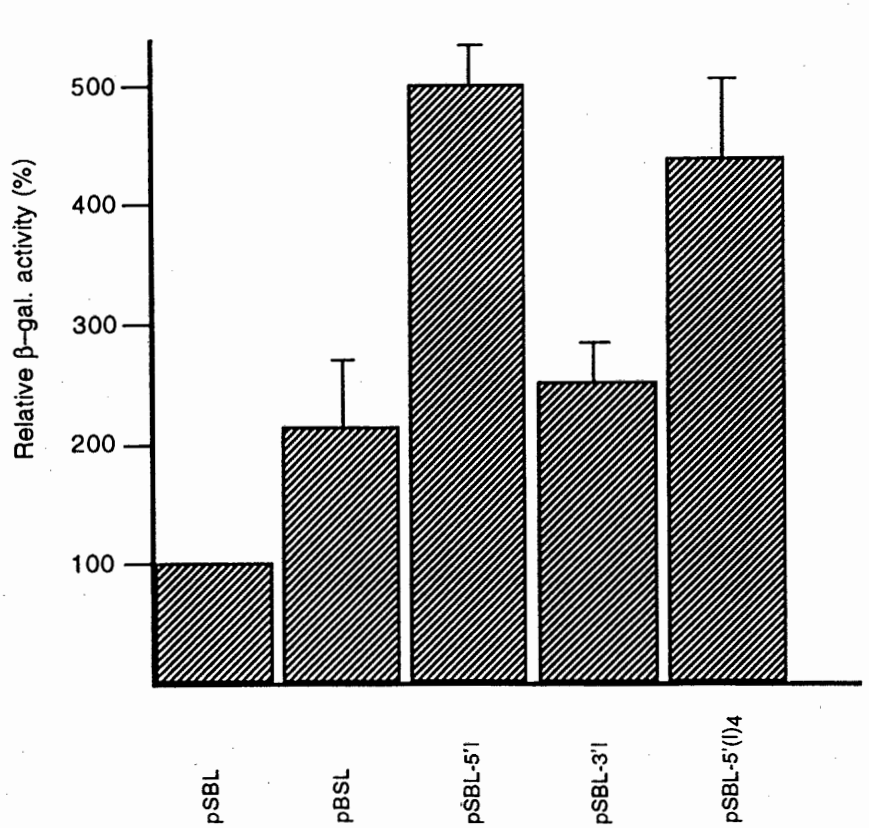
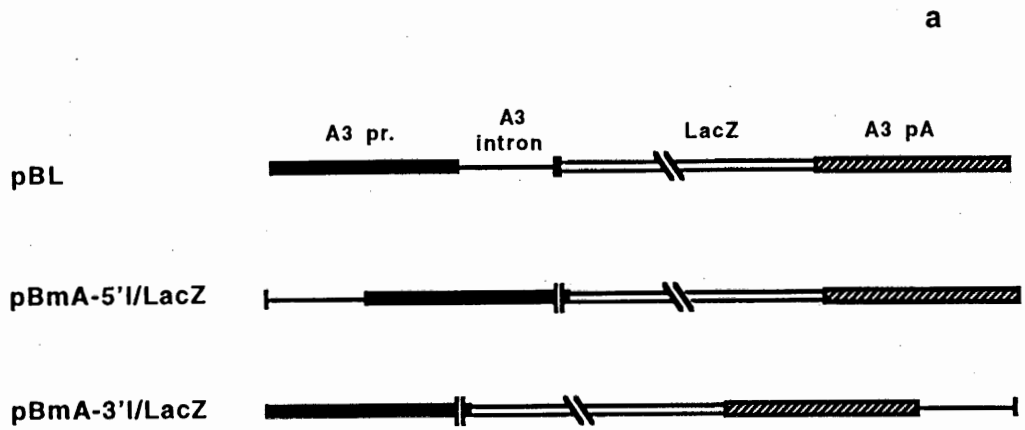
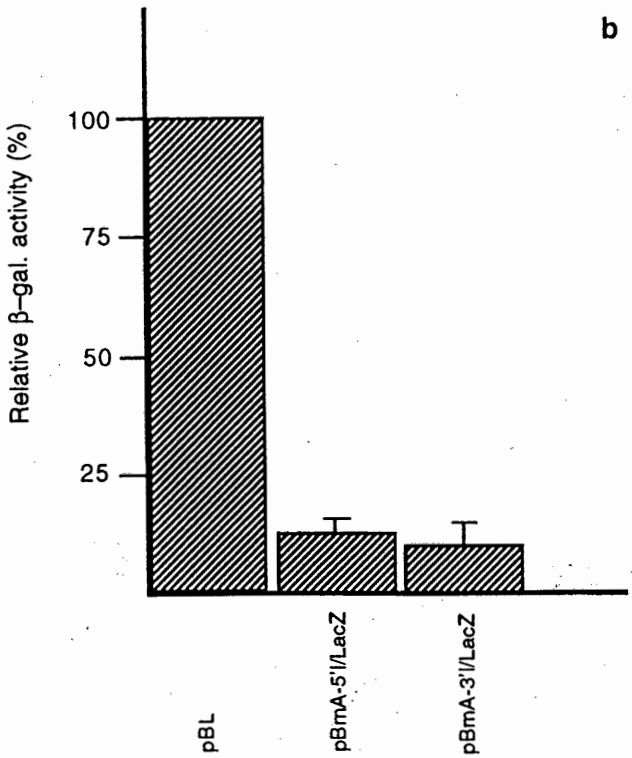

b
Relative β-gal. activity (%)
500
400
300
200
100
pSBL
pBSL
pSBL-5'I
pSBL-3'I
pSBL-5'(I)4

Figure 28. The first A3 intron could not restore the activity of the intronless A3 actin 5' UTR in mouse cells. The A3 first intron was inserted both upstream and downstream of the A3 promoter (**a**). β–galactosidase activities are given relative to the activity obtained with the pBL plasmid (**b**). The β-galactosidase activities were determined twice for each plasmid construct. Error bars indicate standard deviations from the mean.


a
A3 pr.
A3 intron
LacZ
A3 pA
pBL
pBmA-5'I/LacZ
pBmA-3'I/LacZ


b
Relative β-gal. activity (%)
100
75
50
25
pBL
pBmA-5'I/LacZ
pBmA-3'I/LacZ

The insertion of the intron into the transcriptionally inactive pBmA-ΔI/LacZ plasmid either upstream or downstream from the transcription unit could not restore the activity of the A3 promoter. This result confirms our previous findings that the A3 actin promoter can not be utilized by the mammalian transcription apparatus.

V. DISCUSSION

A. Isolation of a stably transformed *Bombyx mori* cell line: identification of novel transcriptional start sites of the A3 cytoplasmic actin gene

The A3 cytoplasmic actin gene is expressed in all tissues of *Bombyx mori* during all developmental stages (Mounier and Prudhomme 1991). Besides ubiquitous expression of the A3 gene, it has also been demonstrated that the A3 mRNA level displays cyclic changes in the silk gland during larval life (Mounier et al. 1991). The changes in mRNA concentration may be a result of different regulatory mechanisms. The mRNA concentration can be regulated at the level of transcription and/or it can also be controlled at the post-transcriptional level by modification of the mRNA stability. The use of alternative promoters and transcriptional start sites often results in mRNA isoforms with different 5' non-translated leader exons which exhibit different stability or translation efficiency (Atwater et al. 1990). Many of the cytoplasmic actin genes analyzed so far have very complex regulation which is usually associated with the use of multiple regulatory elements and alternative promoters. Although previous reports suggested that the A3 actin gene has a less complex promoter organization, our studies demonstrated the existence of at least two earlier undetected mRNA isoforms. The transcription of one of these mRNA isoforms starts ~35 bp upstream from the earlier identified transcriptional start site. It is of interest to note that the upstream start site coincides with the suggested

TATA-box of the downstream initiation site. Although primer extension indicated that the two initiation regions are used approximately with the same frequency in BmN cells, we can not rule out that in other tissues or at different developmental stages, the use of one of these start sites may become predominant. This presumption would also explain the failure of others to detect the upstream start site (Mange et al. 1997). Interestingly, the two initiation regions show a significant sequence similarity to each other, which raises the possibility that they are the result of a recent duplication event. At present, we do not know the significance of such a close association of two efficient transcriptional start sites. It is possible that the alternative 5' leader exons can affect some properties of the A3 actin mRNA (translation efficiency, stability, intracellular transport).

By RT-PCR we also detected transcripts that initiated in the first intron of the A3 actin gene. From the amount of the RT-PCR product we concluded that this initiation site is used less frequently in BmN cells than the two major start sites preceding the first intron. Although we could not recover the 5' ends of these transcripts by 5' RACE, it is possible that some of the minor bands detected by primer extension may represent these mRNAs. At present we do not know whether transcription initiated in the first intron is regulated by the promoter region preceding the intron or whether it is controlled by an alternative promoter. The A-T rich intron contains several sequence elements which fit well with the consensus sequence of the TATA-box. Additionally, putative binding sites of several *Drosophila* homeodomain proteins can also be recognized. The concentration of homeodomain binding sites around the fibroin promoter has been reported (Hui et al.

1989). It has also been suggested that silkgland specific factors interacting with these binding sites may play an important role in transcriptional regulation of the fibroin and other silk protein genes. It would be interesting to examine the use of the intronic start site of the A3 actin gene in silkgland at different developmental stages in relation to the expression of other silkgland specific proteins.

In the studies presented above we used a stably transformed BmN cell line. The cell line was isolated by using a selection cassette containing the *S. alboginer* puromycin *N*-acetyl transferase gene under control of the 5' and 3' transcriptional regulatory elements of the A3 cytoplasmic actin gene. The heterologous *pac* gene made it easier for us to follow the transcripts synthesized under control of the A3 regulatory elements. Detailed characterization of the transformed BmN cell line demonstrated integration of the foreign DNA into a *B. mori* chromosome. Southern hybridization experiments also indicated that both plasmids (pBmA/Pur, pTK-β) used for the transfection are highly amplified resulting in tandem arrays of unrearranged plasmid molecules. Although we could identify minor transcripts synthesized from rearranged transcription units the majority of the *pac* mRNA exhibited the expected structure. It is interesting to note that the polyadenylation of one of the rearranged transcripts was regulated by the SV40 polyadenylation signal (the pTK-β plasmid contains the bacterial β-galactosidase gene under the transcriptional control of the herpes simplex virus thymidine kinase (HSV-TK) promoter and SV40 polyadenylation signal). In contrast, the activity of the HSV-TK promoter was not detected in BmN cells by β-galactosidase cytochemical staining. This

observation is in agreement with previous data that the HSV-TK promoter is not active in insect cells from which the Sp1 transcription factor is missing (McKnight and Tjian 1986; Santoro et al. 1988).

Our work may also have some important practical implications. Recently, Lu et al. described a baculovirus-free insect expression system in which the activity of silkworm A3 actin based expression vectors could be increased by more than three orders of magnitude by using a combined *cis-*, *trans*-activation scheme (Lu et al. 1997). This observation paves the way for the development of an effective non-lytic insect expression system. Besides the strong expression vectors, however, the generation of stably transformed *B. mori* cell lines carrying chromosomally integrated copies of these vectors will also be required for the establishment of such a system. Ours is the first report that describes the isolation and characterization of a stably transformed *B. mori* cell line. The novel *pac*-puromycin selection scheme used in this study might have been an important factor in our success to establish this cell line. A recent report also described generation of stable, puromycin resistant *Spodoptera frugiperda* cell lines using the *pac* gene as a selectable marker (McLachlin and Miller 1997). These data combined with our findings therefore strongly suggest that the cost-effective *pac*-puromycin selection system can be used efficiently to generate stable insect cell lines producing proteins of interest.

B. Isolation of stably transformed mammalian cell lines by using an A3 actin-based selection cassette

The A3 cytoplasmic actin promoter is highly expressed in all tissues of the silkworm during all developmental stages. It has been reported that the A3 actin promoter is also active in several lepidopteran (Lu et al. 1996) and *Drosophila* cell lines (Abraham et al. 1993). Transcriptional regulation of the A3 actin gene has been studied in detail (Mange et al. 1997). Different positive and negative regulatory elements were identified in the promoter region. One of the positive regulatory elements proved to be a serum response element (SRE). SRE motifs are involved in vertebrate actin gene regulation (Frederickson et al. 1989). The *Bombyx* A3 actin gene is the first reported SRE-regulated invertebrate cytoplasmic actin gene. Therefore, it was a good candidate to examine whether its transcriptional signals are capable to express foreign genes in vertebrate cells. Here we describe experiments which prove that the transcriptional regulatory elements of the insect A3 cytoplasmic actin can be used to express the bacterial β-galactosidase gene in mammalian and avian cells in transient transfection assays. In the LMTK$^-$ mouse cell line, the A3 5' UTR showed ~1/3-rd of the activity of the strong SV40 viral promoter, while the A3 polyadenylation signal functioned with the same efficiency as the SV40 polyadenylation signal did.

We also report that an A3 actin gene based selection cassette could be used to establish stably transformed mammalian cell lines. The integration of the A3 plasmid

construct into the host genome was demonstrated by Southern and *in situ* hybridizations. The integrated plasmid molecules frequently exhibited amplification that may indicate the presence of amplification promoting elements in the 5' or 3' UTR of the A3 actin gene. Although it has been previously described that the promoters of several insect genes are functional in mammalian cells (Corces et al. 1981, Burke and Ish-Horowicz 1982, Reeves et al. 1983, Tokunaga et al. 1984), this is the first reported case of the use of an insect selection cassette for isolation of stably transformed mammalian cell lines.

The isolated cell lines stably expressed 3 *hyg* mRNA isoforms. Two of these mRNA forms were generated by the alternative usage of the SV40 and the A3 polyadenylation signals. RT-PCR experiments also proved that the insect splicing signals can be efficiently used by the mammalian splicing apparatus.

Our work also has some practical implications. Earlier, Johnson and his coworkers suggested the use of the A3 actin expression cassette for construction of recombinant insecticide baculoviruses (Johnson et al. 1992). In light of the recent discovery that baculoviruses can efficiently deliver their DNA into mammalian cells (Boyce and Bucher 1996), our data raise questions about the safety of recombinant baculoviruses utilizing A3 actin based expression cassettes. On the other hand, the A3 actin-based selection cassettes have certain characteristics that might be useful in the cellular production technology. The surprisingly high amplification rate of the pBmA/HmB plasmid in the isolated mammalian cell lines suggest the presence of amplification promoting elements within the 5' or 3' UTR of the A3 actin gene. It is

possible that the incorporation of A3 actin based selection cassettes into plasmid constructs containing genes of proteins to be produced would make it easier to establish stably transformed overexpressing mammalian cell lines.

C. The first intron of the *Bombyx mori* A3 actin gene contains an alternative promoter

Previous observations indicated that the *Bombyx mori* A3 cytoplasmic actin and numerous vertebrate immediate-early response genes, including cytoplasmic actin genes, may exhibit similar regulatory mechanisms. Our success to use an A3 actin-based selection cassette to isolate stably transfected mammalian cell lines apparently supported this assumptions. In our promoter analysis experiments presented above, we wished to test this hypothesis further. Additionally, we were also hoping that the heterologous mammalian expression system might provide us the opportunity to analyze the putative intronic promoter of the A3 actin gene. Surprisingly, neither of the earlier identified regulatory elements of the A3 actin promoter were required for expression of the β–galactosidase reporter gene in mammalian cells. However, the presence of the A3 first intron in the plasmid constructs was absolutely essential for transcriptional activity. The position of the intron was also important; it had to be situated immediately upstream from the β–galactosidase gene. In this arrangement the intron could elicit a β–galactosidase activity in mouse cells comparable to the strong SV40 early promoter. By using deletion

constructs we localized the intronic promoter to the second half of the intron. We also determined that the transcription initiates ~72 bp upstream from the 3' splice site. It is tempting to speculate that the identified intronic promoter is the same promoter that controls the synthesis of the minor A3 actin mRNA isoform detected earlier in *Bombyx mori* cells.

Our experiments also identified a short region within the first A3 intron that contains a strong positive transcriptional element. The intron could substantially stimulate the activity of the heterologous SV40 promoter inserted either upstream or downstream from the transcription unit. This observation suggests that the positive element of the A3 intron functions as a transcriptional enhancer in mammalian cells.

VI. CONCLUSIONS

Here we studied the expression and regulation of the 5' and 3' transcriptional signals of the *Bombyx mori* A3 cytoplasmic actin gene in homologous and heterologous expression systems:

i., We have isolated and characterized a stably transformed *Bombyx mori* cell line containing a novel selectable marker gene, puromycin *N*-acetyl transferase, under control of transcriptional regulatory signals from the A3 cytoplasmic actin gene. Integration of the transfected plasmids into the host genome was demonstrated by Southern and *in situ* hybridizations. Establishment and characterization of stably transformed insect cell lines, like the one described here, represents an important step in the development of nonlytic insect expression systems.

ii., By using this cell line we have identified alternative transcriptional initiation sites for the A3 cytoplasmic actin gene. One of these start sites is located ~35 bp upstream from the earlier determined transcription initiation site. The two mRNA start sites are utilized with a similar efficiency in the BmN cell line. In addition, we detected transcripts that initiated in the first intron of the A3 actin gene. These transcripts may be synthesized under control of an alternative promoter. The mRNA isoforms generated by the use of the alternative start sites may exhibit different characteristics (stability etc.), therefore the alternative promoter and initiation site usage may be an important factor in regulating the cyclic changes of the A3 actin mRNA level in the silk gland during larval morphogenesis.

iii., Here we also report that the transcriptional regulatory signals of the *Bombyx mori* A3 actin gene are functional in vertebrate cells in transient transfection assays. Using an A3 actin-based expression cassette, we have isolated stably transformed mammalian cell lines. Isolation of transformed cells was based on expression of the selected marker gene. Stable integration of A3 actin plasmid construct into the host genome was demonstrated. The integrated plasmid molecules frequently exhibited amplification which may indicate the presence of amplification promoting elements in the 5' or 3' UTR of the A3 actin gene. This is the first reported case for the use of an insect selection cassette to establish transformed mammalian cell lines. The analysis of the structure of the mRNA molecules synthesized under control of the insect vector revealed that both the insect splicing and polyadenylation signals function in mammalian cells.

iv., By testing the transcriptional activities of deletion derivatives of the 5' UTR of the A3 actin gene, we demonstrated that the earlier identified promoter elements of the A3 gene are not required for expression in mammalian cells. On the other hand, we proved that the first intron of the A3 actin gene contains an efficient promoter that shows a comparable activity to the SV40 early promoter. The analysis of the promoter region suggests that this is the promoter that regulates the synthesis of the rare A3 actin mRNA isoform identified in *Bombyx mori* cells. Additionally, we also detected a strong enhancer-like element in the A3 intron that could significantly stimulate the activity of the SV40 early promoter in mouse cells.

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