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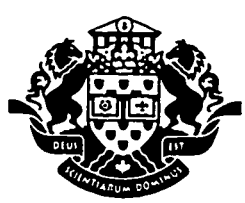

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ESTABLISHMENT AND CHARACTERIZATION OF P19 EMBRYONAL CARCINOMA CELLS HARBORING AN INDUCIBLE STY GENE

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Abstract

In order to establish Myc-Sty inducible clones, I generated a bio-cistronic Lac repressor expression construct (PCJ2LacIN) and subsequently transfected it along with pPOP β-gal into P19 EC cells. One of the clones (CJ2-8) showed 35 fold induction in β-gal activity between un-induced and induced cells.

Subsequently, I also generated an inducible Sty expression vector (pPOPMycSty) containing two Lac operator sequences which is to be bound by Lac repressor to regulate the expression of the target genes at the downstream of the operator sequences. Following the co-transfection of pPOPMycSty, a construct containing PGK-1 promoter and its intragenic region, B17 and PGKNeo and subsequent drug selection, most of the drug resistant colonies turned out to be positive for Myc-Sty although its expression level and inducibility were somewhat variable among the clones.

Myc-Sty from the inducible clone #9 is catalytically active, can dimerize with GST-Sty in vitro, phosphorylated on Tyr in vivo, localized in the nucleus and furthermore, immunofluorescence result showed that it phosphorylates and redistribute SR proteins in the nucleus resulting in affecting alternative splicing.

Western analysis result using α-SR monoclonal antibody (mAb104) recognizing the phospho-epitope common to every SR protein suggested that Sty may selectively phosphorylate SRp30 and SRp55, which were among the proteins found to be interacting with Sty by yeast two hybrid system. Subsequently, double immunofluorescence both for Myc-Sty and EC cell surface antigen (SSEA-1) or neuronal differentiation markers (A60 and HNK-1) indicated that Myc-Sty alone does not seem to give rise to the differentiation

process.

Acknowledgment

I sincerely express thanks to Dr. John Bell and Dr. Michael McBurney for their kind advice and support during the course to this work. I would also like to express thanks to Dr. Peter Duncan, Ninan Abraham, David Stojdl and Alan Chen for their valuable discussion and advice and finally all the members of the cancer research group for their kind help

Dedication

I would like to dedicate this thesis to my family and especially thank my wife and daughter for their endless support and love.

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Introduction

Sty/Clk is a dual specificity kinase

Sty (also known as Clk) was initially identified by cDNA library screening using anti-phosphotyrosine antibody in an attempt to discover noble protein tyrosine kinases (PTKs) which may be involved in the differentiation process (Ben-David *et al*, 1991; Howell *et al*, 1992). It was quite feasible because bacterial cells (*E. coli*) don't express PTKs.

Sty/Clk has a very unique biochemical property that it can auto-phosphorylate Ser/Thr as well as Tyr residues (a dual specificity kinase, Lindberg *et al*, 1992; Douvile *et al*, 1994) suggesting that Sty/Clk may act as a bridge between different signal transduction pathways involving Ser/Thr and/or Tyr specific kinases. Sty/Clk is composed of roughly two major domains; N-terminal regulatory domain and C-terminal catalytic domain. The N-terminal regulatory domain of about 156 a.a contains a putative nuclear localization signal (NLS: Lys-Arg-Lys-Lys-Arg) suggesting that Sty/Clk may be localized in the nucleus and Arg/Ser repeating sequences (RS domain) required for protein-protein interaction. No other characteristic protein motifs were not found based on the cDNA sequences (i.e, SH2, SH3, leucine zipper, myristylation signal and zinc finger domain etc.). Interestingly, Sty/Clk protein with a mutation in the NLS region didn't abolish its nuclear localization completely after the transient transfection with the mutant construct suggesting that Sty/Clk may have multiple nuclear localization signals (Duncan *et al*, 1995). The C-terminal catalytic domain of approximately 300 a.a. is composed of 11 subdomains. Subdomain VI and VIII of Sty/Clk, which are thought to be indicative of Ser/Thr versus Tyr specificity, showed high

homology to many other known Ser/Thr protein kinases. Interestingly, a tryptophan residue found in subdomain XI of all tyrosine kinases is not present in Sty/Clk (Howell *et al*, 1992).

Sty/Clk pre-mRNA is subject to alternative splicing (Duncan *et al*, 1995); Inclusion of the alternatively spliced second exon yields mRNA coding for full length and catalytically active Sty/Clk, while exclusion of the exon causes a frame-shift resulting in the production of the truncated/catalytically inactive form of Sty/Clk (figure 1) respectively. The function of the truncated Sty/Clk is not clear yet but it seems to regulate the kinase activity of the full-length Sty/Clk by forming the heterodimer (Duncan *et al*, 1995). With regard to regulating the kinase activity of Sty/Clk, another interesting feature is its auto-regulation; if the level of full length Sty/Clk protein increases, splicing to yield transcripts for truncated Sty/Clk is favored while if the level of truncated Sty/Clk protein increases, production of the transcripts for full length Sty/Clk increases indicating the kinase activity of Sty/Clk in vivo may be tightly controlled by this auto-regulatory loop (Duncan *et al*, 1997).

Sty/Clk may be implicated in the cellular differentiation

It has been known that many protein kinases are involved in differentiation (Nurse and Bissett, 1981; Basler and Hafen, 1988; Chabot *et al*, 1988; Geissler *et al*, 1988; Baker and Rubin, 1989; Price *et al*, 1989; Schejter *et al*, 1989; Elion *et al*, 1990). Sty/Clk cDNA was originally isolated by screening P19 EC cell cDNA library using anti-phosphotyrosine antibody (Howell *et al*, 1992). Therefore, It was assumed that Sty/Clk may play some important roles in the signaling pathways leading to the (neuronal) differentiation. This notion

was more plausible taking into account the facts that P19 EC cell line used as a model system can be easily differentiated into muscle and neuron following the treatment with DMSO and RA respectively (McBurney *et al*, 1982) and the previous northen analysis also demonstrating that the level of Sty/Clk mRNA in P19 EC cells increased following RA treatment but not following DMSO treatment. Furthermore, expression of Sty/Clk mRNA is ubiquitously and also developmentally regulated (Howell *et al*, 1992). Undifferentiated and rapidly proliferating embryonic stem cells and P19 EC cells produced only two mRNA species of approximately 1.7-Kb (truncated) and 1.8-Kb (full-length) in size. However, differentiated cells produced two additional Sty/Clk transcripts of 3.2 and 5.6-kb in size (Ben-David *et al*, 1991; Howell *et al*, 1992). Interestingly, it was known that those two larger transcripts are incompletely spliced products which can't be translocated into the cytoplasm for translation (Duncan *et al*, 1995) and more interestingly, they were not observed in the leukemic cell lines such as SP10 (myeloma) and P388 (pre-B cell) (Howell *et al*, 1992) suggesting these two larger transcripts are differentiation-specific. Further evidence came from the result that in PC12 cells, over-expression of Sty/Clk led to the initiation of neuronal differentiation process characterized by the neurite outgrowth (Myers *et al*, 1994).

Sty/Clk exists as multiple isoforms and is a member of LAMMER family kinases

Sty/Clk exists as multiple isoforms. To date, many isoforms have been identified in mouse as well as in human: murine Sty/Clk (Ben-David *et al*, 1991; Howell *et al*, 1992), human Clk1 (Johnson and Smith, 1991) ,Clk2 (Hanks *et al*, 1992; Hanes *et al*, 1994), and Clk3 (Hanes *et al*, 1994) but more isoforms are expected to exist. Surprisingly, all of those

isoforms are also subject to the alternative splicing resulting in the production of full-length and truncated protein. It is assumed that each isoform plays a different role at the different developmental stages. Amino acid sequences of these isoforms are not identical but showed high similarity.

Recently, many dual specificity kinases have been identified and then they were classified collectively as LAMMER family kinases (Yun *et al*, 1994). LAMMER family kinases are highly conserved through evolution from yeast to human suggesting that they may have some vital functions in vivo. This family includes Wee1$^+$ (Featherstone and Russell, 1991), a regulator of yeast cell cycle, MEK (MAPK/ERK) kinase (Nakielny *et al*, 1992), KNS1 from S. cerevisiae (Padmanabha *et al*, 1991), AFC1, 2, 3 from Arabidopsis thaliana (Bender and Fink, 1994), Doa from Drosophila melanogaster (Yun *et al*, 1994), human Clk1, 2, 3 and mouse Sty/Clk. In addition to a high homology across the kinase domain, they also share a highly conserved motif (EHLAMMERILGPLP) in a subdomain X of the kinase domain. This region is not conserved among other kinases (Hanks *et al*, 1988) and therefore, it is consider to be responsible for determining substrate specificity (Yun *et al*, 1994).

a) **Doa (Darkner of apricot)**

Doa is a novel protein kinase identified from *Drosophila*. It is a dosage sensitive modifier of the apricot allele (w^a) of the white eye (w) locus (Rabinow and Birchler, 1989). Mutation at the Doa locus introduced by the insertion of the copia retrotransposon into the second intron of the w gene caused normally red eyes to be apricot-colored. Homozygous

mutation of this gene led to the death at the early larval stage of *Drosophila* development indicating this gene is essential for the development (Rabinow and Birchler, 1989). Doa function is also critical to Drosophila eye development in that the organization of pigment cells, bristle cells and photoreceptors were affected by in various mutant classes. Surprisingly, Doa gene also showed stage specific and tissue specific Doa transcripts indicating that expression of LAMMER family kinases may be regulated at the post-transcriptional level (Yun *et al*, 1994).

b) **AFC (Arabidopsis thaliana Fus-Complementing gene)**

AFC was initially identified in a screen to investigate related signal transduction pathways in higher plants (*Arabidopsis thaliana*) to complement a yeast having a mutation in the STE12 mediated signaling pathway. AFC1 induces three STE12-dependent process even in transduction defective yeast strains: mating-specific gene expression in haploid yeast, mating of haploid yeast to yield diploid, and pseudohyphal growth in diploid yeast. AFC1 has no effect on transcription of the STE12 gene and instead is likely to activate the STE12 protein through the direct interaction. However AFC1 has only limited homology to FUS3 and KSS1, endogenous yeast kinase regulators of STE12. AFC1 is not a MAPK homologue but most similar to Sty/Clk family members (41% identical to the kinase domain of Sty/Clk). Interestingly AFC also comprises multi-isoforms (AFC2 and AFC3) which showed 75% and 68% of identity to AFC1 respectively (Bender and Fink, 1994)

c) **KNS 1**

KNS 1 contains an open reading frame of 720 codons. The C-terminal of the predicted protein sequences is similar to that of many other known kinases, exhibiting 36% identity to cdc2 gene product of *Schizosaccharomyces pombe* and 34% identity to cdc28 gene product of *S. cerevisiae*. Mutants with the deletion in the gene grew at the same rate as wild type cells using several different carbon sources, mate at normal efficiencies and sporulate. No defects were discovered in entry into or exit from stationary phase implying that KNS1 gene is not essential for cell growth and a variety of other cellular process in yeast (Pamanabha *et al*, 1991).

Sty/Clk associates with SR splicing factors

The biological roles of most of LAMMER family kinases including Sty/Clk are not clear yet. In an attempt to figure out the possible biological roles of Sty/Clk, Yeast two hybrid system was exploited resulting in the discovery of a series of RNA binding proteins; hnRNP G (Soulard *et al*, 1993), RNP S1 (Schmidt and Werner, 1993), SRp20 (Ayane *et al*, 1991), SRp30a (also known as ASF/SF2, Ge *et al*, 1991; Krainer *et al*, 1991), human SRp55 (Screaton *et al*, 1995) and human SRp75 (Zahler *et al*, 1993b) indicating that Sty/Clk may be involved in alternative splicing event (Colwill *et al*, 1996)

a) **Splicing**

A characteristic of most of the primary transcripts of higher eukaryotes and some transcripts in all eukaryotes is the presence of untranslated intervening sequences (Intron). The number of introns per pre-mRNA varies considerably. Furthermore, within different pre-

mRNAs, the introns are variously distributed and have many different sizes. Introns in higher eukaryotes range from 50% to nearly 90% of the pre-mRNA. The remaining segments (Exons) are joined together to form the finished mRNA molecules. The excision of the introns and the formation of mature mRNA by joining the exons is splicing (figure 2). Introns are extremely precisely spliced out of pre-mRNA. A one-nucleotide slippage in a splice point would shift the reading frame on the 3' side of the splice to give an entirely different amino acid sequence. Such fidelity invariably is achieved by the recognition of particular base sequences. Comparison of the 5' exon-3' intron and 5' intron- 3' exon boundaries of a number of hnRNAs in diverse species of eukaryotes revealed some consensus sequences. The consensus sequences at the 5' end of vertebrate introns is AGGUAAGU, at the 3' end of introns, the consensus sequences is a stretch of ten pyrimidines (U or C) followed by any base and then by C and ending with the invariant AG. Introns also have an important internal site located between 20 and 50 nucleotide upstream of the 3' splice site called branch point. Interestingly, mutations in each of these three critical regions led to the aberrant splicing. Some forms of thalassemia, a group of hereditary anemia characterized by the defective synthesis of hemoglobin was caused by the aberrant splicing (Spritz *et al*, 1981).

Even before splicing was accomplished in vitro, several observations led to the suggestion that snRNAs assisted in the splicing reaction. Firstly, the short consensus sequence at the 5' end of introns (CAG/GUAAGU) was found to be complementary to a sequence near the 5' end at the U1 snRNA, secondly, snRNAs were found associated with hnRNPs in the nuclear extracts. The snRNAs are associated in the nucleus with six to ten proteins to form small nuclear ribonucleoprotein particles. Finally, antisera from patients with

auto immune disease, Systemic Lupus Erythromatosus (SLE) contain antibodies against a protein that is common to U1, U2, U4 and U5 snRNPs found to have great specificity for one or another of the individual snRNP. These specific antisera have been widely used in characterizing components of the splicing reaction. For example, when antisera that is specific for U1 snRNP was added to an in vitro splicing mixture, the splicing reaction was interrupted confirming the importance of the U1 snRNP in splicing reaction. Furthermore, U2 snRNP base-pairs with branch-point regions of an intron. In yeast, base-pairing between U2 RNA and the branch site occurs over six bases. However, in higher eukaryotes where the branch point sequence is less highly conserved, therefore the association of U2 snRNP with pre-mRNA is assisted by an auxiliary protein called U2AF (Zamore and Green, 1989 and 1991) which binds to the pyrimidine rich region near the 3' splice site. U2AF interacts with RNA through an RNP motif and is thought to interact with other proteins required for splicing through a domain containing repeats of the dipeptide serine-arginine (SR motif). The snRNAs in the U4 and U6 snRNPs base-pair over an extended complementary region, this complex then associates with U5 snRNP bound to the 3' splice site. The U4/U6/U5 complex then associates precisely via protein-protein interactions with the previously formed complex comprising pre-mRNA base-paired to U1 and U2 snRNPs. The resulting high molecular weight (60 S) ribonucleoprotein complex is called a spliceosome. After formation of the spliceosome, extensive rearrangements occur in the pairing of snRNAs and the pre-mRNA. The U4 and U6 dissociate from each other and U6 snRNA that is just 5' to the sequence that interacts with the branch point in pre-mRNA. U1 is thought to dissociate from the 5' splice site in pre-mRNA after U5 base-pairs with exon sequences flanking the splice sites. The

arranged spliceosome then catalyzes two consecutive transesterfication reactions that result in RNA splicing. After the second transesterfication reaction, the ligated exons are released from the spliceosome while the lariat intron intermediate remains associated with the snRNPs. This final intron-snRNPs complex is unstable and dissociates. The individual snRNPs released can participate in a new cycle of splicing and the lariat intron is degraded by some ribonucleases.

b) **Alternative splicing**

Recently it was found that a group of proteins (non snRNP proteins) other than snRNPs are components of spliceosome complex (Vijayraghavan *et al*, 1989; Reed, 1990). Main characteristic of these non-snRNP proteins is that they have RNP motif responsible for RNA-binding (Burd and Dreyfuss, 1994) as well as repeats of the dipeptide Ser-Arg (SR motif) for protein-protein interaction (Roth *et al*, 1991; Zahler *et al*, 1992; Zou and Manley, 1993; Fu, 1995). These proteins are collectively called SR proteins due to that motif. SR proteins comprise a family of evolutionally conserved pre-mRNA splicing factors (Zahler *et al*, 1992). The primary amino acid sequences of these proteins are highly related and they share an N-terminal RNA recognition motif and a C-terminal domain of variable length that consists almost exclusively of alternating Ser-Arg residues.

Many animal cells express a set of SR proteins of similar molecular size: 20 (Ayane *et al*, 1991), 30, 40, 55 (Roth *et al*, 1991), and 70-75 kD (Zahler *et al*, 1993b). The 30-kD band contains two distinct polypeptides, SRp30a and SRp30b, which have also been described as SF2/ASF (Ge *et al*, 1991; Krainer *et al*, 1991; Tacke *et al*, 1992) and

PR264/SC35 (Fu and Maniatis, 1990, 1992) respectively. Individual SR proteins isolated from vertebrates and invertebrates function similarly as essential splicing factors when tested in depletion and reconstitution assays (Ge *et al*, 1991; Krainer *et al*, 1991; Mayeda *et al*, 1992; Zahler *et al*, 1992) indicating that a degree of redundancy in their function however, SR proteins have distinct specificity with regard to alternative spice site selection (Zahler *et al*, 1993a) and it was also found that a single SR protein commits different pre-mRNAs to the splicing with a high substrate specificity (Fu, 1993). These SR proteins are implicated in alternative splicing event which is a common mechanism of regulating gene expression in eukaryotes. This splice site selection is dependant on the type, amount (Fu and Maniatis, 1993; Zahler *et al*, 1993a) and phosphorylation status of SR proteins however this alternative splicing is strictly regulated for instance, in different cell types, at different stages during development, and at early and late stages of infections by SV40 and adenovirus. Changes in SR protein expression have been demonstrated at the transcription level during B cell development (Ayane *et al*, 1991), insulin and mitogen induction (Diamond *et al*, 1993), and T cell activation by phytohematoglutinin (Screaton *et al*, 1995). Furthermore over-expressed two SR proteins, ASF/SF2 and SC35 affected alternative splicing in vivo in diverse ways (Wang and Manley, 1995) although they showed equivalent activities in vitro, affecting alternative 5' and 3' splice site selection with adenovirus E1a pre-mRNA; over-expressed ASF/SF2 gave rise to shifts in alternative splicing. SC35 over-expression also altered E1a splicing but the pattern was distinct from that detected with ASF/SF2, indicating that the two proteins can function differently in vivo. Furthermore it was also found that SC35 and ASF/SF2 specifically interact with both the U1snRNP-70K and with the 35 kD

subunit of U2AF (U2AF35) (Wu and Maniatis, 1993). These observation led to the suggestion that SC35 and other members of SR proteins may function in splice site selection by acting as a bridge between components (U1 snRNP and U2AF) bound to the 5' and 3' splice sites and the protein-protein interactions mediated by SR proteins may also play an important role in the regulation of alternative splicing. More recently it was also revealed that SR proteins are required for the first step of spliceosome assembly (Fu, 1993) and interaction of the U1 snRNP with the 5' splice site of the pre-mRNA and individual SR proteins have distinct abilities to promote interaction of U1 snRNP with alternative 5' splice junctions as well (Zahler *et al*, 1995). These results suggest that SR proteins direct 5' splice site selection by regulation of U1 snRNP assembly onto pre-mRNA. In addition, the disruption of two SR proeins (ASF/SF2 and SRp55) led to the defect in the viability of a chicken B-cell line DT40 (Wang *et al*, 1996) and the development of Drosophila (Ring and Lis, 1994) at the early stage respectively, which suggests that alternative splicing by SR proteins is an important mechanism to regulate the differentiation and development and furthermore, supports the notion that Sty may involved in the differentiation process.

As described earlier, alternative splicing is also regulated by phosphorylation of SR proteins. In vitro, Sty /Clk phosphorylated ASF/SF2 on Ser residue located within its RS domain (Colwill *et al*, 1996a and 1996b) and it was found that Ser/Arg rich domain in N-terminal regulatory domain of Sty/Clk itself is critical for the binding to SR proteins (Colwill *et al*, 1996a). Furthermore, immunofluorescence studies showed that a catalytically inactive form of Sty/Clk is co-localized with SR proteins in nuclear speckles which is considered to be the storage sites of splicing components while over-expression of active Sty/Clk caused

the despecklization and the redistribution of SR proteins within the nucleus (Colwill *et al*, 1996a) suggesting that Sty/Clk directly regulates the activity and compartmentalization of SR proteins in the nucleus by the phosphorylation of SR proteins.

There are some other protein kinases considered to phosphorylate SR proteins in vivo. The first kinase activity identified, U1 70K kinase is associated with the small nuclear ribonucleoproteins (snRNPs) (Woppmann *et al*, 1993) although the cDNA for U1 70K kinase has not been cloned yet. The second kinase, SRPK1, was initially cloned based on the its ability to phosphorylate SC35 (Gui *et al*, 1994a). It is a serine kinase which is regulated by cell cycle, phosphorylates SR proteins and induces the disassembly of nuclear speckles (Spector *et al*, 1993a and 1993b) in permeabilized cells similarly to Sty/Clk (Gui *et al*, 1994a). Interestingly, over-expression of SRPK1 inhibited splicing of β-globin pre-mRNA in vitro indicating that SR proteins phosphorylated by over-expressed SRPK1 affect the splicing (Gui *et al*, 1994a). SRPK1 and Sty are 32% identical across kinase domain, including key amino acids that are likely to be involved in substrate specificity and phosphorylate the same SR proteins in vitro (Colwill *et al*, 1996a). Furthermore according to the recent study, SRPK1 has the higher specificity toward ASF/SF2 than Sty. In vitro, Sty phosphorylated Ser-Arg, Ser-Lys or Ser-Pro sites, whereas SRPK1 had a high preference for Ser-Arg (Colwill *et al*, 1996b) suggesting that Sty has a broader substrate specificity than SRPK1 and those two protein kinases may play different roles in regulating SR splicing factors in vivo.

Many evidences that phosphorylation/dephosphorylation of SR proteins is also critical for spliceosome formation and splice site selection have been accumulated. Protein

phosphatase 1 (PP1) either inhibited the formation of spliceosome complex at the early step (Mermoud *et al*, 1992 and 1994) or switched splicing to the proximal 5' splice site (Cardinali *et al*, 1994). Addition of phosphorylated SR proteins relieved this inhibition in spliceosome assembly (Mermoud *et al*, 1994), implying that the function of SR proteins is regulated by phosphorylation and the expression level, and spliceosome assembly requires protein phosphorylation. However, catalysis of pre-mRNA splicing requires dephosphorylation steps to proceed, which was confirmed by the finding that inhibitors of protein phosphatase 2A (PP2A) blocked the first step of the catalysis during which the 5' splice site is cleaved, while inhibitors of both PP1 and PP2A blocked this step and the second step of 3' splice site cleavage and exon ligation (Mermoud *et al*, 1992; Tazi *et al*, 1992).

P19 EC cell

In culture, pluripotent murine embryonal carcinoma cell line, P19 can differentiate into many types (McBurney *et al*, 1982) similar to those normally found in embryos and therefore have been used to investigate some developmental events as a model system. Central to the understanding of embryonic development are mechanisms of the commitment of early embryonic cells to form divergent cell types resulting in a fetus. P19 EC cells provide a system in which drugs can be used to manipulate the formation of tissues which normally comprise the fetus.

a) **Neuronal differentiation of P19 EC cells**

Retinoic acid (greater than 5×10^{-7} M) for as little as 48 hr efficiently induces the neuronal differentiation process resulting in the development of neurons, fibroblasts,

astroglia and microglia cell types normally derived from the neuroectoderm (Jones-Villeneuve *et al*, 1982 and 1983). The neurons were initially identified by their morphology and by the appearance of neurofilament within the cytoplasm. Neuronal nature of these cells was further documented by showing that their cell surfaces displayed tetanus toxin receptors, and a neuronal cell marker. Several days before the appearance of neurons, there was a marked decrease in the amount of an embryonal carcinoma surface antigen and at the same time, there was also a substantial decrease in the volumes of individual cells. The efficiency of RA induced-differentiation was dependent on the status of P19 cells in the cell cycle but critically dependent on the ratio between the number of cells and the moles of RA in the culture medium (Berg and McBurney, 1990). P19 cultures plated at lower cell density were more efficiently induced to differentiate than cultures containing cells at higher cell density. This difference is not due to cell to cell contact but may be related to the rapid metabolism of RA by the cells plated at the higher density. The majority of neurons formed by the treatment of P19 with RA contained GABA, glutamic acid decarboxylase and GABA transaminase. Neuropeptide Y and somatostatin were less frequently found and both were partially co-expressed with GABA and with one another. Smaller number of cells were positive for tyrosine hydroxinase, DOPA decarboxylase, serotonin, calcium gene-related peptide, gelatin and substance P. The variety and proportions of cells having different transmitter types were reproducible and varied very little over 40 days in culture except for cells containing enkephalin which were abundant only in mature cultures of 32 days or more. Synapsis formed between neurons and some contained both small clear and large dense core vesicles within the presynaptic bouton because GABA, neuropeptide Y an somatostatin are abundant in P19-

derived neurons, in embryonic neurons and in rostral regions of the mammalian CNS (Staines *et al*, 1994) indicating that the developmental events occurring in P19 cell culture are close to those of embryonic neuroectoderm.

b) **Instability of transfected genes in P19 cells**

Introduction of recombinant genes into mammalian cells in culture has been a very important step in understanding the molecular mechanisms of various cellular process but unfortunately the efficiency which plasmids containing recombinant genes are expressed following stable integration into genomes of embryonal carcinoma cells is extremely low (McBurney *et al*, 1994b) although the plasmids can be easily and efficiently transfected into P19 cells. Therefore it is so difficult to isolate stable clones of cells expressing genes. Even in clonal populations derived from the transfected cells, the introduced genes were expressed in some but not all cells (McBurney *et al*, 1994b).

The mechanism responsible for this loss of gene expression is not clear yet but Schmidt-Kastner *et al.* performed a series of experiments to investigate the mechanism (Schmidt-Kastner *et al*, 1996). First, they transfected P19 cells with a construct harboring *E. coli* β-gal gene and examined the expression of the gene in the clonal populations of cells. Cells both carrying and expressing β-gal gene decreased in number at a rate of approximately 0.02 events per cell and per cell division. Interestingly, these β-gal gene non-expressing cells were of two types, some lost the transfected gene itself while others contained inactivated genes. The inactivation turned out to be irreversible and was at the level of transcription initiation but not associated with increased DNA methylation which is a known mechanism

partly responsible for this mechanism in many cases (Schmidt-Kastner *et al*, 1996). Furthermore, since transfected DNAs integrate into the host genome as tandem arrays, the gene loss and inactivation seen in EC cells may be analogous to the repeated gene- induced inactivation seen in lower eukaryotes.

c) **Enhancement of integration of the transfected gene into genomes of P19 by B17**

It was demonstrated that a construct (B17) carrying the promoter and intragenic region of murine PGK-1 gene were expressed with high efficiency in P19 cells (McBurney *et al*, 1994a and 1994b). This enhanced expression was related to increased copy number of plasmid DNA stably integrated into the genomes of P19 cells and furthermore, the increased high copy number seemed to result from enhanced ligation of transfected plasmids because co-transfected plasmids were also integrated in increased numbers. The enhanced integration and expression of the transfected plasmids required active transcription through an intragenic region of PGK-1(B17), perhaps resulting in more recombinogenic plasmid DNAs. However ironically, subsequent studies showed that these recombinogenic plasmid DNAs incorporated into the genome were also excised efficiently from the host genome by recombination enhanced by transcription through the tandem repeated sequences of the transfected plasmid DNAs (McBurney *et al*, 1994b).

Inducible system

The introduction of heterologous genes into cultured mammalian cells or tissues is fundamental for understanding the biochemistry, genetics and functions of genes and gene

products. Therefore, the ability to regulate the transcription of transduced foreign genes in mammalian cells and transgenic animals will facilitate the analysis of gene function during embryonic development and cellular differentiation and for the studies of gene therapy. This is much more important in which the gene product is toxic to the cells or must be maintained at appropriate levels. Ideally, regulation of expression of the delivered gene should be in an on or off manner; when turned on, gene expression should be highly induced and when turned off, it should be silenced. Attempts to control gene activity by various eukaryotic promoters responsive to, for example, heavy metal (Brinster *et al*, 1982; Mayo *et al*, 1982; Searle *et al*, 1985), interferon (McCormick *et al*, 1984), heat shock (Nouer, 1991) or hormones (Hynes *et al*, 1981; Lee *et al*, 1981; Klock *et al*, 1987; Israel and Kaufman, 1989) have generally suffered from the leakiness of the inactive state, low level of induction or from pleiotropic effects caused by inducers used for the systems such as elevated temperature, metal ion or glucorticoid hormone action. In search for better inducible systems that do not rely on endogenous control elements, several groups demonstrated that two prokaryotic inducible promoters function in mammalian cells.

The tetracycline inducible system is based on regulatory elements of the Tn10-specified tetracycline-resistant operon of *E.coli* (Hillen and Wissman, 1989) in which transcription of resistance-mediating genes is negatively regulated by the tetracycline repressor (TetR). Recently by combining TetR with the C-terminal domain of VP16 from Herpes simplex virus (HSV) known to be essential for the transcription of the immediate early genes (Triezenberg *et al*, 1988), a hybrid transactivator (tTA) that stimulates minimal promoters fused to tetracycline operator sequences (tetO) was generated. However, in order

to exploit this system, cell lines that stably express tTA must first be established. For this difficult purpose, a conventional transfection method such as calcium phosphate co-precipitation may not be efficient for the transfer of tTA gene into the cell of interest. Furthermore, efficient expression of tTA is toxic to the cells probably due to the squelching effect (Gill and Ptashne, 1988) generated from the VP16 transactivation domain in tTA. Recently, many groups developed new vector systems to control the expression of tTA gene itself in an inducible manner to overcome the squelching effect of tTA.

The other prokaryotic inducible promoter used extensively is Lac promoter in Lac operon of *E.coli* (Riggs *et al*, 1970; Miller and Reznikoff, 1980) . Genes encoding enzymes important for lactose metabolism (lacZYA) are under the negative control of a repressor tetramer consisting of 38.6 kD subunits encoded by lacI gene. This protein can specifically bind to both allo-lactose and to a unique operator sequence (lacO) positioned immediately downstream of the lac promoter (lacP). In vitro, under standard conditions (I=0.5 M [pH 7.4], 24 °C), the equilibrium association constant governing the binding of repressor to lacO is 10^{13} M^{-1} (Riggs *et al*, 1970). Binding of repressor to lacO significantly inhibits transcription from lacP. Normally, the rate of lac transcription is regulated by the concentration of allo-lactose or an analogue such as isopropyl β-D-thiogalactoside (IPTG). These compounds can function as inducers of lac operon transcription and appear to act by binding to repressor and decreasing the affinity of the latter for lacO, thereby allowing increased transcription of the lac operon. In vitro, under the standard condition, the equilibrium association constant for the repressor-IPTG is approximately 3 X 10^{10} M^{-1}, i.e, 300-fold lower than that of free repressor for lac O. After the initial conceptual introduction of this system as an useful inducible system

in mammalian cells, three basically different approaches have been described: i) prevention of transcription initiation by properly placed lac operators at promoter sites (Brown *et al*, 1987; Hu and Davidson, 1987; Figge *et al*, 1988; Deuschle *et al*, 1989; Fuerst *et al*, 1989), ii) blockage of transcribing RNA polymerase II during elongation by lac repressor/operator complex (Lac R/O) (Deuschle *et al*, 1990), and iii) activation of a promoter responsive to a fusion protein between LacR and activation domain of virion protein 16 (VP16) of herpes simplex virus (HSV) to reduce the basal expression of target genes in the absence of inducers and maximize the induction level in the presence of inducers (Labow *et al*, 1990; Baim *et al*, 1991). However, at present, the utility of LacR/O-based systems in mammalian cells is limited since the inducer isopropyl-β-D-thiogalactoside (IPTG) , despite its rapid uptake (about 4 hr) and intracellular stability (Wyborsky and Short, 1991) acts rather slowly and inefficiently resulting in only moderate induction. Currently Tet inducible system has been superseding lac inducible system because of the specificity of TetR for its operator sequence as well as the high affinity (Takahashi *et al*, 1986) of tetracycline for TetR and well-studied chemical and physiological properties of tetracycline and the development of new tetracycline inducible system to adopt the advantage of retroviral vector system in order to deliver genes of interest more efficiently into any mammalian cells (Idia *et al*, 1996; Paulus, *et al*, 1996).

The main object of my project is to identify biological substrates of Sty in vivo and to check the possibility that Sty alone can give rise to the differentiation of P19 cells. In order to answer these two question, establishment of Sty stable clones is indispensable but according to the previous studies, long term constitutive over-expression of Sty was cytotoxic to the cells. Therefore to overcome this problem, I exploited Lac inducible promoter system.

Figure 1. Alternative splicing of Sty pre mRNA
Inclusion and exclusion of alternatively spliced exon II results in the full length catalytically active and truncated catalytically inactive protein respectively.

Figure 2. Illustration of the Splicing reaction
Phosphorylation of SR proteins and other proteins (snRNPs) is required for the spliceosome assembly. Once spliceosome complex containing U1, U2, U4/U6.U5 snRNPs is formed, at least two separate dephosphorylation events are required for two consecutive transesterfication reacion. with PP1 necessary for the first transesterfication and PP2A for the second one. Dephosphorylation of U1 70 kD protein may also be required for the first catalysis step. Black dot indicates the branch point.

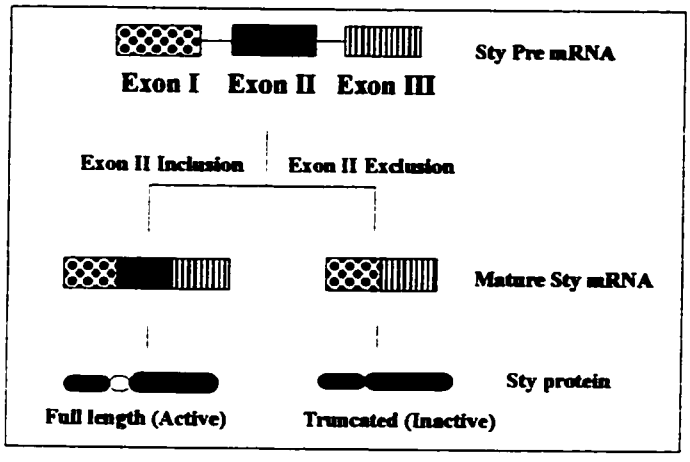


Figure 1.

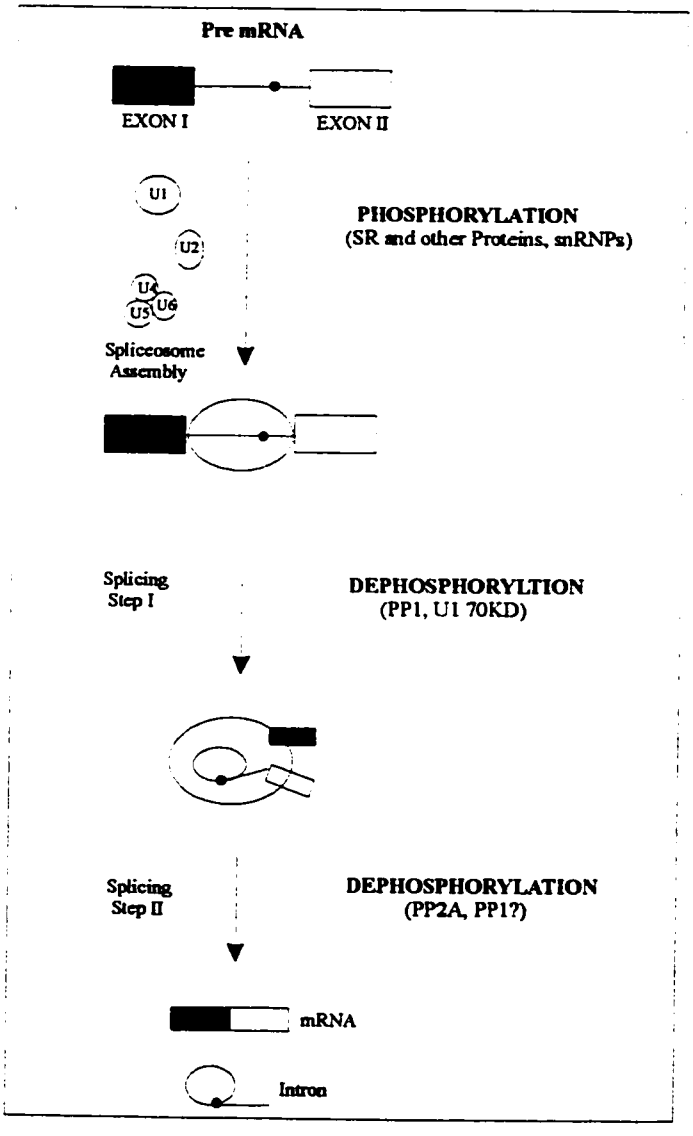


Figure 2.

PART I

Establishment of P19 Lac R expressing cell line

Materials and Methods

Construction of Lac repressor expression vector, PCJ2LacIN

Lac repressor expression vector, PCJ2LacIN, was generated by the insertion of PGK-1 promoter, NLS (nuclear localization signal) (Roberts *et al*, 1987) and Lac repressor gene of the parental vector, PGKLacIN (Hannan *et al*, 1993) into another mammalian expression vector PKJ 74 containing IRES (Gurtu *et al*, 1996) and a hygromycin resistant gene. A fragment comprising PGK-1 promoter, NLS and Lac repressor gene was amplified from PGKLacIN by the conventional polymerase chain reaction (PCR) using the following two primers: upstream (5'-ctcgaggaattcacc-3') and downstream (5'-atcgatctgcagctta-3'). Briefly, PCR reaction was carried out with 25 pmoles of each primer, 0.2 mM of each dNTP, 1,25 units of *Taq* polymerase in 50 mM KCl, 10 mM Tris-HCl, pH 8.4 and 2.5 mM $MgCl_2$. PCR conditions were 94 °C, 5 min; 65 °C, 2 min; 72 °C, 3 min for 1 cycle and subsequently 94 °C, 1 min; 65 °C, 2 min; 72 °C, 3 min for 25 cycles and finally 94 °C, 1 min; 65 °C, 2 min; 72 °C 10 min for 1 cycle. Amplified product (approximately 1.8 kb in size) was resolved by 1 % agarose gel electrophoresis (1%) and ethidium bromide staining. This fragment was subsequently digested with XhoI and Cla I and subcloned into PKJ 74 previously digested with Xho I and Cla I.

P19 EC cell culture and stable transfection with PCJ2LacIN

P19 EC cells were maintained in α-MEM supplemented with 10 % serum (FCS: DBS=3:1). For transfection to obtain the stable cell line expressing Lac R, cells were trypsinized, counted and plated out at a density of 1 x 10^6 cells in 100 mm dish and next day, 5 μg of each construct; PCJ2LacIN, pPOP-βgal and B17, was added to 500 μl of 0.25 M $CaCl_2$ and then 500 μl of 2 X BES buffer (pH 6.89) was added to the DNA mixture drop by drop and incubated for 20 min at RT to allow precipitates to form. The precipitates were added to the cells with rotating the dishes and incubated at 37 °C. 9 hrs later, cells were washed with cold PBS three times and fed with the fresh media. Next day, transfected cells were trypsinized and 1/10 cells were plated out and selected with hygromycin (200 μg/ml). 2 weeks later, about 60 drug resistant colonies were picked and expanded for further experiment. Each clone was trypsinized, split into 3 dishes; one dish of the cells were saved for further experiments, another dish was IPTG-untreated and the other one was induced with 5 mM IPTG for 15 hr and subject to X-gal staining to find the best inducible Lac repressor expressing clones preliminarily. X-gal staining was performed as follows: first cells were washed with cold PBS three times, fixed with 2 % formaldehyde, let them at room temperature for 30 min, washed again with PBS and finally incubated with X-gal solution (0.1 M phosphate buffer, 0.04 % X-gal, 1 mM $MgSO_4$, 2 mg potassium ferrocyanide and 1 mg potassium ferricyanide) 37 °C for several hours. Finally several clones were selected for β-gal assay to determine the best Lac repressor expressing cell line.

β-gal assay and establishment of CJ2-8 Lac R stable cell line

For β-gal assay, cells were washed with cold PBS three times, scraped with the

rubber policeman with 200 µl of sucrose cell harvest buffer (0.25 M sucrose, 100 mM $NaPO_4$ buffer pH 7.0 and 100 mM EDTA) per 100 mm dish, transferred to a fresh microfuge tube and subject to repeated freezing and thawing three times and centrifuged. Protein was quantified by protein assay kit (Bio-Rad) and 20 µg of protein was added to 800 µl of assay buffer (100 mM Tris-Cl pH 7.5, 100 mM β-Mercaptoethanol and 10 mM $MgSO_4$) and subsequently mixed with 200 µl of ONPG stock solution (4 mg/ml) resolved in the assay buffer. Samples were run on spectrophotometer using the kinetics program (420 nm, 30 min, Beckman DU40 spectrophotometer)

Results

Construction of Lac repressor expression vector

The parental Lac repressor expression vector PGKLacIN (Hannan et al, 1993) has no drug resistance gene in it required for the selection and also no suitable restriction enzyme site between Lac repressor gene and PGK-1 polyadenylation signal. In order to generate a bi-cistronic LacR expression vector containing NLS-fused to Lac repressor gene (Hu and Davidson, 1991), IRES and a drug resistant gene and SV40 poly A^+, the sequences containing PGK-1 promoter, nuclear localization signal (NLS) (Roberts et al, 1987) and Lac R gene were PCR amplified using two PCR primers which were tagged with XhoI and ClaI respectively to facilitate subcloning from the PGKLacIN and subsequently the PCR product (about 1.8 kb) was cloned into the linearized PKJ 74 with Xho I and Cla I previously. PKJ 74 contains internal ribosome entry site (IRES) (Gurtu et al, 1996), and a hygromycin

resistance gene. Therefore the resulting bi-cistronic expression vector PCJ2LacIN (figure 3) has some advantages that Lac R gene and a hygromycin resistant gene are driven by a common PGK-1 promoter yielding one transcript encoding two polypeptides at the same time and therefore, subsequent selection with hygromycin can automatically ensure the expression of LacR in cells. In addition, NLS in PCJ2LacIN also allows the Lac repressor to translocate into the nucleus for its function. It is a very important feature in that prokaryotic cells don't have nuclear membrane and Lac repressor is originally a protein from bacteria therefore, without the NLS, the translocation of Lac repressor may be substantially interrupted.

Establishment of Lac R stable cell line

Following the co-transfection with PCJ2LacIN and pPOPβ-gal and the selection with hygromycin (200μg/ml), the best LacR stable cell line was determine simply by performing β-gal assay (figure 4). pPOPβ-gal co-transfected with PCJ2LacIN contains two lac operator sequences which is to be bound by Lac repressor protein to repress the expression of β-gal gene downstream of it. According to the assay, a stable clone called CJ2-8 showed very low level of basal β-gal activity (almost similar to the level of un-transfected P19 cells) in the absence of IPTG, while when it was induced with 5 mM IPTG for 15 hrs, it showed about 35-fold induction in β-gal activity compared with that of uninduced one indicating that in the absence of IPTG, CJ2-8 cells produced functional and enough amount of Lac repressor to repress the expression of β-gal gene tightly in nucleus and also 5 mM of IPTG can efficiently induce the expression of β-gal gene.

Figure 3. Construction of Lac R expression vector, PCJ2LacIN
a) The sequence comprising PGK-1 promoter, nuclear localization signal (NLS) and Lac R cDNA sequence was amplified from the PGKLacIN by PCR using two primers tagged with Xho I and Cla I restriction enzyme site respectively. Approximately 1.8 kb DNA band amplified was isolated from the 1% agarose gel and digested with both Xho I and Cla I. b) PKJ 74, it was also digested with Xho I and Cla I. the isolated DNA fragment contains internal ribosome entry site (IRES), a hygromycin resistant gene and SV 40 polyadenylation signal. These two DNA fragments were ligated to generate a final Lac R expression vector, PCJ2LacIN.

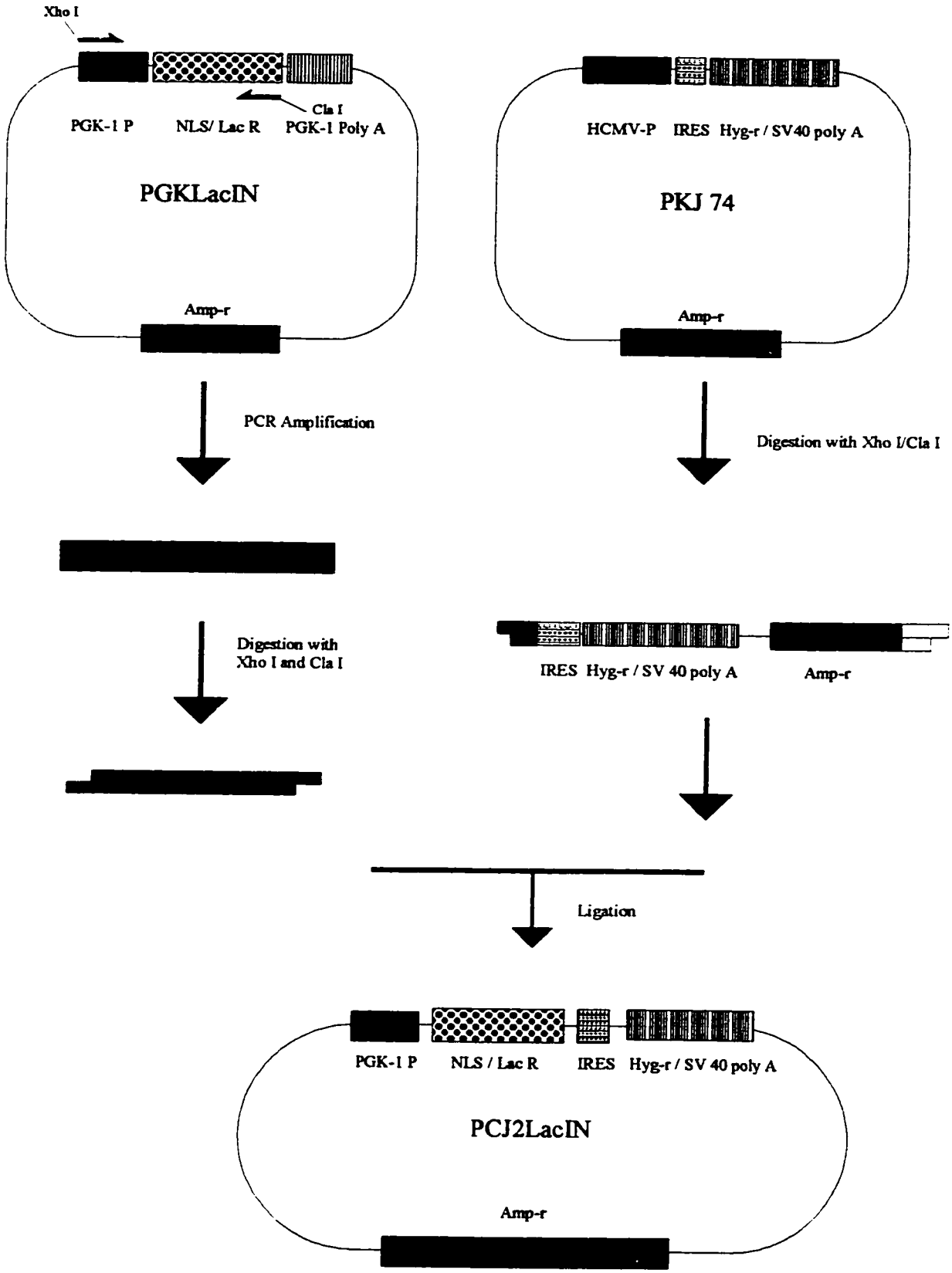

Xho I
Cla I
PGK-1 P
NLS/ Lac R
PGK-1 Poly A
PGKLacIN
Amp-r
HCMV-P
IRES
Hyg-r / SV40 poly A
PKJ 74
Amp-r
PCR Amplification
Digestion with Xho I/Cla I
Digestion with
Xho I and Cla I
IRES
Hyg-r / SV 40 poly A
Amp-r
Ligation
PGK-1 P
NLS / Lac R
IRES
Hyg-r / SV 40 poly A
PCJ2LacIN
Amp-r

Figure 3.

Figure 4. β-gal assay for a P19 Lac R expressing cell line, CJ2-8
In order to check the expression of Lac R and the induction level of β-gal gene in pPOPβ-gal construct, β-gal enzyme assay was performed. **1** indicates CJ2-8 cells treated with 5 mM IPTG for 15 hrs, **2** indicates CJ2-8 cell lines untreated with IPTG. **3** represents un-transfected P19 cells as a negative control. Approximately 20 μg of total lysates from the each sample was added to the reaction solution (800 μl assay buffer and 200 μl ONPG) and analyzed by spectrophotometer (30 min, 420 nm).

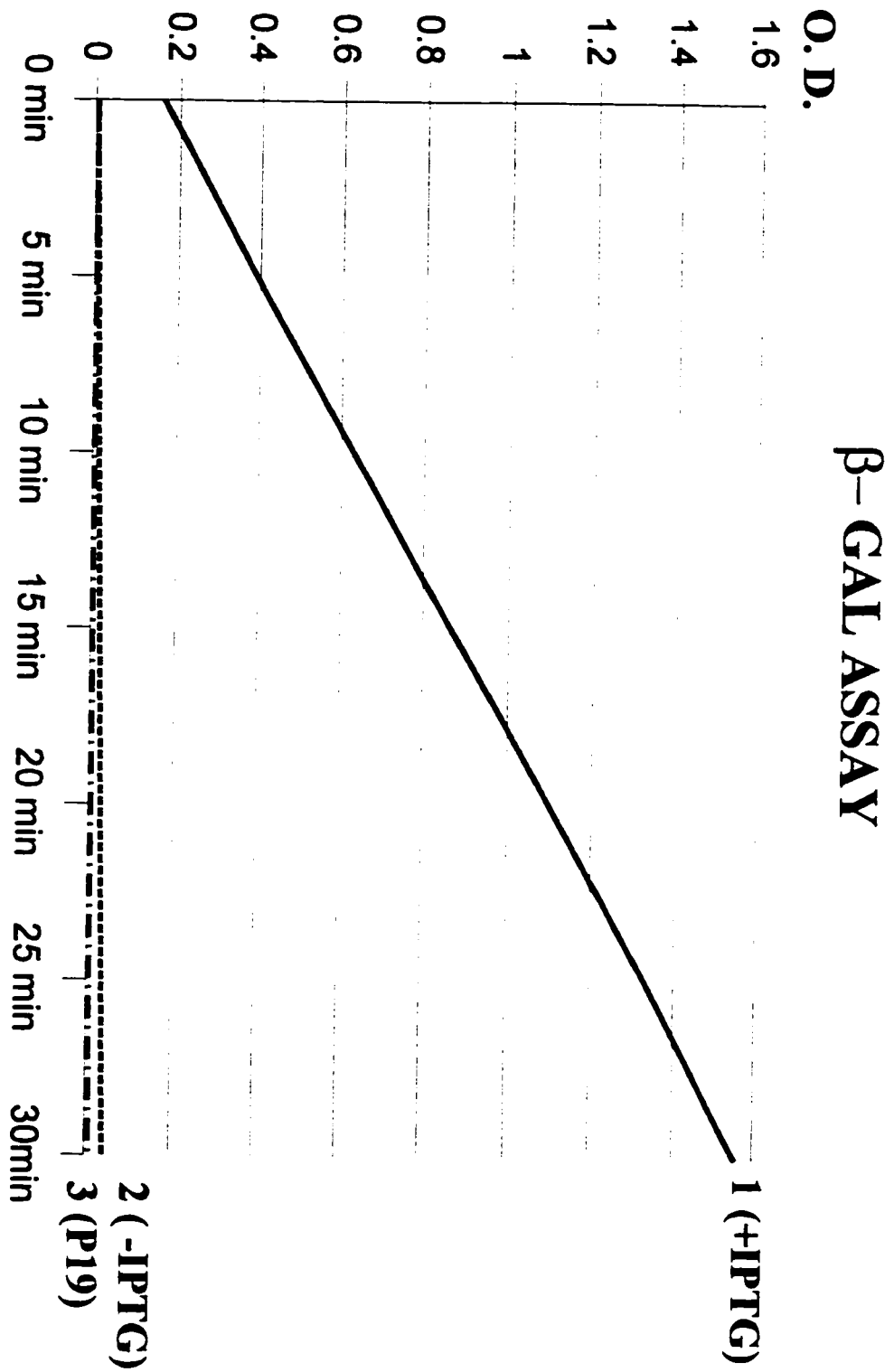

β– GAL ASSAY
O. D.
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 min
5 min
10 min
15 min
20 min
25 min
30min
1 (+IPTG)
2 (-IPTG)
3 (P19)

Figure 4.

PART II

Establishment of Myc-Sty inducible clones in CJ2-8 cells

Materials and Methods

Construction of Sty/Clk expression vector pPOPMycSty

In order to generate an inducible Sty/Clk expression vector containing two Lac operator sequences, first, Myc-Sty cDNA fragment was isolated by the digestion of PECEMyCSty with Bgl I and Sst I, blunt-ended with T4 DNA polymerase and subsequently cloned into the Sma I site of pPOP (Hannan et al, 1993), a target vector with two Lac operator sequences separated by 40 bps which are bound by Lac repressor resulting in the repression of gene expression downstream of PGK-1 promoter.

Maintenance of Lac repressor cell line (CJ2-8) and transient transfection with pPOPMycSty

CJ2-8 cells were always maintained in the α-MEM (FCS:DBS=3:1) supplemented with hygromycin (200 μg/ml) to make the cells express Lac repressor proteins continuously. For the transient transfection with pPOPMycSty to verify the expression vector, pPOPMycSty can express the inducible Myc-Sty protein of the expected size, CJ2-8 cells were trypsinized and split into three dishes at a density of 1 X 10^6, the next day 30 μg of pPOPMycSty construct was mixed with 1 ml of 0.25 M $CaCl_2$ and subsequently 1 ml of 2 X BES buffer was added gradually to the DNA/$CaCl_2$ mixture and let them to precipitate for 20

min and finally 1ml of precipitate was added into one dish of cells drop by drop with rotating the dish gently and remaining 1 ml of it was added to another dish. 8 hrs following the addition of precipitates, cells were washed with cold PBS three times to remove the excess precipitates and then fed with the fresh media supplemented hygromycin (200 μg/ml). 15 hrs before the harvest, one dish of the transfected cells were treated with 5 mM IPTG, 24 hrs after the transfection the cells were washed with PBS again and harvested with SDS-sample buffer for the western blot analysis.

Western blot

About 100 μg of proteins obtained from each sample above were resolved by 10 % SDS-PAGE, proteins were transferred to nitrocellulose membrane and blocked with 5 % skim milk in TBST (TBS+0.05% Tween 20) for 1 hr. Anti-Myc Ab (9E10) was added at 1:100 for 2 hr incubation at room temperature. Membranes were then washed with TBST three times and incubated with a 1:5000 dilution of horseadish peroxidase conjugated goat α-mouse secondary antibody (Bio-Rad) for 1 hr and washed with TBST, three times and subsequently developed using chemiluminescence reagent (KPL).

Stable transfection with pPOPMycSty into CJ2-8 cells

Two sets of CJ2-8 cells were transfected with 7.5 μg of (pPOPMycSty, PGKNeo and B17) and PGKNeo (CJ2-8Neo) alone respectively by the calcium phosphate method. 24 hrs after the transfection, cells were selected with both hygromycin (200 μg/ml) and G418 (400 μg/ml). 15 days later, drug resistant colonies of cells from the first set of transfection

were picked and expanded for western blot analysis and for further characterization of the stable clones. Each clone was split into three small dishes; one for further expansion, another for no induction and the other for IPTG-induction. One dish for the induction was treated with 5 mM IPTG overnight and next day, cells were harvested with SDS-sample buffer supplemented with 5 % BME. Each sample was analyzed by 10 % SDS-PAGE and western blot using α-Myc monoclonal Ab (9E10) as a primary antibody and HRP-conjugated secondary antibody (Bio-Rad) and detected with the chemiluminescence kit (KPL).

Results

Construction of inducible Sty expression vector, pPOPMycSty

An inducible Myc-Sty expression vector, pPOPMycSty was generated simply by cloning the Bgl I and Sst I fragment encompassing Myc epitope tagged Sty cDNA into the Sma I site of the parental vector, pPOP which has 5' PGK-1 promoter and two Lac operators separated by 40 bps and multi cloning site (MCS) and 3' PGK-1 poly A^+ tail (figure 5). The mammalian PGK 1 gene is X linked and encodes the enzyme 3-phosphoglycerate kinase. Several features of the gene suggested that the use of its promoter to drive the expression of other genes would have significant advantages in cell culture work and for use in transgenic animals; The PGK 1 gene is a housekeeping gene which is expressed at high levels in virtually all cell types and is comparable in the activity to the viral promoters such as SV40 and RSV. However unlike these viral promoters the PGK promoter is highly active in embryonic tissues

Figure 5. Construction of inducible Myc-Sty expression vector, pPOPMycSty
a). PECEMycSTY vector, it was digested with Bgl I and Sst I to isolated Myc-Sty cDNA fragment and blunt ended. b). pPOP was digested with SmaI. The Bgl I/Sst I fragment was cloned into the Sma I site of pPOP. The resulting Myc-Sty expression vector, pPOPMycSty contains the inducible promoter containing two lac operator sequences separated by 40 bp and Myc-Sty cDNA at the downstream of it therefore, the expression of Myc-sty is regulated by Lac repressor and an inducer (IPTG).

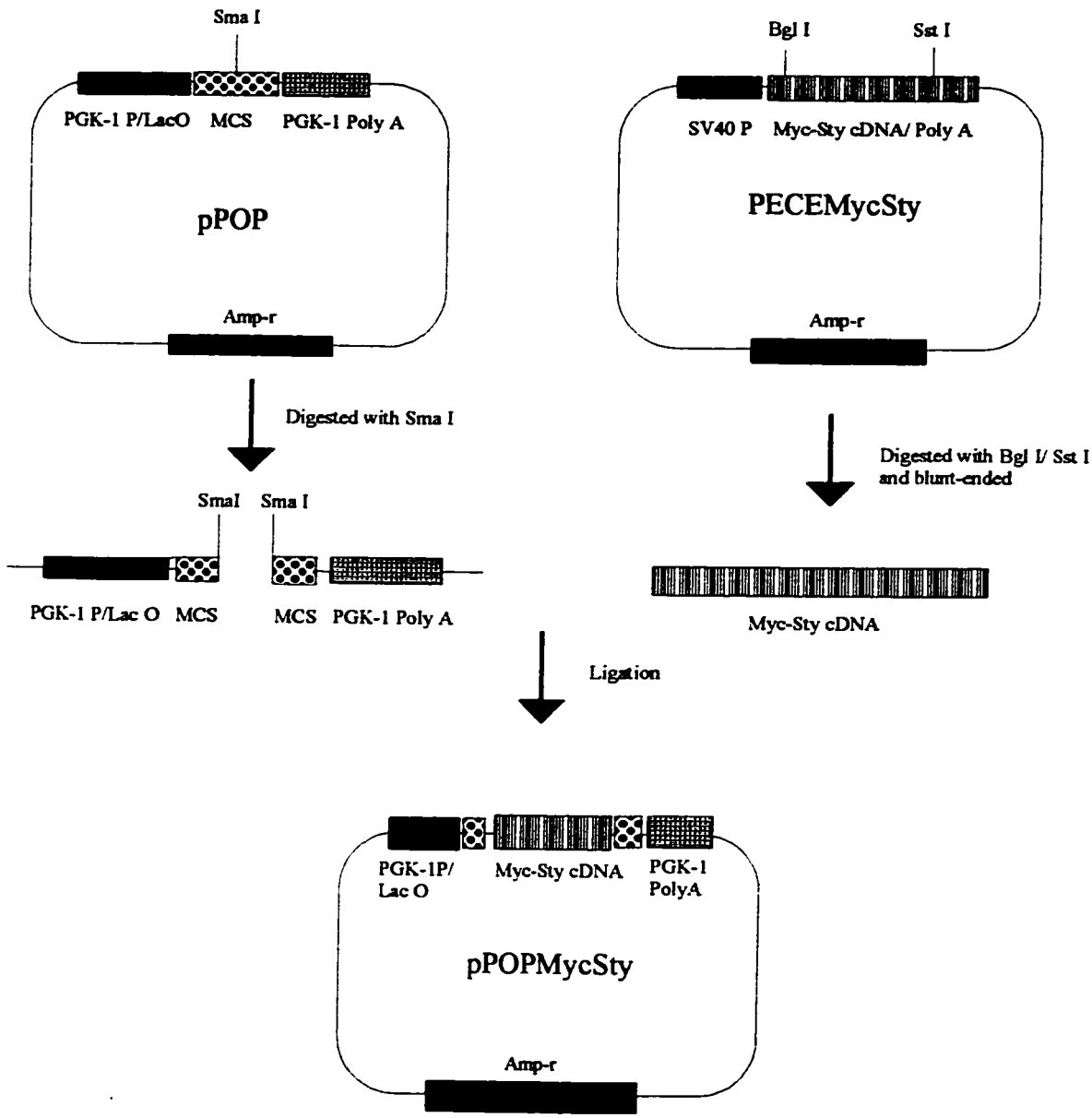

Sma I
PGK-1 P/LacO
MCS
PGK-1 Poly A
pPOP
Amp-r
Digested with Sma I
SmaI
Sma I
PGK-1 P/Lac O
MCS
MCS
PGK-1 Poly A
Bgl I
Sst I
SV40 P
Myc-Sty cDNA/ Poly A
PECEMycSty
Amp-r
Digested with Bgl I/ Sst I and blunt-ended
Myc-Sty cDNA
Ligation
PGK-1P/ Lac O
Myc-Sty cDNA
PGK-1 PolyA
pPOPMycSty
Amp-r

Figure 5.

and in particular, in mouse embryonic stem (ES) cells (Riele et al, 1990; Mortensen et al, 1991).In this context, PGK-1 promoter can be also suitable in P19 cells. Another interesting feature of pPOPMycSty, Myc-Sty inducible expression vector, is to have two lac operator sequences separated by 40 bp within the promoter region. It was demonstrated that this distance allowed this vector to achieve 90-98% of maximal inhibition and this repression was efficiently relieved by the inducer, IPTG (Hannan et al, 1993). Myc sequence allows Myc-Sty to be detected by α-human Myc monoclonal antibody (9E10). Initially no antibody against Sty was available but in the middle of my project, the antibody recognizing N-terminus of Sty was established therefore it could be also exploited for some western blot analysis to detect both Myc-Sty and endogenous Sty.

Transient transfection of pPOPMycSty into CJ2-8 cells

To verify that pPOPMycSty can express the inducible protein of expected size, CJ2-8 cells were transiently transfected with pPOPMycSty using the calcium phosphate method. Comparing expression level of MycSty between IPTG-untreated and treated for 15 hrs, as shown in figure 6, IPTG-untreated cells showed slightly repressed Myc-Sty expression but relatively high basal expression, while IPTG-treated ones showed enormously induced Myc-Sty protein of expected size(approximately 68 kD in size). The relatively high basal expression in transient transfection can be explained taking into account enormous copies of Myc-Sty gene may be expressed at the same time in a small portion of cells when they were transfected transiently. Despite of the relatively high basal expression of Myc-Sty, the result

Figure 6. Transient transfection of pPOPMycSty into CJ2-8 cells
To check that pPOPMycSty can express Myc-Sty of the right size (about 68 kD) and the inducibility of its expression in CJ2-8 Lac R expressing cell line, transient transfection was carried out. Lane 1 represents un-transfected CJ2-8 cells as a negative control, lane 2 represents CJ2-8 cells transfected with PECEMycSty construct as a positive control, lane 3 showed CJ2-8 cells transfected with pPOPMycSty construct but un-induced with IPTG and finally lane 4 indicates CJ2-8 cells transfected with pPOPMycSty followed by induction with 5 mM IPTG for 15 hrs. 50 μg of total lysates from each sample was analyzed by 10% SDS-PAGE and immunoblotted with α-Myc monoclonal antibody (9E10).

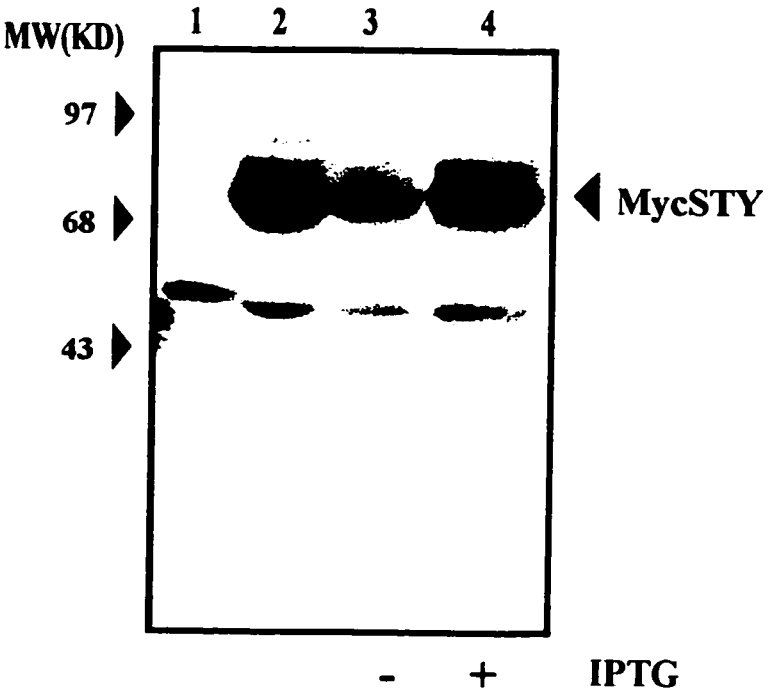


Figure 6.

indicated that reasonable amount of the Lac R proteins are expressed accompanied by the continuous drug selection in CJ2-8 cells and functioning efficiently to repress the expression of the target genes. In addition, it also indicates that the repression exerted by the repressor is relieved efficiently with IPTG.

Establishment of Myc-Sty stable clone in CJ2-8 cells

Many previous stable transfections with pPOPMycSty had failed before the final Sty inducible clones were obtained. Initial co-transfection only with pPOPMycSty and PGKNeo into CJ2-8 cells to obtain Sty inducible clones didn't work; sometimes there were no drug resistant colonies at all while cells transfected with PGKNeo alone resulted in many G418 and hygromycin resistant colonies (CJ2-8Neo) and sometimes, although drug resistant colonies were obtained, they did not express Myc-Sty at the high level demonstrating that excessively over-expressed Sty was likely to be toxic to the cells or expression of Myc-Sty cDNA was so unstable in P19 cells that it could not be maintained stably after the transfection. The phenomenon that the gene on transfected plasmids into P19 EC cells are not stably expressed and even in clonal populations derived from transfected cells, introduced genes are expressed in some but all cells, was already demonstrated quite well. To check the first possibility, the electroporation as a transfection method was exploited because it has been known that the electroporation generally delivers lower copy number of gene of interest than calcium phosphate method but it didn't work. Therefore, the second possibility of co-transfection with B17 and pPOPMycSty and PGKNeo was tested because it was previously found that

B17, a construct containing the promoter and intragenic regions of murine PGK-1 gene, increased the efficiency of the stable transfection in P19 EC cells significantly by perhaps enhancing the stable integration of increased copies of transfected gene into the genomes. It turned out to be successful resulting in a lot of drug resistant colonies indicating B17 may significantly increase the stable transfection efficiency in P19 cells. The resistant colonies were tested for the Sty expression.

Western blot analysis for the expression of Sty in the inducible clones

As shown in figure 7, those inducible clones showed the highly inducible Myc-Sty expression in most clones and even in pooled clones in the presence of IPTG compared with that of un-induced cells although the basal level expression in the absence of IPTG was somewhat variable. Interestingly the clones induced with IPTG produced very broad Myc-Sty bands suggesting that over-expressed Sty is so active that it auto-phosphorylates at multiple sites while uninduced clones showed relatively compressed bands at the different positions implying that the kinase activity of Sty is regulated by its own expression level.

Figure 7. Western blot analysis for Myc-Sty inducible clones
After the co- transfection with pPOPMycSty, B17, and PGKNeo, the drug resistant colonies were picked and expanded for the western analysis to identify the best Myc-Sty inducible clones. Lane 1, as a negative control, indicates un-transfected CJ2-8 cells, lane 2 indicates, as a positive control, CJ2-8 cells transfected with PECEMycSty construct. Lane 3, 5, 7, 9, 11 and 13 represent un-induced clone #9, #11, #15, #19, pooled clones 1 and 2 respectively. Lane 4, 6, 8, 10, 12 and 14 represent induced clone #9, #11, #15, #19, pooled clones 1 and 2 respectively. 50 μg of protein from each sample were run on 7.5% SDS-PAGE and subsequently immunoblotted with α-Myc antibody.

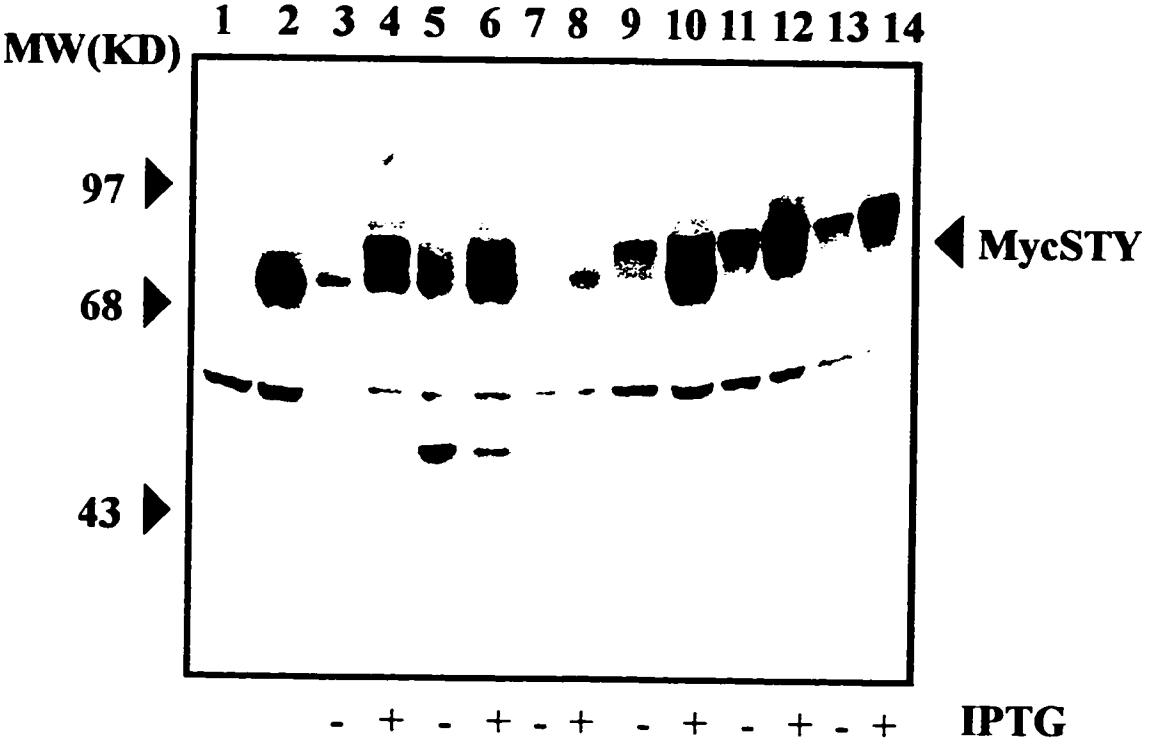


Figure 7.

Part III

Characterization of Myc-Sty from inducible clones

Materials and Methods

In vitro kinase assay

Cells were lysed in cell lysis buffer (20 mM Tris-Hcl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM NaF, 2 mM sodium phosphate, 500 μM sodium vanadate, 200 μg/ml PMSF, 2 μg/ml aprotinin and 5 μg/ml leupeptin) for 30 min on ice. Lysates were cleared by centrifugation and supernatant was immunoprecipitated with α–Sty Ab recognizing N-terminal of Sty (1:100 dilution) overnight at 4 °C. Next day, immunoprecipitates were incubated with protein G-conjugated beads (Gamma-plus) washed with the cell lysis buffer, three times for 45 min at 4 °C followed by washing and quick centrifugation to remove the binding of non-specific protein. Immunoprecipiates were assayed for kinase activity in kinase buffer (20 mM HEPES, pH 7.1, 10 mM $MgCl_2$, 2 mM $MnCl_2$ containing 10 μi of [γ^{32} P]ATP for 30 min at room temperature. Reactions were inactivated by the addition of 2 X SDS-sample ample buffer with 5 % β-Mercaptoehtanol followed by boiling for 5 min. Samples were resolved by 7.5 % SDS-polyacrylamide gel electrophoresis and analyzed by phosphoimager (Molecular dynamics Inc.).

Indirect double immunofluorescence for both MycSty and SR proteins

Approximately 2 x 10^5 cells were plated out on 0.1% gelatin-coated coverslips, next

day, washed with phosphate-buffered saline(PBS) and fixed in -20 °C methanol for 10 min at -20 °C. Following the hydration of cells in PBS, rabbit α-Myc Ab (1/100 dilution) and mouse α-SR Ab (1/2 dilution) in PBS containing 0.3 % Triton X-100 were added to the fixed cells followed by washing with cold PBS to remove non-specific antibody binding. Subsequently fluorescein isothiocyanate-conjugated α-mouse and Texas red-conjugated α-rabbit secondary antibodies were incubated with cells for 1 hr in humid chamber. The coverslips were washed with cold PBS three times. Finally the coverslips were mounted on the slide glass along with anti-fade solution and then observed under the microscope (40X or 100X magnification, Zeiss)

Subcellular fractionation of clone #9 cells

When generating cytoplasmic and nuclear extracts, cells were first lysed at 4°C by gently pipetting afer 5 min in hypotonic buffer (20 mM HEPES. pH 7.9, 10 mM KCl, 0.1 mM sodium vanadate, 1 mM EDTA, 10 % glycerol, 0.5 mM PMSF, 1μg/ml aprotinin, 1μg/ml leupeptin, 1 mM DTT) with 0.2 % NP-40. After centrifugation at 4 °C (13,000 rpm in microfuge) for 10 sec supernatant was collected as cytoplasmic extracts and then mixed with SDS-sample buffer. Nuclear extracts were prepared by resuspension in SDS-sample buffer followed by sonication for 20 sec at 4 °C and boiling for 5 min, run on 10 % SDS-PAGE and probed with α-Myc mAb.

Phosphorylation on Tyr of Sty in vivo

Two sets of P19 cells were transfected with PECEMycSty construct by the calcium

phosphate method in the same manner as mentioned earlier and then only one dish of the transfected cells were incubated with 50 μM sodium vanadate (a phosphatase inhibitor) overnight. On the other hand, two dishes of the inducible Sty clone # 9 and #11 were plated out, grown in the media supplemented with both hygromycin and G 418 and incubated with 50 μM sodium vanadate overnight but only one dish of cells were also induced with IPTG overnight. Subsequently the cells were harvested with the extraction buffer supplemented with protease and phosphatase inhibitors at 4 °C for 30 min, the supernatant was cleared by centrifugation for 10 min and immunoprecipitated with α-Sty polyclonal antibody for overnight. Next day, Sty/ antibody complexes were mixed with the protein G conjugated γ-plus beads for 1 hr, washed with the extraction bead, extracted with 2 X SDS-sample buffer supplemented with 5 % β-Mercaptoethanol (5 %) and finally boiled for 5 min. The protein extracts spun down were loaded onto 10 % SDS-PAGE, transferred to the nitrocellulose membrane at 0.2 Amp overnight, blocked with 5% skim milk in TBST, blotted with α-phosphotyrosine antibody (1:1000 dilution) for 1 hr at room temperature, probed again with α-mouse HRP-conjugated secondary antibody (Bio-Rad, 1:5000 dilution) for 1 hr at RT and developed with chemiluminescence reagent (KPL).

GST-Sty binding assay

GST-Sty binding reactions were performed basically as described elsewhere (Duncan *et al*, 1995). Briefly, bacterially expressed GST or GST-Sty was coupled to Glutathion-Sepharose 4B beads (Pharmacia) in immunoprecipitation buffer (same buffer as that used in kinase assay) after sonicating bacterial cells on ice for 20 sec, three times. Approximately

10 μg of GST or GST-Sty coupled to beads was used in each binding experiment. Sty-stable clone #9 cells expressing Myc-tagged Sty protein were grown in the presence of 5 mM IPTG for 1 day, subject to lysis with the same immunoprecipitation buffer and then mixed with GST or GST-Sty beads. Incubation was carried out for 2 hr at 4 °C on a rotating platform. The beads were washed with the immunoprecipitation buffer three times and proteins were eluted with SDS-sample buffer supplemented with 5% β-Mercaptoethanol. Bound proteins were analyzed by SDS-PAGE, transferred to nitrocellulose and subsequently immunoblotted with anti-Myc mAb (9E10).

Triton X-100 cell extraction of clone #9 cells

One set of clone #9 cells were grown in the absence of IPTG, three sets of clone #9 cells were grown in the presence of IPTG (5 mM) for 1 day, 2 day, and 4 day respectively and as a negative control for Myc-Sty and endogenous Sty, B7 cells (null for Sty), were also cultured. Cells were washed with cold PBS and harvested with extraction buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 % Triton X-100, 2 mM NaF, 2 mM sodium pyrophosphate, 500 μM sodium vanadate, 200 μg/ml PMSF, 2 μg/ml aprotinin and 5 μg/ml leupeptin) on ice for 30 min. After centrifugation at 13,000 rpm at 4 °C for 10 min, supernatant was mixed with 2 X SDS- sample buffer and precipitates were also mixed with the SDS-sample buffer followed by sonication for 20 sec at 4 °C. The samples were boiled and quantified by protein assay kit (Bio-Rad) and resolved on SDS-PAGE followed by western blotting using α-Sty antibody.

Results

In vitro kinase assay

To prove that Myc-Sty produced from the inducible clones was functional protein, in vitro kinase assay was performed. In vitro kinase assay was a prerequisite for further experiments in that for initial transfections without B17, there were no drug resistant colonies and it could be assumed that Myc-STY expressed from the inducible clones is not catalytically active. However, the result clearly demonstrated that Myc-Sty protein from the clones was catalytically active and also highly inducible (figure 8). Surprisingly, endogenous Sty immnoprecipitated with α-Sty Ab (N-terminal specific) showed very little kinase activity compared with that of Myc-Sty suggesting that at the normal state, either expression level of endogenous Sty and/or its kinase activity is relatively low because the kinase activity may be subject to tight regulation possibly through the interaction with other regulatory proteins (i.e truncated Sty). In parallel with the kinase assay, the same samples were analyzed for western blot with α-Myc monoclonal antibody or α-Sty antibody (figure 9). The results indicated that the kinase activity is proportional to the amount of Myc-STY protein expressed in each clone and especially clone #9 expressed higher amount of Myc-STY than endogenous STY although its basal expression is also relatively high while clone # 15 and #19 showed extremely low basal expression of Myc-STY but they also expressed less amount of endogenous Sty indicating that all cells of those clones didn't seem to express Myc-Sty. I think this phenomenon may be due to the mosaic expression of exogenous genes

Figure 8. In vitro kinase assay using Myc-Sty from inducible clones
To verify that Myc-Sty protein expressed the inducible clones is catalytically active or not, in vitro kinase assay was performed. Cells were harvested with the Triton X-100 based extraction buffer supplemented with the protease inhibitors (aprotinin and leupeptin) and phosphatase inhibitors (sodium vanadate and NaF) and cleared by centrifugation. Supernatant was incubated with α-Sty antibody for overnight, re-incubated with the protein G beads and subject to the kinase assay. The samples were analyzed by 7.5% SDS-PAGE followed by the phosphoimager scanning. Lane 1 indicates CJ2-8 cells as a negative control, lane 2 and 3 indicate P19 cells transfected wih PECEMycSty and pPOPMycSty respectively as positive controls. Lane 4, 6, 8 represent un-induced clone 9, 15 and 19 respectively while lane 5, 7, 9 represent induced clone 9, 15 and 19 respectively.

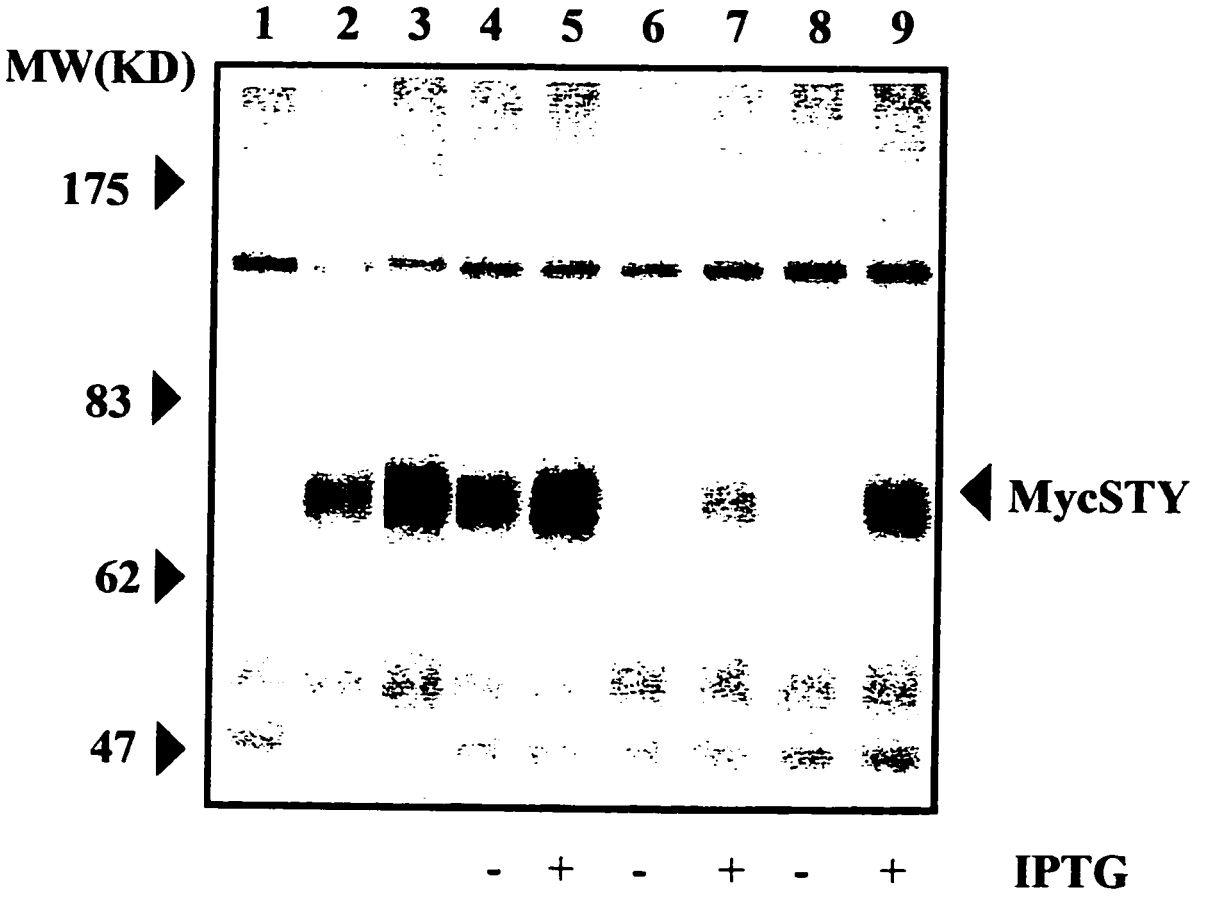
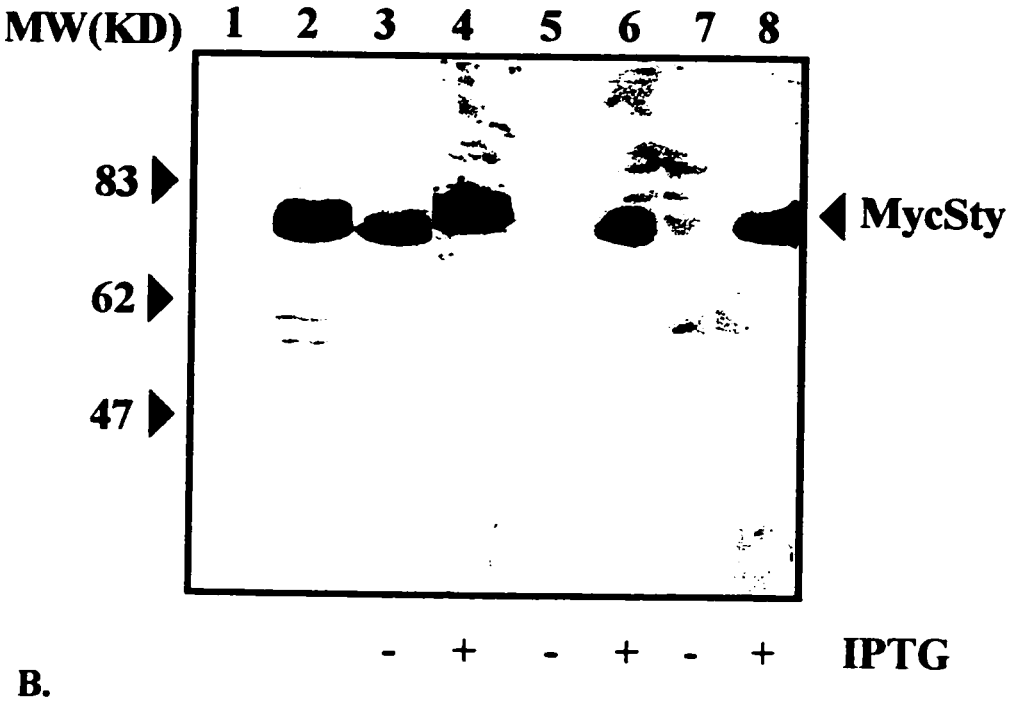


Figure 8.

Figure 9. Western blot analysis to correlate the kinase activity and Myc-Sty expression level and to compare the relative expression level of Myc-Sty and endogenous Sty
This is a parallel experiment with the in vitro kinase assay; one dish of cells were used for the in vitro kinase assay and the other one was used for this western analysis. A) 50 μg of total protein from each sample was loaded on 10% SDS-PAGE gel and subsequently immunoblotted with α-Myc monoclonal antibody (9E10). Un-transfected CJ2-8 cells (lane 1), CJ2-8 cells transfected with PECEMycSty (lane 2), clone # 9 cells without IPTG (lane 3), clone #9 cells with IPTG (lane 4), clone #15 cells without IPTG (lane 5), clone #15 cells with IPTG (lane 6), clone #19 cells without IPTG (lane 7) and clone #19 cells with IPTG (lane 8). B) the same blot was stripped with the stripping buffer and re-probed with α-Sty antibody for the second western analysis.

A.


MW(KD)
1 2 3 4 5 6 7 8
83
62
47
MycSty
- + - + - +
IPTG

B.

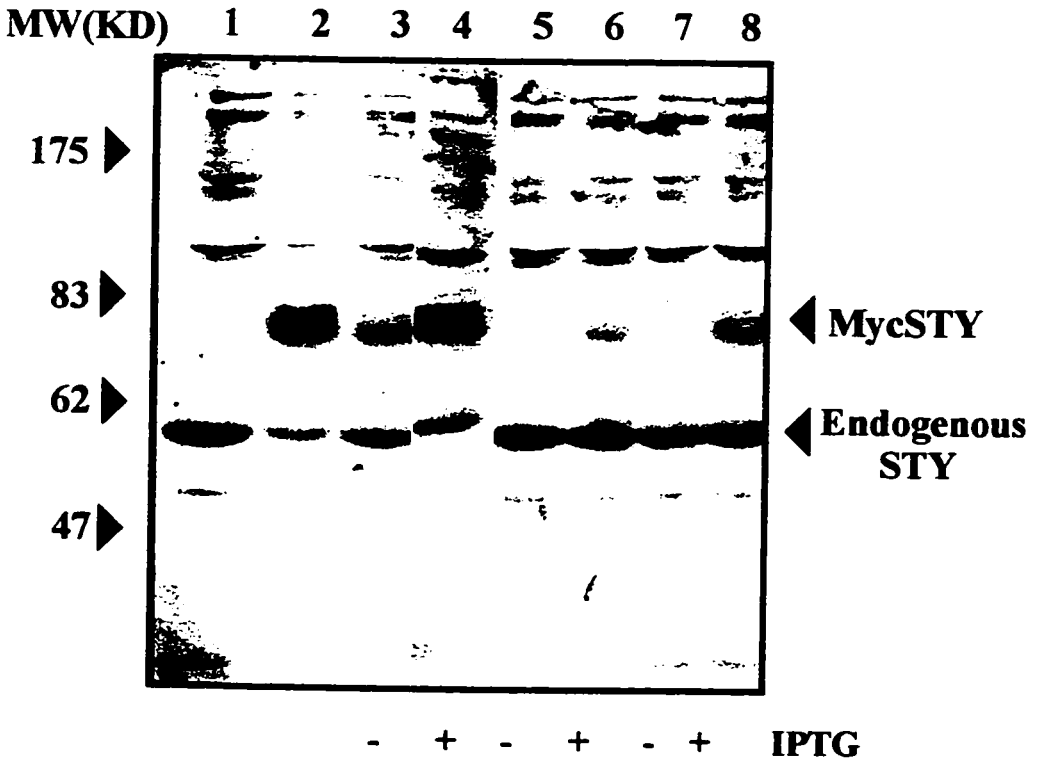

MW(KD)
1 2 3 4 5 6 7 8
175
83
62
47
MycSTY
Endogenous
STY
- + - + - +
IPTG

Figure 9.

in P19 cells even in the continuous drug selection as mentioned earlier or may be also due to the difference in the copy number of Sty gene and/ or the expression level of Lac repressor in the individual clones. Interestingly, the level of endogenous Sty in clone #9 was lower than those in clone #15 and #19 clones suggesting that Sty expression may be regulated by the auto-regulatory loop (Duncan *et al*, 1997).

Double immunofluorescence for Myc-Sty and SR proteins

As described above, in vitro kinase assay result demonstrated that Myc-Sty from the inducible clones is catalytically active but I also had to prove that over-expressed Myc-Sty can phosphorylate SR proteins and despecklize them in vivo so I performed double immunofluorescence both for Myc-Sty and SR proteins as a functional assay. IPTG- induced clone # 9 cells showed the diffused pattern of SR proteins in the nucleus of the positive cells, which is indicative of SR proteins phosphorylated by the Sty protein in the nucleus while IPTG-induced CJ2-8 Neo cells and un-induced clone #9 cells (data not shown) did not demonstrate positive cells for Myc-Sty and no redistribution of SR proteins (figure 10). The result showed that the induced Myc-Sty was localized in nucleus and despecklized SR proteins indicating that it is a functional protein kinase which can phosphorylate and redistribute SR protein resulting in affecting the alternative splicing in vivo. However, according to the immunofluorescence, the percentage of positive cells showing intensive red staining in the nucleus was not 100 % but only about 5 % indicating that Sty gene was inactivated or lost consistently with the previous finding of instability of transfected genes in

Figure 10. Indirect double immunofluorescence both for Myc-Sty and SR proteins
Cos-1 cells were grown on the coverslips and transfected with PECEMycSty construct. CJ2-8Neo cells were grown in the presence of IPTG for 24 hr while one set of clone #9 cells were cultured in the absence of IPTG and the other set of cells were grown in the presence of IPTG for 24 hr. Cells were fixed with - 20 °C methanol and probed with rabbit α-Myc monoclonal antibody and mouse α-SR monoclonal antibody (mAb 104) and subsequently with Texas red and FITC-conjugated secondary antibodies. Panel A and B indicates COS-1 cells for Myc-Sty (red) and SR (green) respectively. Panel C and D indicates CJ2-8Neo cells for Myc-Sty and SR respectively. Panel E and F represents IPTG-induced clone #9 cells for Myc-Sty and SR respectively.

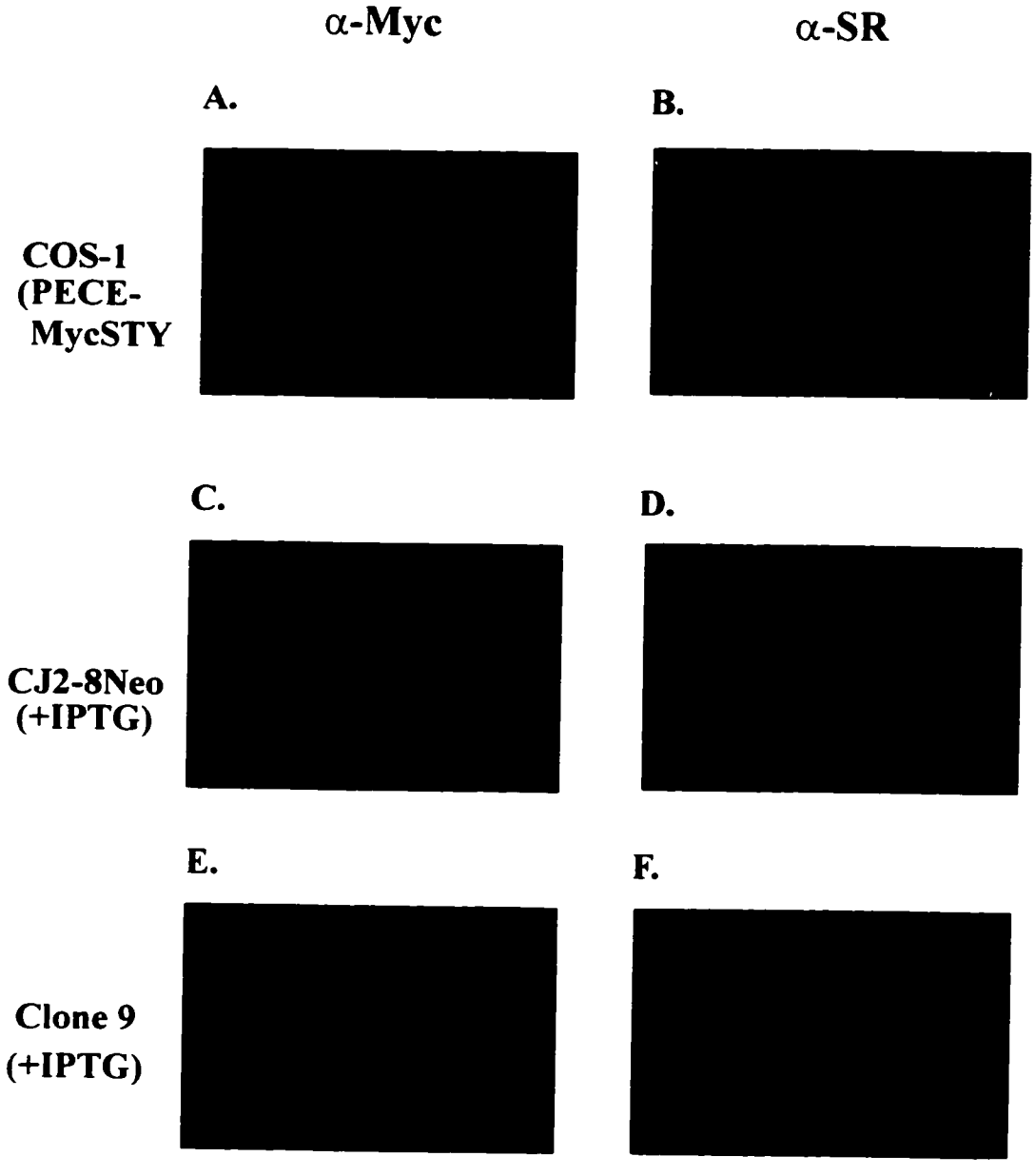


Figure 10.

P19 EC cells.

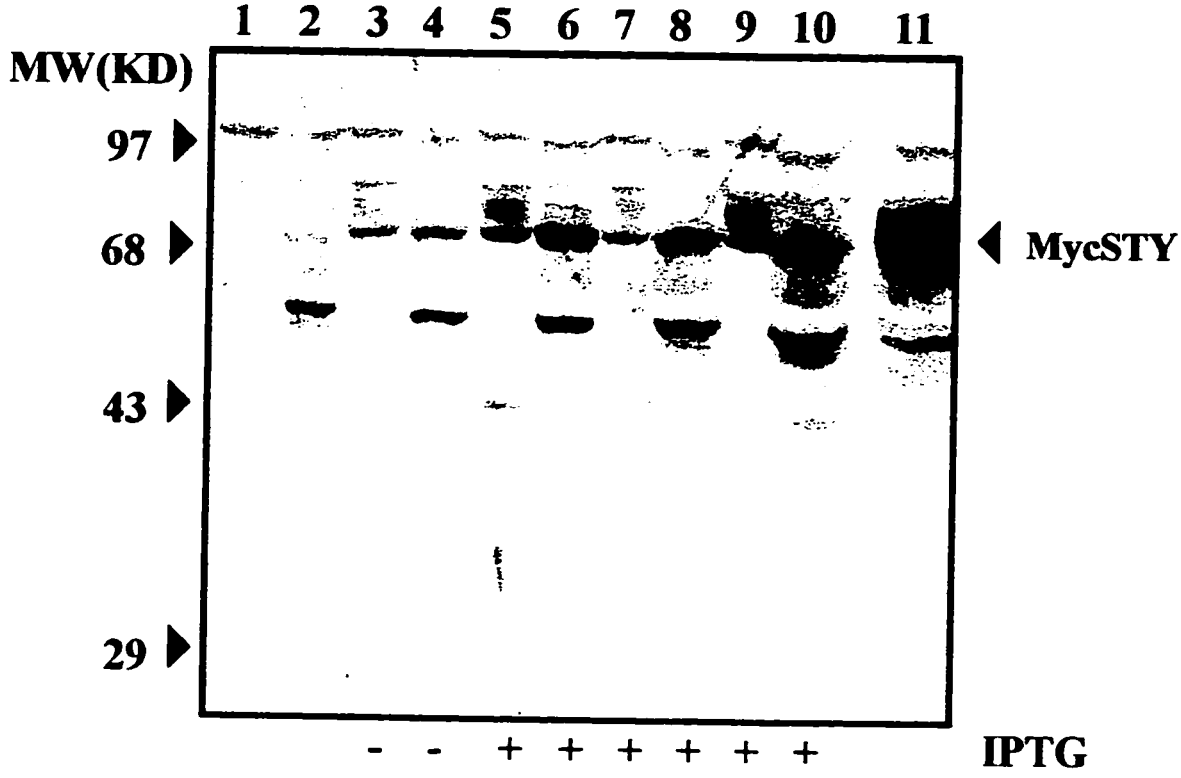
Subcellular fractionation of clone #9 cells

The immunofluorescence result clearly showed Sty is localized in the nucleus but the result also showed some staining in the cytoplasm which was not distinguishable from the background. Therefore, to confirm the nuclear localization of Myc-Sty especially during four day time course, cytoplasmic and nuclear extracts from clone #9 cells were subject to the western analysis. The result clearly showed Myc-Sty is localized predominantly in nucleus, which reached the highest expression level on the day 4, throughout the time course but interestingly, some Myc-Sty is also found in cytoplasm (figure 11A). I assume that it may be due to the detergent (0.2 % NP 40) used for the fractionation. Actually 0.2 % NP 40 is used to retrieve some extractable proteins from the nucleus and therefore some over-expressed Sty was extracted but the possibility that the minor portion of Sty may also be present in cytoplasm for unknown function should not be ruled out. In order to rule out the possibility of contamination from the nuclear Myc-Sty, the same blot was stripped and re-probed with anti-SR protein Ab (mAb104). Bands detected by mAb104 lined up only with proteins in nuclear extracts verifying no contamination (figure 15). Additionally, figure 11B showed that endogenous Sty is absolutely localized in the nucleus for its function.

Sty is phosphorylated on Tyr in vivo

Figure 11. Subcellular fractionation of the inducible clone #9
A) Clone #9 cells were grown in the absence (lane 3 and 4) or in the presence of 5 mM IPTG for 1 day (lane 5, 6), 2 day (lane 7, 8) and 4 day (lane 9, 10) respectively. Lane 1 and 2 indicates CJ2-8Neo as a negative control while lane 11 represents CJ2-8 cells transfected with PECEMycSty as a positive control for Myc-Sty protein. Clone #9 and CJ2-8Neo cells were subject to subcellular fractionation and then split into cytoplasmic (lane 1, 3, 5, 7 and 9) and nuclear (lane 2, 4, 6, 8 and 10) fractions. 50 μg of each protein was run on 10% SDS-PAGE and then detected by immunoblot with α-Myc monoclonal antibody (9E10). B) P19 cells were also fractionated into the cytoplasmic (lane 1) or nuclear (lane 2) portion and then immnoblotted with α-Sty polyclonal antibody.

A.


1 2 3 4 5 6 7 8 9 10 11
MW(KD)
97
68
43
29
MycSTY
- - + + + + + +
IPTG

B.

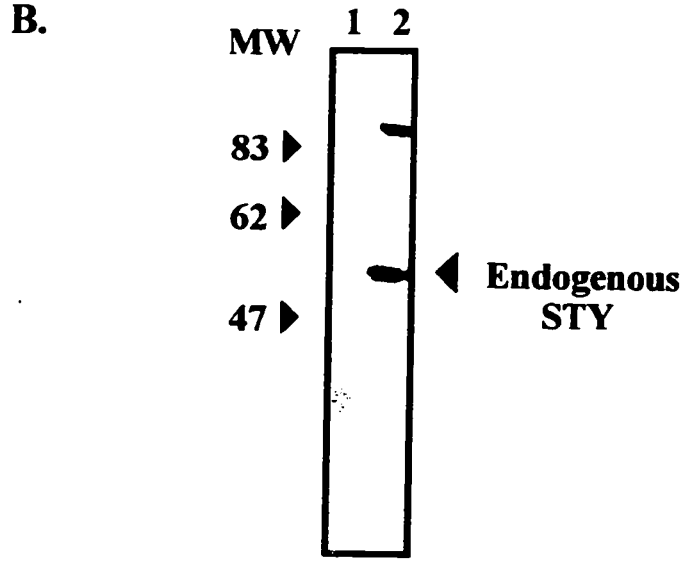

MW
1 2
83
62
47
Endogenous
STY

Figure 11.

Figure 12. Myc-Sty is phosphorylated on Tyr in vivo
P19 cells were transfected with PECEMycSty construct and grown overnight in the absence (lane 1) or presence (lane 2) of 50 μM sodium vanadate. Clone #9 and #11 cells were also treated with 50 μM sodium vanadate overnight. Lane 3 and 5 indicate un-induced clone #9 and #11 cells respectively while lane 4 and 6 represents their counterparts induced with IPTG for 15 hrs respectively. Every sample was first precipitated with α-Sty polyclonal antibody. Immunoprecipitated Sty proteins were run on 10% PAGE and detected by immunoblot analysis with α-phosphotyrosine antibody.

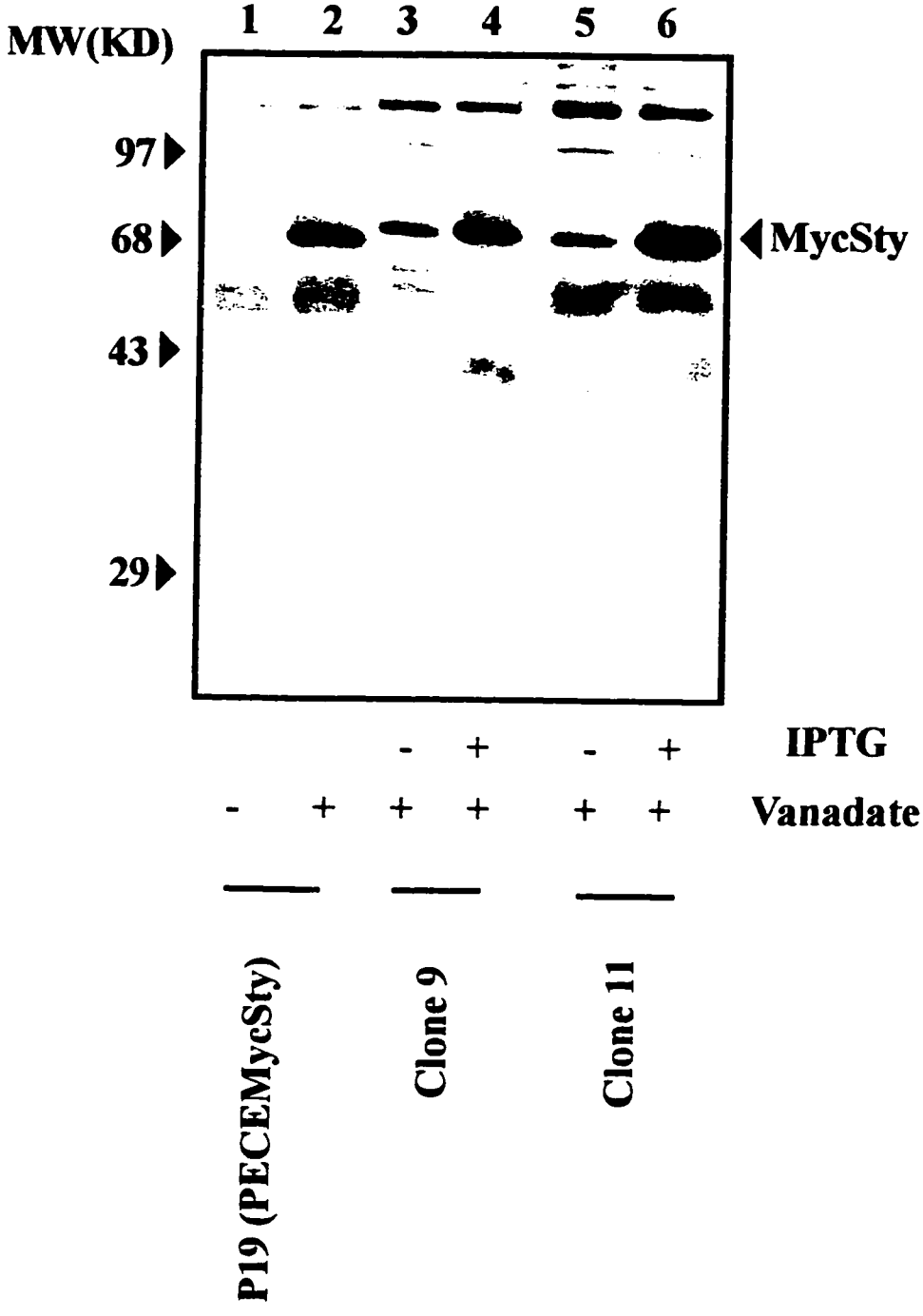

MW(KD)
1
2
3
4
5
6
97
68
43
29
MycSty
IPTG
Vanadate
P19 (PECEMycSty)
Clone 9
Clone 11

Figure 12.

The next question that was addressed was whether Myc-Sty from the inducible clone was phosphorylated on Tyr residue by itself as demonstrated previously (Duncan *et al*, 1995). In COS-1 cells transfected transiently with PECEMycSty construct, Myc-Sty was phosphorylated on Tyr residue only in the presence of a phosphatase inhibitor (sodium vanadate) implying that the phosphorylation on Tyr may be extremely transient and possibly involved in another very important regulatory mechanism in vivo. Figure 12 also revealed that Myc-Sty from the clone #9 and clone #11 which were immunoprecipitated with α-Sty polyclonal antibody recognizing N-terminus common to both Myc-Sty and endogenous Sty was phosphorylated on Tyr in vivo and Tyr-phosphorylated Myc-Sty was also inducible when compared to the level of the un-induced cells. However, the same immunoprecipitates didn't reveal any endogenous Sty which was phosphorylated on Tyr at the high level similarly to Myc-Sty suggesting that the kinase activity of endogenous Sty is subject to the tight regulation.

GST-Sty binding assay with Myc-Sty in vitro

In order to demonstrate that Myc-Sty from an inducible clone #9 can form a dimer with Sty, (because it is assumed that Sty should dimerize through its RS domain for the kinase activity) I carried out binding assay using GST or GST-Sty coupled to beads. As shown in Figure 13, Myc-Sty was bound only to GST-Sty while there was no binding activity for GST alone consistently with the previous result (Duncan *et al*, 1995). The positive control band is Myc-Sty protein immunoprecipitated with α-Myc Ab. The result indicates that this Myc-Sty can

Figure 13. Myc-Sty can dimerize with GST-Sty in vitro
CJ2-8 cells were transfected with PECEMycSty construct (lane 1) and clone #9 cells were induced with 5 mM IPTG for 15 hrs (lane 2 and 3). On the other hand, lane 4 indicates total cell lysate from CJ2-8 cells transiently transfected with pPOPMycSty. Cells were harvested with the extraction buffer and cleared by centrifugation. Supernatant was subject to immunoprecipitation with α-Myc antibody (9E10) (lane 1) or GST coupled to Sepharose (lane 2) or GST-Sty coupled to Sepharose (lane 3). Purified Myc-Sty was visualized by 10% SDS-PAGE followed by the immunoblot with α-Myc monoclonal antibody (9E10).

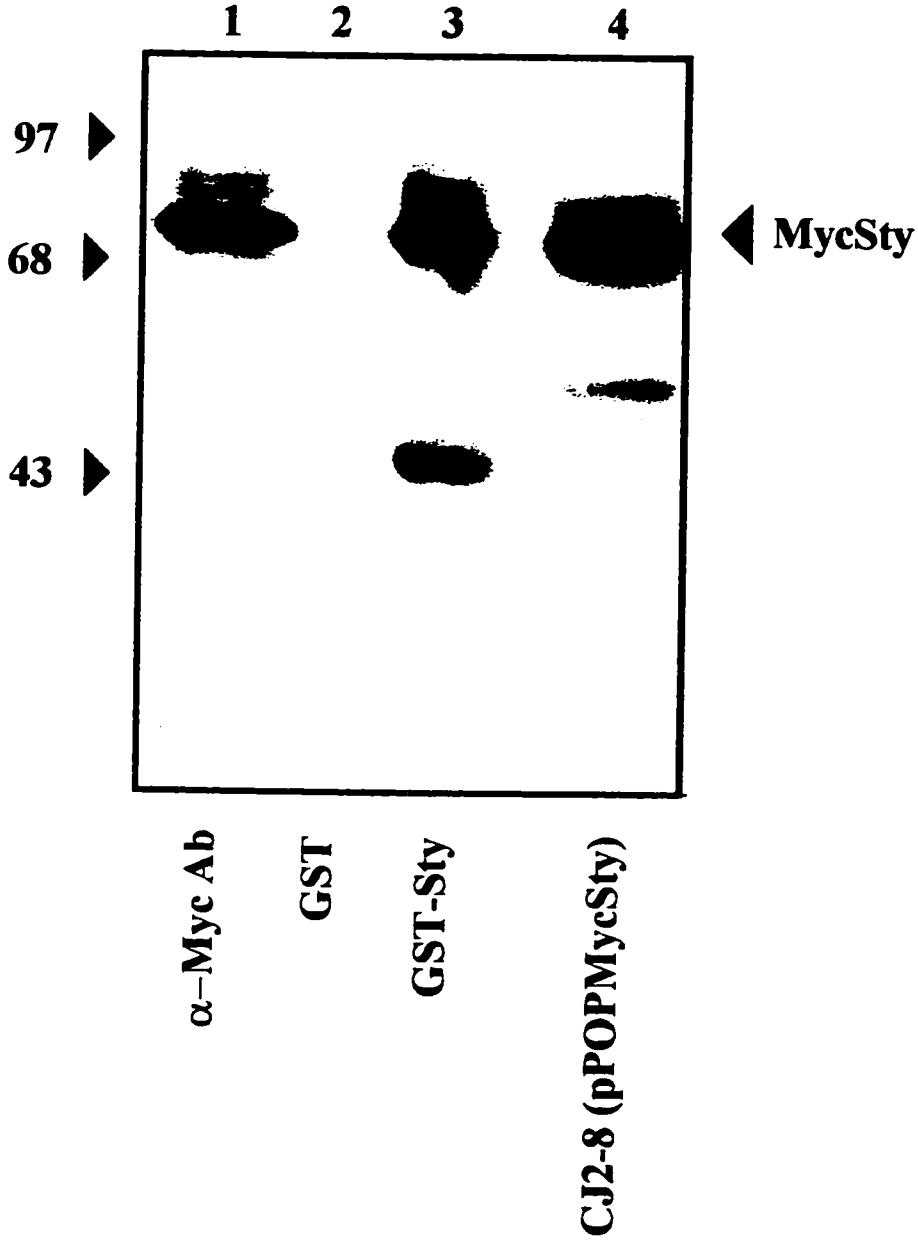


Figure 13.

dimerze with Sty for its function and the interaction may be quite specific.

Different solubility of Myc-Sty and endogenous Sty in Triton X-100.

It was previously demonstrated that full length Myc-Sty transiently transfected into COS-1 cells, upon Triton X-100 based extraction, was present absolutely in the detergent soluble fraction while catalytically inactive and truncated Sty was localized in the insoluble fraction (Duncan *et al*, 1995). This indicates that only catalytically active full length Sty may phosphorylate SR proteins, releases SR proteins and itself released from the nuclear speckles and redistributes them in the nucleus.Only redistributed Sty was extracted by Triton X-100 and collected in the detergent soluble fraction. Figure 14 showed compared with endogenous Sty, relatively more Myc-Sty was positioned in the detergent soluble fraction. This is more evidence that the kinase activity of endogenous Sty is being tightly controlled in some ways (perhaps by interaction with truncated Sty or other proteins) although endogenous Sty itself has the intact protein structure of a catalytically active kinase while Myc-Sty may be actively phosphorylating SR proteins because it is expressed at a higher level, and therefore is fully active. It may be released from association with other proteins which exists in vivo to regulate the kinase activity, resulting in its being extracted by Triton X-100. One more interesting thing is that lane 10 in figure 14 showed that Myc-Sty from the cells induced with IPTG for 4 days was positioned only in the detergent soluble fraction suggesting that the expression level of Sty may reach the peak on the day 4 and the kinase activity of Sty may be correlated with its expression level consistently with the result from the subcellular fractionation where Myc-Sty also reached the peak expression level on day 4

Figure 14. Time course western blot for comparison of the solubility in Triton X-100 between Myc-Sty and endogenous Sty

Clone #9 cells were grown in the absence of IPTG (lane 2 and lane 6) or in the presence of IPTG for 1 day (lane 3, 7), 2 day (lane 4, 8) and 4 day (lane 5, 9). Lane 1, and 6 indicates B7 cells (null for Sty) as a negative control both for Myc-Sty and endogenous Sty. Cells were extracted with Triton X-100 based extraction buffer and separated into the detergent soluble (lane 1, 2, 3, 4, and 5) and insoluble (lane 6, 7, 8, 9, and 10) fractions. Proteins were run on 7.5% PAGE and subsequently visualized by the immunoblot with α-Sty polyclonal antibody and chemiluminescence.

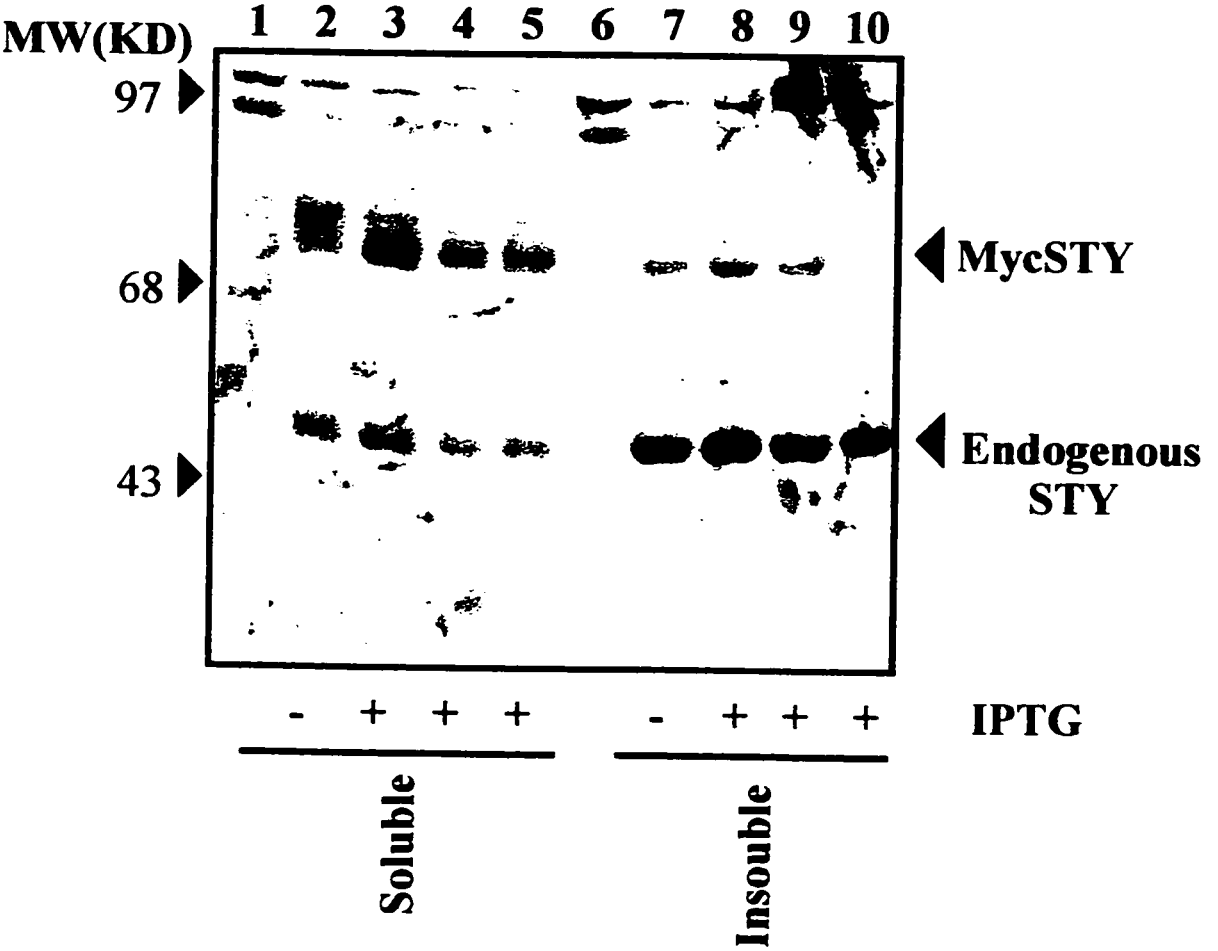

MW(KD)
1 2 3 4 5 6 7 8 9 10
97
68
43
MycSTY
Endogenous STY
- + + + - + + +
IPTG
Soluble
Insouble

Figure 14.

PART IV

Investigation of biological substrates and function of Sty in vivo

Materials and Methods

Western blot analysis

CJ2-8Neo cells were cultured with 5 mM IPTG for 2 days and these cells were trypsinized, plated out at the density of 1 X 10^6 cells in 100 mm dish and incubated with 5 mM IPTG for two more days. On the other hand, clone # 9 cells were induced with IPTG for 1 day, 2 days or 4 days with continuous replating before harvesting and at the same time , one dish of clone #9 cells were grown in the absence of IPTG. The cells were harvested with extraction buffer and separated into cytoplasmic and nuclear fractions. Each fraction was mixed with 2 X SDS sample buffer, boiled with 2 X SDS sample buffer and quantified by protein assay kit (Bio-Rad). 50 μg of protein from each sample was run on 10% SDS-PAGE and immunoblotted with α-SR antibody (mAb104) after transferring the gel onto the membrane and being blocked with 5% skim milk in TBST. The same blot was stripped with the stripping buffer for 30 min at 55 °C and probed with α-Man antibody (known to be a nuclear membrane protein) for a loading control.

Indirect double immunofluorescence

i) double immunofluorescence for Myc-Sty and EC cell surface antigen marker SSEA-1:

Firstly, as a negative control, P19 cells were plated out in three 100 mm culture dishes and treated with RA (1×10^{-6} M) for 2 days. The cells were trypsinized and grown in several 60 mm bacterial culture dishes in the absence of RA to allow the cells to form aggregates. Next day, cells were spun down briefly and replated on the coverslips coated with 0.2 % gelatin and then allowed them to grow for 2 days. Secondly, as a positive control, P19 EC cells were plated out on the coverslips and allowed to grow until the cells reached to 70 % confluence. Thirdly, CJ2-8Neo cells were initially plated on 100 mm culture dish, incubated with IPTG for 2 days and then replated on the coverslips and allowed to grow in the media containing IPTG for another 2 days. Fourthly, one set of # 9 cells were plated out onto the 100 mm culture dish, grown for 2 days in the absence of IPTG and replated onto the coverslips and grown for 2 more days in the absence of IPTG. Finally the other set of # 9 cells were induced with IPTG for 2 days and plated on the coverslips followed by the induction with IPTG for another 2 days. Each culture prepared above was washed with cold PBS three times, fixed with cold methanol at -20 °C for 10 min and rehydrated with PBS again. The fixed cells were blocked with 5 % FBS in PBS to reduce the background and probed with the antibody mixture of rabbit α-Myc antibody (1:100 dilution) and mouse α-SSEA-1 antibody (1:2 dilution) in PBS containing 0.3 % Triton X-100 and incubated at 4 °C overnight. Next day, cells were washed with cold PBS three times and probed with FITC-conjugated α-Mouse secondary antibody and Texas-red conjugated α-rabbit secondary antibody (each secondary antibody was diluted at the ratio of 1:20). The cells were incubated at 37 °C humid chamber for 1 hr followed by gentle washing with cold PBS and mounted onto the slide glass along with antifade solution, sealed to avoid hydration and finally

examined under the fluorescence microscope.

ii) double immunofluorescence for Myc-Sty and neuronal differentiation marker (A60)

The procedure for the immunofluorescence was same. α-A60 was used to detect the neuronal differentiation marker present in the nucleus. It should be also noted that in this case, P19 cells acted as a negative control and differentiated P19 cells with RA acted as a positive control.

iii) double immunofluorescence for Myc-Sty and neuronal differentiation marker HNK-1

It was performed by the exactly same procedure as that described above except that α-HNK-1 was used as a primary antibody to detect another neuronal differentiation marker in the cell membrane. Undifferentiated P19 cells were a negative control while the differentiated ones were a positive control.

Subcloning of clone #9 cells

Clone #9 cells were plated out at a very low density (approximately 500 cells) in 15 cm tissue culture dish and maintained in the media containing hygromycin (300 μg/ml) and G 418 (400 μg/ml) for one week. Isolated colonies were picked and grown for 2 days. Cells were split into two small dishes for western blot to check the expression of Myc-Sty in the subcloned cells. One dish was cultured in the absence of IPTG while the other dish was treated with 5 mM IPTG for 15 hrs to induce the expression of Myc-Sty. Cells were harvested with 2 X SDS sample buffer and boiled and then analyzed by 12.5 % SDS-PAGE followed by immunoblotting with α-Myc antibody (9E10). In parallel to this, same cells are plated on two sets of the coverslips for the detection of Myc-STY by immunofluorescence;

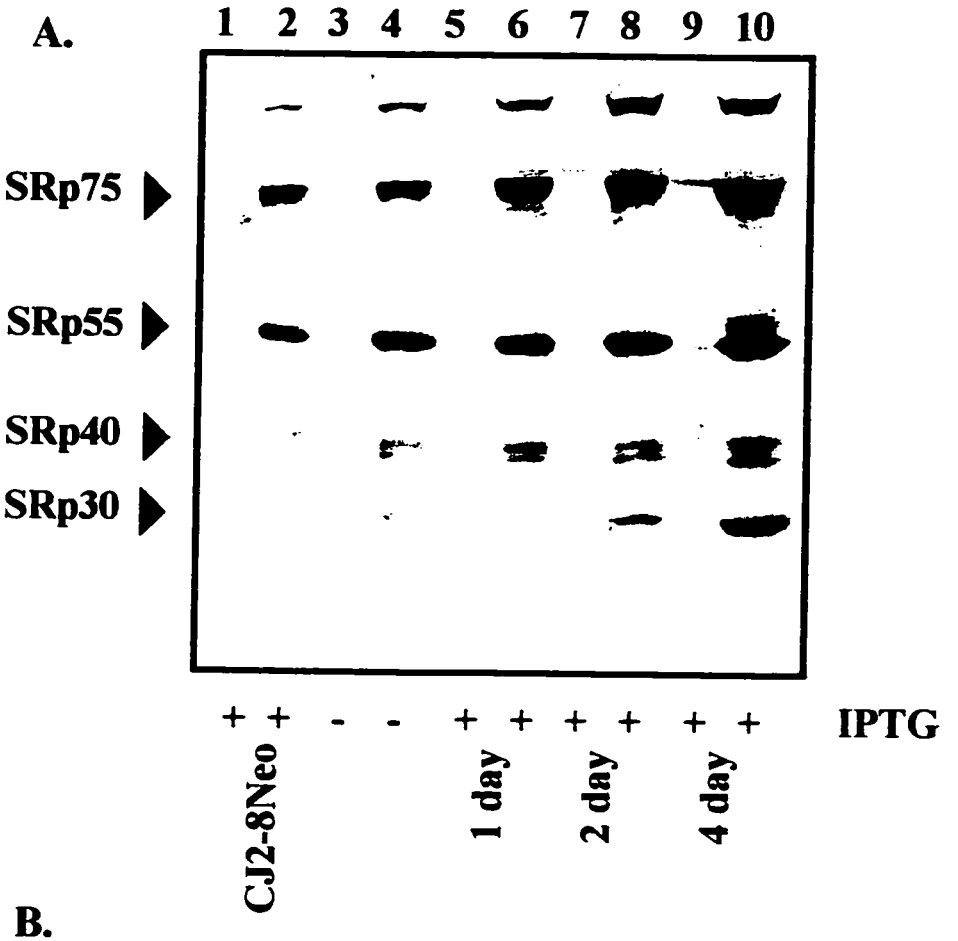
one coverslip for repression and the other one for the induction. Cells on one coverslip were incubated with 5 mM IPTG for 15 hrs. Cells were fixed with -20 °C methanol and probed with α-Myc antibody (9E10) and finally probed with a-mouse FITC-conjugated secondly antibody. Cells were mounted on the slide glass and observed for the expression of Myc-Sty

Results

SRp30 and SRp55 may be the physiological substrates of Myc-Sty in vivo

To identify substrates of Sty in vivo, I took the advantage of α-SR monoclonal antibody (mAb104) (Mayeda et al, 1992; Gui et al, 1994b) because it was known that the antibody recognizes the phospho-epitope which is common to virtually all SR proteins. I assumed that if Sty phosphorylates only a subset of SR proteins in vivo, the clones induced with IPTG should show some significant difference in the phosphorylation of the subset of SR proteins. Figure 15A showed that only SRp30 and SRp55 in cells grown in the presence of IPTG for 4 days seemed to be selectively phosphorylated by over-expressed Sty at the high level in vivo implying that these two SR proteins may be substrates of Sty. This is consistent with two previous results where the expression level of Sty reached the highest point on the day 4 (figure 11). Additionally figure 15B for the western blot with α-Man antibody (nuclear membrane protein) demonstrated that almost equal amount of protein was loaded in each lane. However this result is somewhat controversial because the percentage of the Myc-Sty positive cells is approximately 25% due to the mosaic expression of Myc-Sty in the inducible

Figure 15. SR proteins selectively phosphorylated by Myc-Sty in vivo
A) One set of clone #9 cells were cultured in the absence of IPTG (lane 3 and 4) or the other sets of cells were grown in the presence of IPTG for different hours (1 day: lane 5 and 6, 2 day: lane 7 and 8, 4 day : lane 9 and 10). As a negative control, CJ2-8Neo cells (lane 1 and 2) were also grown in the presence of IPTG for total 4 days to avoid the possible effect of IPTG on the result. Cells were harvested with the lysis buffer and then separated into the cytoplasmic (lane 1, 3, 5, 7 and 9) or nuclear (lane 2, 4, 6, 8, 10) fractions. 50 μg of each protein was analyzed by 10% SDS-PAGE and subsequent immunoblot with α-SR monoclonal antibody (mAb104). B) the blot above was stripped with the stripping buffer and then reprobed with α-Man antibody to verify that the same amount of protein was loaded in each lane.


A.
1 2 3 4 5 6 7 8 9 10
SRp75
SRp55
SRp40
SRp30
+ + - - + + + + + +
IPTG
CJ2-8Neo
1 day
2 day
4 day

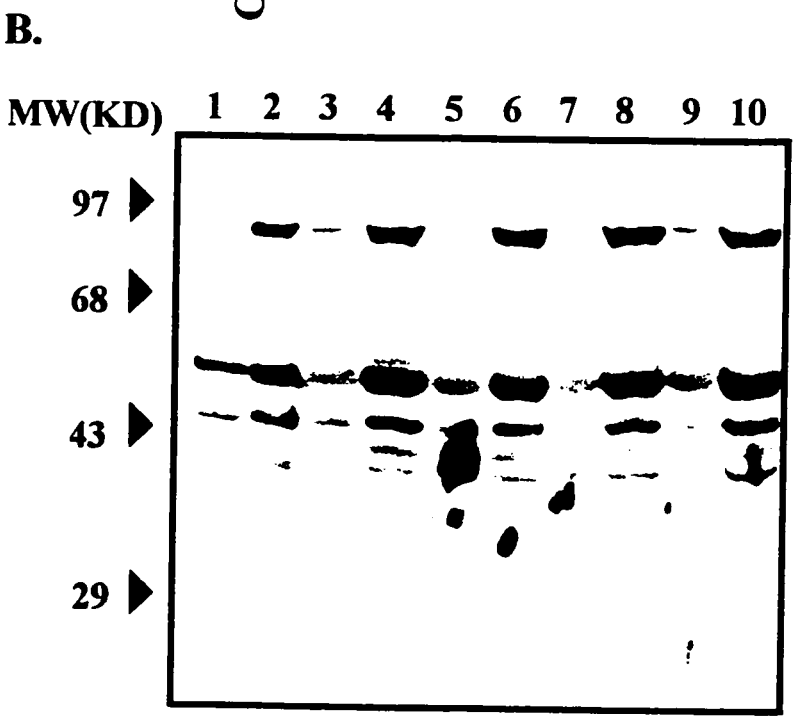

B.
MW(KD)
1 2 3 4 5 6 7 8 9 10
97
68
43
29

Figure 15.

clone #9. Despite of the low percentage of positive cells, I argue it is quite reasonable because of following four previous findings; first yeast two hybrid system isolated these two SR proteins as proteins interacting with Sty (Colwill *et al*, 1996a), secondly, both SRp30a (ASF/SF2) and SRp55 are crucial for cell viability and the development (Ring and Lis, 1994; Wang *et al*, 1996) and finally after the treatment of P19 with RA, incompletely spliced forms of Sty mRNA accumulated in the nucleus. Over-expression of these two SR proteins also led to the production of unspliced or incompletely spliced mRNA species (Wang and Manley, 1995).

Double immunofluorescence for Myc-Sty and neuronal differentiation and embryonic stem cell markers

Previous studies suggested that Sty/Clk may be involved in differentiation (Howell *et al*, 1992; Myers *et al*, 1994). The question I raised was whether Sty/Clk alone can give rise to the differentiation in the inducible clone without any other treatment known to be required for P19 differentiation. As in figure 16, I performed a series of indirect double immunofluorescence for Myc-Sty and SSEA-1 (EC cell surface marker) (Steuer et al, 1990; Smolich and Papkoff, 1994; Slack et al, 1995; Lin et al, 1996; Ling and Neben, 1997), A60 (neuronal differentiation marker in nucleus) (Mullen et al, 1992; Wolf et al, 1996) or HNK-1 (neuronal differentiation marker on cell membrane) (McBurney et al, 1988). This approach seemed to be very feasible because the number of positive cells in inducible cell lines was relatively low and EC cell and neuronal differentiation markers described above were well

Figure 16. Double immunofluorescence both for Myc-Sty and EC cell surface antigen (SSEA-1) or neuronal differentiation markers (A60 and HNK-1)
To differentiate P19 cells into the neurons, P19 cells were grown in the media supplemented with RA for 2 days, trypsinized, transferred to bacterial culture dishes for the aggregation without RA for 1 day split into three dishes and subsequently plated out on the coverslips. Clone 9 cells were first cultured for two days with or without IPTG, trypsinized , replated onto the coverslips and grown for another 2 days with or without IPTG. Cells were fixed with -20 °C methanol, probed with rabbit α-Myc antibody and mouse α-SSEA-1, α-A60 or α-HNK-1 antibody and then probed with texas red and FITC-conjugated secondary antibodies. Red staining represents Myc-Sty and green staining represents each marker protein. Panel A represents P19 cells for SSEA-1. Panel D and G indicate differentiated P19 cells for A60, and HNK-1 respectively. Panel B, E and H indicate clone #9 cells grown in the presence of IPTG for Myc-Sty while panel C, F and I represent clone #9 cells for SSEA-1, A60, and HNK-1 respectively.

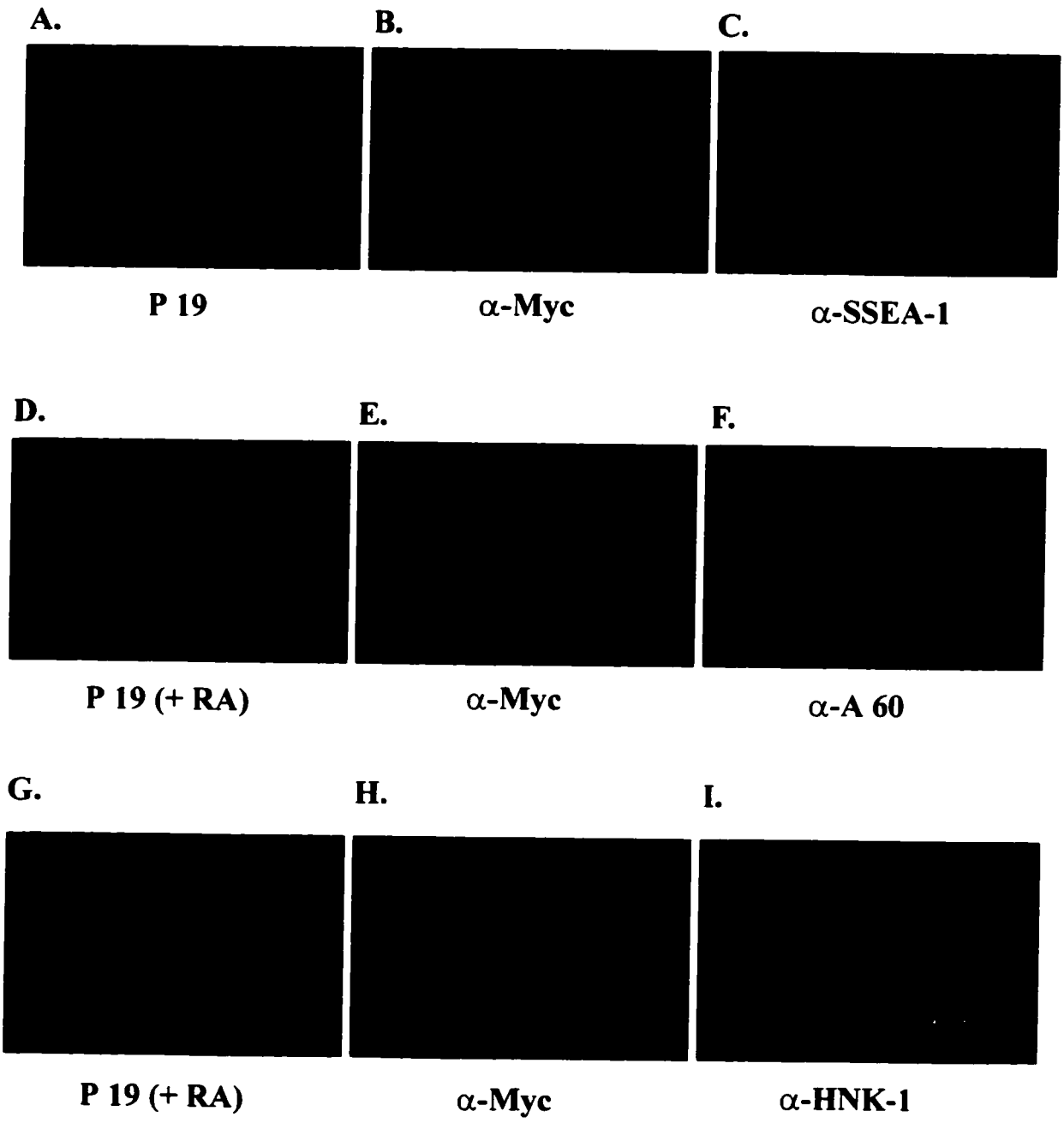


Figure 16.

characterized and can be used as a valuable criteria to verify the neuronal differentiation of P19 cells; typically, differentiated P19 cells show significantly reduced EC cell surface antigen such as SSEA-1 while they show enormously increased a series of neuronal differentiation marker proteins such as A60 and HNK-1. For the double immunofluorescence for Myc-Sty and a neuronal differentiation marker A60, whole clone #9 cells including positive cells for Myc-Sty didn't show any positive signals for A60 (figure 16, panel F) while for the double immunofluorescence for Myc-Sty and another neuronal differentiation marker HNK-1, positive cells for Myc-Sty were not exactly matched with positive cells for HNK-1(figure 16, panel I). On the other hand, for the immunofluorescence for Myc-Sty and SSEA-1, Myc-Sty positive cells showed extremely reduced expression of EC cell marker on the cell membrane suggesting that Myc-Sty positive cells seemed to lose some characteristics of embryonal carcinoma cells and differentiate. However, even negative cells for Myc-Sty (figure 16, panel C) and CJ2-8Neo cells (data not shown) showed greatly reduced expression of SSEA-1, EC cell surface antigen suggesting that CJ2-8Neo and inducible clone #9 cells were already slightly differentiated. It was confirmed by the result from the staining of CJ2-8 Neo and induced clone #9 cells with α-HNK-1 antibody, which showed that CJ2-8Neo cells expressing no Myc-Sty (data not shown) and negative cells of induced clone #9 cells for Myc-Sty were also producing positive signal for the HNK-1 neuronal differentiation marker (figure 16, panel I). In conclusion, based on these results, Sty alone dose not seem to induce the differentiation in P19 EC cells.

Subcloning of the inducible clone #9 cells

I finally tried subcloning to isolate a really stable Myc-Sty inducible clone. About 73 colonies were picked and checked for Myc-Sty expression in each subcloned cell line by immunofluorescence. Some clones showed about 60-70 % of the positive cells and others showed variable expression level but I couldn't find an inducible clone showing 100% positive cells by this subcloning. The expression of Myc-Sty in these subcloned cells was confirmed by the western blot as in figure 17. About 20 subcloned colonies out of 73 expressed high amount of My-Sty and remaining colonies expressed just detectable amount of Myc-Sty or no Myc-Sty (figure 17). According to this result, original inducible clone #9 cells contained about 25% Myc-Sty positive cells on average. Interestingly, this number is higher than number of MycSty-positive cells showing the intensive signal seen in figure 10 and figure 16 indicating some other cells showing no intensive positive signal do express Myc-Sty and furthermore possibly each cell in whole cell population is induced with IPTG to a different degree due to the different amount of Lac repressor protein and the different copy number of Sty gene in the individual cell. It seems to be consistent with ineffectiveness of IPTG in terms of induction previously claimed by many researchers and with the instability of the transfected genes in P19 EC cells.

Figure 17. Western blot for subcloned Myc-Sty inducible clones
A) First 5 subcloned cells out of clone #9 cells were grown in the absence (lane 3, 5, 7, 9 and 11) or their counterparts grown in the presence (lane 4, 6, 8, 10 and 12) of 5 mM IPTG respectively. Lane 1 and 2 represents CJ2-8 cells and CJ2-8 cells transiently transfected with PECEMycSty respectively. B) another 4 sets of subcloned cells were also cultured in the absence (lane 3, 5, 7 and 9) or in the presence (lane 4, 6, 8 and 10) of 5 mM IPTG. Lane 1 and 2 indicates CJ2-8 cells transiently transfected with PECEMyc-Sty and CJ2-8 respectively. Cells were harvested with SDS-sample buffer and analyzed by the immunoblot with α-Myc monoclonal antibody.

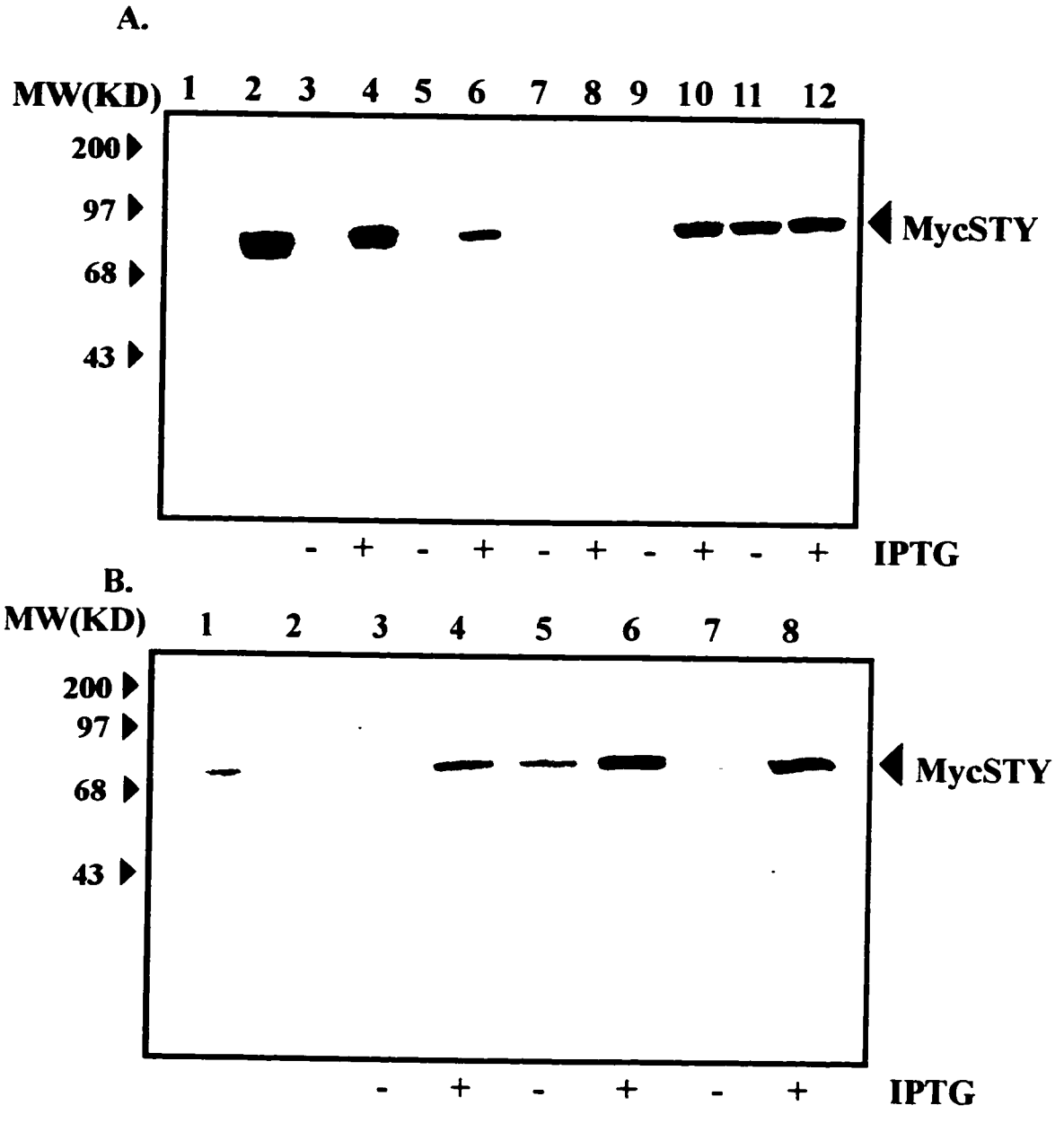

A.
MW(KD)
1 2 3 4 5 6 7 8 9 10 11 12
200
97
68
43
MycSTY
- + - + - + - + - +
IPTG
B.
MW(KD)
1 2 3 4 5 6 7 8
200
97
68
43
MycSTY
- + - + - +
IPTG

Figure 17.

Conclusion

Sty/Clk is a dual specificity kinase which auto-phosphorylates Ser/Thr as well as Tyr residue. Interestingly, Sty/Clk also interacts with and phosphorylates SR proteins resulting in the redistribution of the SR proteins in the nucleus, which may affect alterative splicing. However it's not clear which SR proteins are selectively phosphorylated by Sty/Clk and furthermore, what's the biological outcome of the differential phosphorylation of the SR proteins by Sty/Clk in vivo. With regard to the latter question, it was assumed strongly that Sty/Clk may be implicated in the signaling pathways leading to the differentiation process.

In order to address those two questions, I made a decision to exploit a Lac inducible promoter system. Initially, to establish the system, I generated a bi-cistronic Lac repressor expression construct, PCJ2LacIN containing 5'-PGK-1 promoter, NLS, Lac repressor gene, IRES, hygromycin resistant gene and the SV 40-polyadenylation signal-3'. This construct has some significant advantages; first, NLS allows the Lac repressor protein translated in the cytoplasm to translocate into the nucleus for its function, which is very important feature because Lac repressor is a protein from bacteria and bacteria don't have nuclear membrane therefore, without the NLS, the translocation of the protein may be significantly interrupted. Secondly, IRES allows two proteins to be produced from one transcript therefore maintaining cells in the media supplemented with hygromycin can ensure the expression of Lac repressor in cells. Following the generation of PCJ2LacIN, I transfected PCJ2LacIN into P19 EC cells resulting in a Lac R expressing cell line called CJ2-8. β-gal assay result demonstrated about 35 fold induction between un-induced and induced cells of CJ2-8, and more

importantly, the basal expression of β-gal was extremely low in the absence of IPTG indicating that Lac repressor in these CJ2-8 cells can suppress the gene expression sufficiently as well as the suppression can be relieved by IPTG efficiently.

Subsequently I also generated an inducible Myc-Sty expression construct, pPOPMycSTY containing two Lac operator sequences separated by 40 base pairs. Following the transfection of pPOPMycSTY, B17 and PGKneo into CJ2-8 cells and drug selection, the drug resistant colonies were tested for the expression and inducibility of Myc-Sty. Most of the colonies were positive for the Myc-Sty expression although their expression level and inducibility were somewhat variable. Initially three inducible clones (#9, #15, and #19) were chosen for further experiment.

In vitro kinase assay results also showed that Myc-Sty is catalytically active. Myc-Sty from the inducible clones produced the broad bands indicating that it is catalytically active and therefore phosphorylated at multiple sites while endogenous Sty showed the compact protein bands indicating it is not fully active and therefore phosphorylated only at the limited sites. Triton X-100 extraction experiment supported this notion further; over-expressed Myc-Sty is predominantly present in the detergent soluble fraction while endogenous Sty is predominantly present in the insoluble fraction. One the other hand, the indirect double immunofluorescence both for Myc-Sty and SR proteins demonstrated that it phosphorylates SR proteins and results in the despecklization in the nucleus indicating that Myc-Sty is localized in the nucleus and functional. Subsequent experiments demonstrated that Myc-Sty is phosphorylated on Tyr as well as specifically can dimerize with GST-Sty coupled to Sepharose beads in vitro.

Interestingly, western blot analysis using clone #9 cells and α-SR monoclonal antibody (mAb104) indicated that two SR proteins (SRp30 and SRp55) seem to be selectively phosphorylated by Myc-Sty. However this result is strongly arguable because the number of positive cells for Myc-Sty in clone #9 is approximately 25 %, which was a previously known inherent problem of P19 EC cells. Additionally, as mentioned earlier, SRp30 contains two distinct SR protein of similar size but it's not visualized clearly which one was phosphorylated at the high level by this western blot.

On the other hand, indirect double immunofluorescence both for Myc-Sty and EC cell surface antigen (SSEA-1) and neuronal differentiation markers (A60 and HNK-1) suggested that Myc-Sty alone does not seem to give rise to the differentiation process although it may be a part in the signaling pathways leading to the differentiation or induce the differentiation only in combination with other appropriate conditions.

Finally, I tried subcloning of the inducible clone #9 cells hopefully to find a really stable inducible clone out of them, but unfortunately I couldn't obtain it perhaps due to the instability of the transfected Sty gene in P19 EC cells. However interestingly, I found that 20 subclones out of 73 expressed reasonably high amount of Myc-Sty, which was higher than number of positive cells (about 5%) showing intensive nuclear staining for Myc-Sty. It suggested that each cell in the same inducible clone expresses different amount of Myc-Sty, which could not be clearly detected by the conventional immunofluorescence, perhaps because each cell responds to the IPTG to a different degree due to the different level of repressor or the different copy number of the Sty gene in the individual cell.

Discussion and future direction

Sty/Clk interacts with SR proteins and phosphorylates them resulting in the disassembly of nuclear speckles which contain small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP splicing factors (SR proteins) and act as storage sites for splicing factors suggesting that Sty may influence alternative splicing. The phosphorylation and redistribution of these SR proteins seem to be very crucial to the spliceosome formation and splice site selection in vivo. SRPK1, another protein kinase, also phosphorylates and despecklizes SR proteins in the nucleus affecting alternative splicing. Actually, over-expressed SRPK1 inhibited the splicing in vitro. The phosphorylation by SRPK1 is dependant on cell cycle; it controls the intranuclear distribution of splicing factors in interphase cells and the reorganization of nuclear speckles during mitosis. However, there has been no evidence that Sty/Clk is regulated by cell cycle and furthermore, Sty/Clk has a broader substrate specificity than SRPK1 suggesting that they play different roles in regulating SR proteins in vivo.

In spite of these findings, we don't know which SR proteins are phosphorylated by Sty/Clk although the initial yeast two hybrid system identified a series of SR proteins (SRp20, SRp30, and SRp55) interacting with Sty and ASF/SF2 was shown to be phosphorylated by Sty in vitro. Additionally we don't know the biological outcome of the phosphorylation of these SR proteins by Sty either although it was strongly assumed that Sty may be implicated in the differentiation based on results from many previous studies (Howell *et al*, 1992; Myers *et al*, 1994).

In order to address these two main questions, I started to establish Sty inducible

clones in P19 EC cells because it was known that long term over-expression of Sty was cytotoxic to the cells. P19 cells used as a model system is very valuable in that they are easily maintained in serum supplemented media, efficiently transfected by any conventional transfection method, and most importantly differentiated into muscle and neuron by DMSO and retinoic acid (RA) respectively (McBurney *et al*, 1982). Interestingly, I used a construct containing the promoter and intragenic sequence of PGK-1 gene, B17 for the stable transfection to establish Sty inducible clones. Consistently with previous results (McBurney *et al*, 1994a and 1994b), the use of B17 really increased the efficiency of stable transfection with pPOPMycSty. Actually, for my initial transfections without B17, sometimes there were no drug resistant colonies obtained or sometimes, there was no stable expression of Myc-Sty although some drug resistant colonies could be obtained.

Myc-Sty produced from the inducible clones turned out to be catalytically active, dimerize with GST-Sty coupled to Sepharose beads, phosphorylate on Tyr residue , more importantly phosphorylate SR proteins and induce the disassembly of nuclear speckles. As described earlier, Sty was identified using α-phosphotyrosine antibody and auto-phosphorylated on Tyr in bacterial cells. However, interestingly only over-expressed Myc-Sty is phosphorylated on Tyr while endogenous Sty may not be phosphorylated on Tyr suggesting that the kinase activity of endogenous Sty is tightly regulated. Furthermore tyrosine-phosphorylation of even Myc-Sty can be detected only in the presence of sodium vanadate (Na_3VO_4), phosphatase inhibitor suggesting that tyrosine phosphorylation is extremely transient and may play some important regulatory roles.

The evidence that endogenous Sty is not fully active although it has intact protein

structure was demonstrated in some experiments comparing endogenous Sty and Myc-Sty from the inducible clones. For example, western blot for Triton X-100 soluble and insoluble fraction showed that endogenous Sty was predominantly positioned in the detergent insoluble fraction indicating that endogenous Sty is not active fully and bound by other protein complexes (for example, nuclear speckles) in the nucleus therefore could not be extracted by the detergent while most of Myc-Sty was catalytically active and therefore could be detected in the detergent soluble fraction. Furthermore over-expressed Sty showed very broad protein bands while endogenous Sty produced relatively compressed bands suggesting that the latter is subject to very tight regulation and therefore is showing very limited auto-phosphorylation at the limited sites.

I tried to answer the question which SR proteins are phosphorylated by Sty in vivo. Western analysis result strongly suggest that SRp30 and SRp55 may be the physiological substrates of Sty however, this result is somewhat controversial because the percentage of positive cells for Myc-Sty in the inducible clone #9 is about 25% and the phosphorylation and dephosphorylation of SR proteins may be very dynamic process which is processed very quickly in vivo. However this result is very plausible taking into account some previous findings; yeast two hybrid screening identified those two SR proteins as proteins interacting with Sty. On the other hand, over-expression of these two SR proteins resulted in the incompletely or unspliced pre-mRNA somewhat consistently with the production of two larger transcripts of Sty following the differentiation of P19 EC cells with RA, and that both SRp30 and SRp55 are indispensable for the chicken cell viability as well as the Drosophila development (Ring and Lis, 1994; Wang et al, 1996) respectively. This does make sense in

that these two SR proteins play a role both in constitutive and in alternative splicing and furthermore the alternative spicing is a very important mechanism to regulate a series of cellular process such as development and differentiation, which was demonstrated in the Drosophila Sex determination quite intensively (Boggs *et al*, 1987).

Next question I raised was that Sty alone can give rise to the differentiation in P19 EC cells without any other treatment including the aggregation of the cells. According to the double immunofluorescence result Sty alone does not seem to induce the differentiation. It is not surprising taking into account that the differentiation is a very complex process in which the expression of many genes should be orchestrated to initiate and proceed the differentiation and also requires some delicate culture condition such as cell-to-cell contact. The possibility that the biological effect exerted by Sty is only a part of the whole differentiation process, therefore the over-expression of Sty alone was not enough to induce the differentiation or Sty may induce differentiation process only when combined with some appropriate conditions should not be ruled out.

Actually in my lab, to figure out the biological function of Sty, the disruption of three murine isoforms of Sty is being tried to generate Sty knockout mice. This approach is very valuable in dissecting and understanding the complicated developmental process because it is assumed that each isoform may play a different role at the different developmental stages.

The real problem with this inducible system I established is the instability of Myc-Sty cDNA in the inducible clones. This phenomenon is a unique feature of P19 EC cells. P19 cells, compared with other cell lines, are easy to transfect but in proportion to the high transfection efficiency, the transfected genes are also easily lost and/or inactivated. Initially

Myc-Sty in clone #9 was much higher than endogenous Sty but later the situation changed . To study the aspect of the phosphorylation of SR proteins by Sty and their effect on the alternative splicing, it is more desirable to establish the inducible system in other cell lines. Furthermore, many researchers also claimed that IPTG used as an inducer is not efficient and the tetracycline inducible system is better than the Lac inducible one in terms of increasing the induction level. The tetracycline system is worth while trying to figure out the proteins interacting with Sty other than RNA binding proteins with regard to the regulation of Sty and correlation with other signaling pathways. Interestingly, it was demonstrated that in PC 12 cells, the expression and the activity of MAPK after Sty over-expression was increased. It requires further intensive studies but it suggested Sty may also influence other signaling pathways through the alternative splicing or the direct protein-protein interaction. Finally, the studies to understand Sty should also be paralleled at the protein level to understand Sty further. For example, previously, Sty mRNA level was increased following the differentiation of P19 cells with RA but there is no evidence about that at the protein level. Additionally, the study to find transcripts governed by Sty will also be interesting and should be done.

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Abbreviation

Ab: Antibody

ASF: Alternative splicing factor

ATP: Adenosine tri-phosphate

β-gal: β–galactosidase

BES: N,-N-Bis (2-hydroxyethyl)-2 aminoethanol sulfonic acid

BME: β-Mercaptoethanol

cDNA: Complementary deoxy ribonucleic acid

DBS: Donor bovine serum

dNTP: Deoxy nucleoside tri-phosphate

DTT: Dithiothreitol

EDTA: Ethylene diamine tetra acetic aid

EC: Embryonal carcinoma

ES: Embryonic Stem

FBS: Fetal bovine serum

FCS: Fetal calf serum

FITC: Fluorescein isocyanate

GST: Glutahtione s-transferase

HEPES: N-2-hydroxyethyl pipetrazine-N'-2 ethane sulfonic acid

HRP: Horsheadish peroxidase

Hyg: Hygromycin

IPTG: Isopropyl β- D-thiogalactoside

IRES: Internal ribosome binding site

Lac O: Lactose operon operator

Lac R: Lactose operon repressor

mAb: Monoclonal antibody

Neo: Neomycin

NLS: Nuclear localization signal

NP40: Nonidet P-40

ONPG: O-nitrophenyl β-D-galactopyranoside

PAGE: Poly acrylamide gel electrophoresis

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PGK: Phosphoglycerate kinase

PMSF: Phenyl methyl sulfonyl fluoride

RT: Room temperature

SDS: Sodium dodecyl sulphate

Ser: Serine

Tet O: Tetracycline operator

Tet R: Tetracycline repressor

Thr: Threonine

tTA: Tetracycline transactivator

Tyr: Tyrosine

U2AF: U2 snRNP auxiliary factor

X-gal: 5-Bromo 4-chloro 3-indolyl β-D-galactopyranoside

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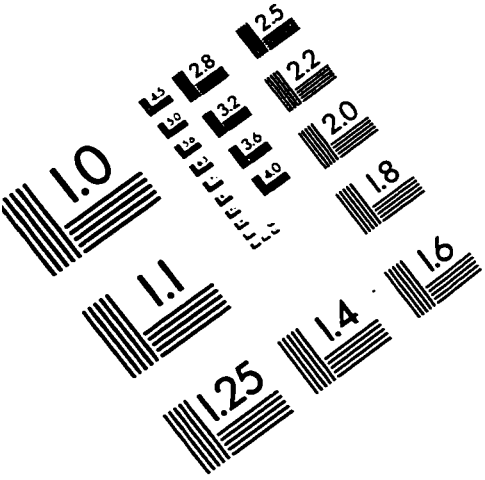
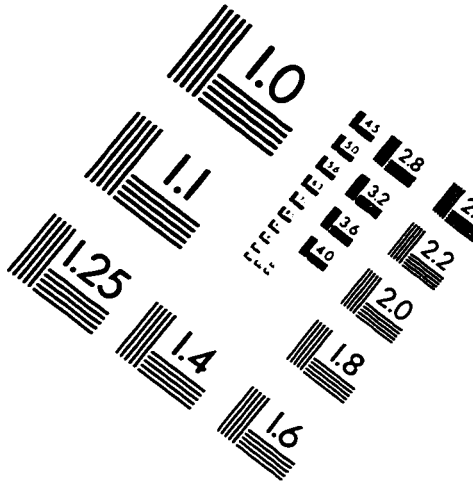
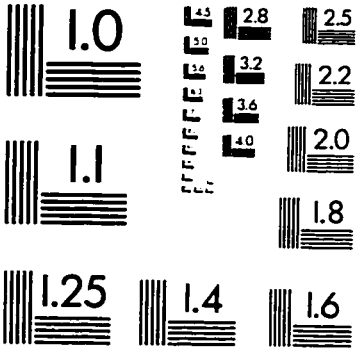
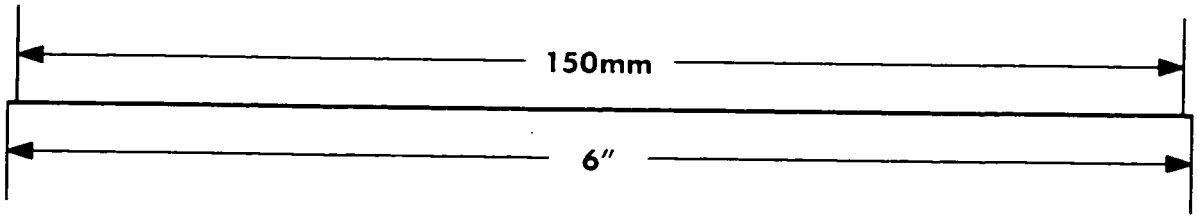
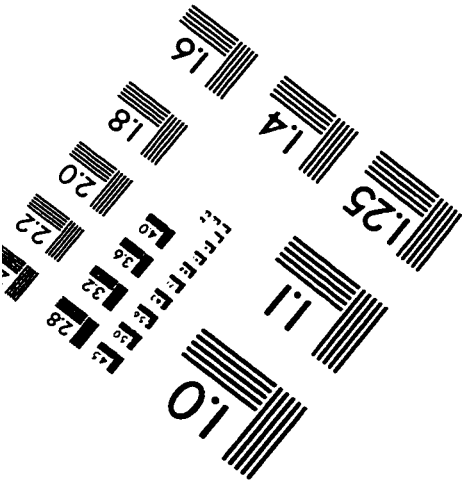
IMAGE EVALUATION TEST TARGET (QA-3)

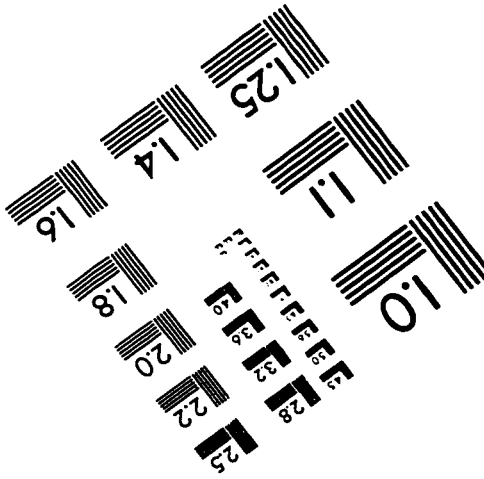












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