CHAPTER V

EXPRESSION OF HUMAN DNASE I IN *DICTYOSTELIUM DISCOIDEUM*

5.1 Introduction: human DNaseI

To check the versatility of the secretory vector [pA15S(1)] and *D. discoideum* as an expression system, human DNase I was cloned in the newly designed vector [pA15S(1)] and expressed in *Dictyostelium discoideum*. DNase I is a Ca^{2+}/Mg^{2+} dependent endonuclease, which is ubiquitously expressed in mammalian tissues and is phylogenitically highly conserved.

5.1.1 Characteristics

(Rauch *et al.*, 1997; Schroter *et al.*, 1996; Oliveri *et al.*, 2001)

DNase I, a secretory protein, is stored in zymogen granules in the nuclear envelope. It is localized primarily in tissues of the digestive system. Highest levels have been found in urine, but is also relatively abundant in semen and saliva. It's catalytic activity involves endonucleolytic cleavage of DNA to 5'-phosphodinucleotide and 5'-phosphooligonucleotide end products. Divalent cations, particularly calcium or magnesium are required for the catalytic activity. Among other functions, DNase I seems to be involved in cell death by apoptosis. It also binds specifically to G-actin and hence blocking the actin polymerization.

5.1.2 Structure (gene/protein)

(Yusada *et al.*,1990; Yusada *et al.*,1995; Yusada *et al.*,1995)

DNase I gene is approximately 3.2 kb long comprising nine (I-IX) exons separated by eight introns. It contains an open reading frame of 849 bp encoding a polypeptide of 260 amino acids with extensive homology to bovine DNase I (77%).

Two possible in-frame initiation codons (ATG) are present upstream of the nucleotides coding for the N-terminal leucine of DNase I. The nucleotides between the first ATG and the N-terminal leucine encode a sequence with a hydrophobic core of 12 amino acids which is a secretory signal sequence. A stop sequence immediately follows the nucleotides encoding the C-terminal lysine.

Length of the unprocessed protein is 282 aa with a molecular weight of 31.433 kD. It contains four cysteines and two potential N-linked glycosylation sites. The active site histidine is well conserved between bovine and DNase I. Two six

stranded ß-pleated sheets are packed against each other to form the core of the protein, which is surrounded by eight alpha helices and six turns.

Salient features of DNase I structure

Key	From	To	Length	Description
Signal	1	22	22	
Chain	23	282	260	DNaseI
Disulfide	123	126		
Disulfide	195	231		Essential for Enzymatic activity
Act_site	100	100		
Act_site	156	156		
Carbohydrate	40	40		N-linked (GLCNAC)
Carbohydrate	128	128		N-linked (GLCNAC) (Potential)

Numbers refers to amino acid residues from N- to C-terminal

5.1.3 Gene sequence:

Accession # NM_005223

gggagctCTGAAGATCGCAGCCTTCAACATCCAGACATTTGGGGAGACCAAGAT
GTCCAATGCCACCCTCGTCAGCTACATTGTGCAGATCCTGAGCCGCTATGACA
TCGCCCTGGTCCAGGAGGTCAGAGACAGCCACCTGACTGCCGTGGGGAAGC
TGCTGGACAACCTCAATCAGGATGCACCAGACACCTATCACTACGTGGTGAGT
GAGCCACTGGGACGGAACAGCTATAAGGAGCGCTACCTGTTCGTGTACAGGC
CTGACCAGGTGTCTGCGGTGGACAGCTACTACTACGATGATGGCTGCGAGCC
CTGCGGGAACGACACCTTCAACCGAGAGCCAGCCATTGTCAGGTTCTTCTCC
CGGTTCACAGAGGTCAGGGAGTTTGCCATTGTTCCCCTGCATGCGGCCCCGG
GGGACGCAGTAGCCGAGATCGACGCTCTCTATGACGTCTACCTGGATGTCCA
AGAGAAATGGGGCTTGGAGGACGTCATGTTGATGGGCGACTTCAATGCGGGC
TGCAGCTATGTGAGACCCTCCCAGTGGTCATCCATCCGCCTGTGGACAAGCC
CCACCTTCCAGTGGCTGATCCCCGACAGCGCTGACACCACAGCTACACCCAC

GCACTGTGCCTATGACAGGATCGTGGTTGCAGGGATGCTGCTCCAAGGCGCC
GTTGTTCCCGACTCGGCTCTTCCCTTTAACTTCCAGGCTGCCTATGGCCTGAG
TGACCAACTGGCCCAAGCCATCAGTGACCACTATCCAGTGGAGGTGATGCTG
AAGTGAggg

5.1.4 Therapeutic applications

(Shak *et al.*, 1990)

As a therapeutic, DNase I is available under the name Pulmozyme (Genentech, USA). It is used to reduces the viscosity of Cystic fibrosis sputum by hydrolyzing the extracellular DNA released by degenerating leukocytes that accumulate in response to infection. Cystic fibrosis (CF) is an inherited disorder characterized by thick mucus secretions in the lungs. Retention of this mucous in the airways contributes to reduced pulmonary function and chronic lung infections in CF patients. Respiratory complications are the major cause of death in CF. The average life expectancy of CF patients is 29 years. It is not known why the airways of these patients remain persistently infected with bacteria such as *Pseudomonas aeruginosa* despite antibiotic therapy. It has been suggested that factors such as impaired clearance of secretions due to their increased viscosity (possibly due to an ion-transport defect), or reduced effectiveness of amino-glycoside antibiotics from reversible binding of polyvalent anions in the secretions, may predispose to bacterial persistence. A plausible molecular mechanism for these secondary phenomena was proposed long back. Experiments in 1950s and 1960s revealed that DNA is present in very large amounts (3-14 mg/ml) in purulent, but not in uninfected lung secretions. White blood cells mobilize to fight the chronic lung infections- characteristic of CF patients. DNA is released by WBC and accumulates in the lungs. DNA, an extremely viscous polyanion may contribute both to the increased viscosity of lung secretions and to the reduced effectiveness of aminoglycoside antibiotics. Lung secretions incubated in vitro with partially purified bovine DNase I show a large reduction in viscosity. Based on these observations, bovine pancreatic DNase I (Dornavac or Dornase) was approved in USA for human use in 1958. However, severe adverse respiratory reactions did occasionally occur, perhaps as a consequence of allergic reactions to a foreign protein. Despite extensive structural homology there is significant divergence at the amino acid level with only 77% overall identity. Except for one region in the ß sheet structure, almost

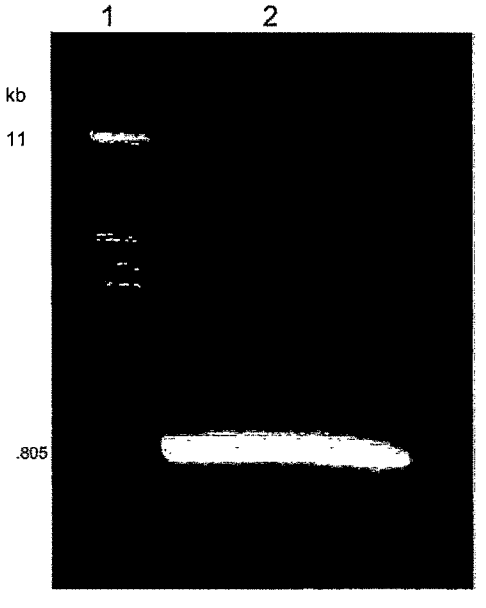
all of the difference between the human and the bovine proteins are located in hydrophilic regions on the surface of the molecule. Thus there is sufficient divergence between bovine and human DNase I in potentially immunogenic regions to suggest that some of the adverse reactions to the bovine protein could be due to an immune response to a foreign protein.

Pulmozyme is the first biologic agent developed and clinically tested specifically for management of CF symptom. It is an aerosolized recombinant human DNase I. in the test tube, Pulmozyme acts to break down DNA molecules. In the body, the exact mechanism by which Pulmozyme works has not been confirmed. It is possible, however, that the product breaks down the DNA molecules in CF patient's lung secretions.

5.2 Construction of pA15S(1)DNase I

First step in the expression of DNase I in *D. discoideum* was cloning of DNase I cDNA into pA15S(1). cDNA for DNase I cloned in pGEXTDNase I was a gift from Prof. Dingermann. In order to isolate DNase I from pGEXTDNase I, it was PCR amplified **(Section 2.3.11)**. Twentymer primers MR04 and MR05 **(Section 2.1.2)** were designed for *SacI* site - 5' primer located at the end of the endogenous secretory signal of DNase I and 3' primer at the end of DNase I gene. *SacI* site was incorporated in the amplified DNase I since *SacI* site present in the MCS of pA15S(1) was planned to be used for cloning of cDNA of DNase I.

Amplification of DNase I was confirmed by the presence of 793 bp band on the gel **[Fig. V(a), lane 2]**. Amplified DNA was eluted from gel, purified using Quagene DNA elution kit and ligated using poly[A] tail of the recovered band and poly[T] region of pGEM-T (Promega) vector. The new vector formed was henceforth named as pMR#7 (3.793kb). *E. coli* cells were transformed with pMR#7 by electroporation method **(Section 2.3.1.2)** and blue/white selection of transformants was done on amp-Xgal-IPTG plates **(Section 2.3.12)**. Many white colonies were obtained, out of which 12 were screened for the insert by digestion with *SacI*. To check for any point mutation in DNase I during PCR, DNase I along with HP1:HP2



5.2 Fig V(a) - PCR amplified DNase I gene from pGEXTDNase I on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: PCR amplified DNase I from pGEXTDNase I

was sequenced from pMR#7 **(section 2.3.14)** and showed no mutation (data not shown).

After confirming the sequence of DNase I gene in pMR#7, pMR#7 was digested with *SacI* to release the DNase I gene of 793 bp **[Fig. V(d), lane 2]**. DNase I gene was gel eluted and purified using Quagene DNA elution kit. Purified DNA was ligated **(Section 2.3.10)** to *SacI* digested and dephosphorylated pA15S(1) **[Fig. V(d), lane 3]**. The new vector formed was henceforth named as pA15S(1)DNase I (6.8 kb) **[Fig. V(b)]**. *E. coli* cells were transformed with pA15S(1)DNase I by electroporation method **(Section 2.3.1.2)**. Since there was no antibiotic selection for the recombinants, colonies obtained on ampicillin plates were replica plated for colony blot assay **(section 2.3.13)**. DNase I gene radiolabelled with ^{32}P by nick translation was used as a probe for detecting recombinants **[Fig. V(c)]**.

Positive clones containing DNase I insert in pA15S(1)DNase I were further confirmed by RE digestion **(section 2.3.5) [Fig. V(d)]**:

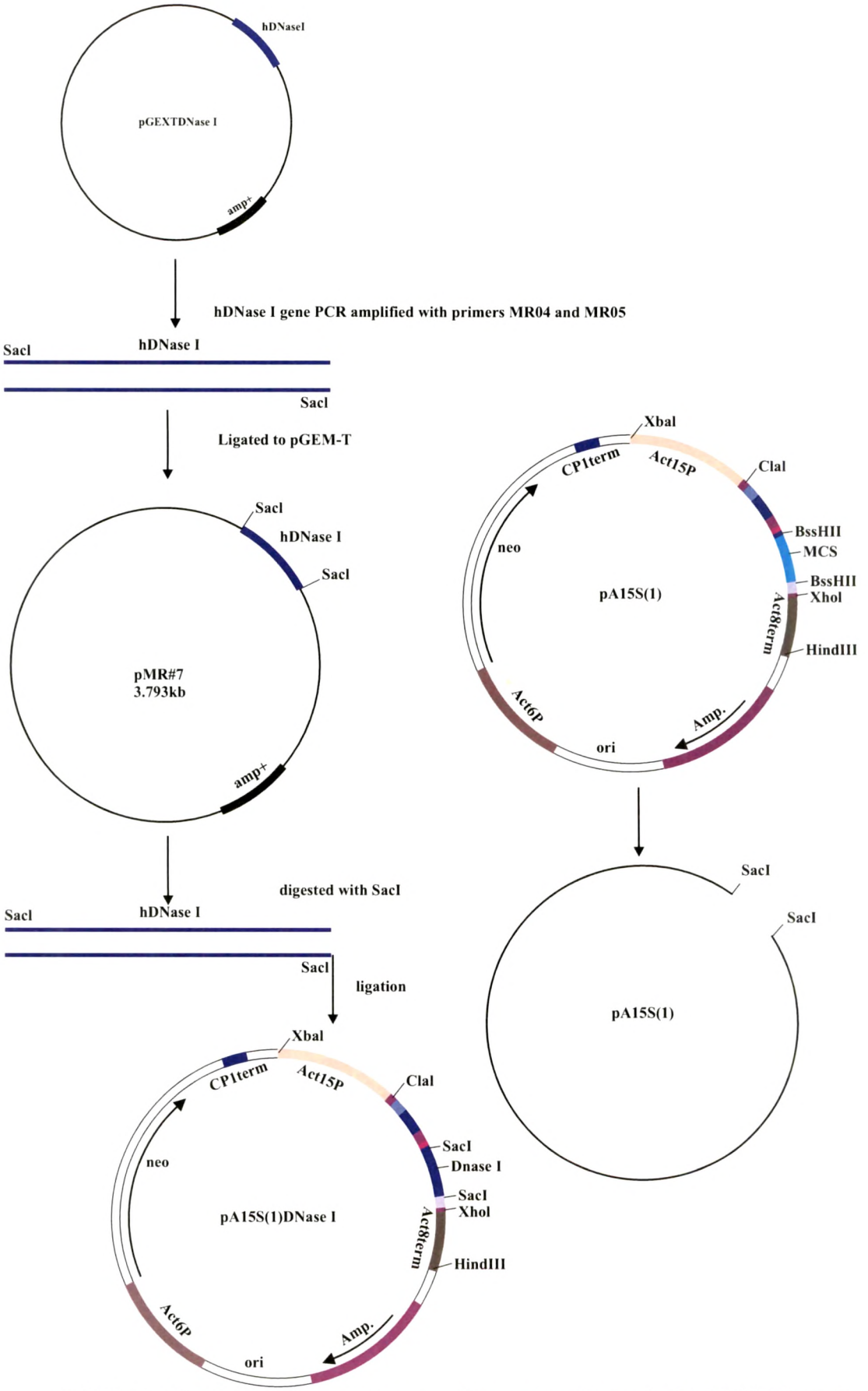
i) *SacI*: *SacI* was used for cloning of DNase I in pA15S(1)DNase I. Release of two fragments of 6 kb and 793 bp from pA15S(1)DNase I on digestion with *SacI* confirmed the presence of DNase I in pA15S(1)DNase I **[Fig. V(d), lane 4]**.

ii) *NarI*: one *NarI* site is present in the DNase I sequence and one *NarI* site is in the pA15S(1) backbone. Release of lower fragment of 705 bp from pA15S(1)DNase I on digestion with *NarI* confirmed the correct orientation of insert in pA15S(1)DNase I **[Fig. V(d), lane 5]**.

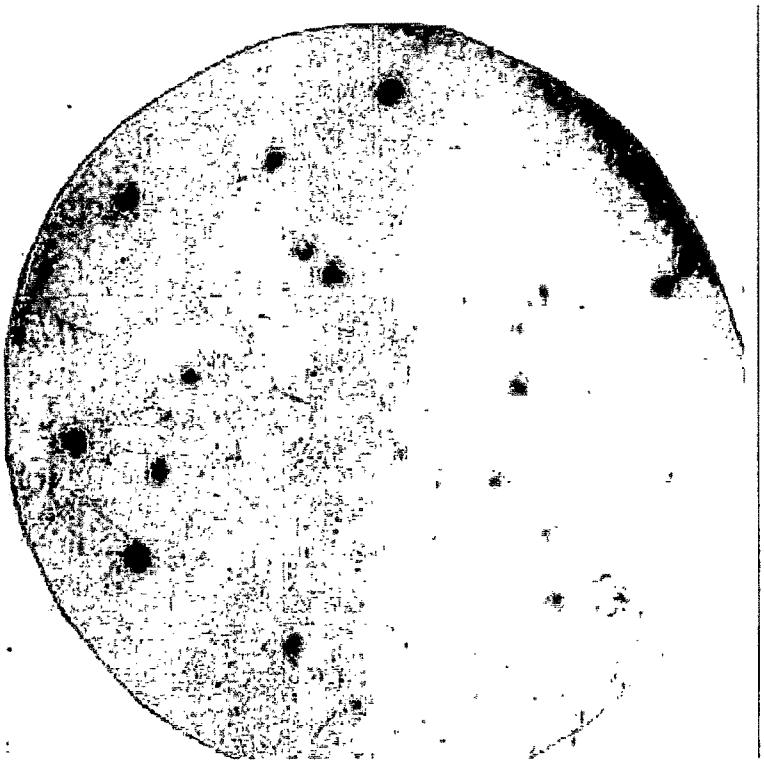
To further confirm the DNase I sequence and its reading frame in pA15S(1)DNase I, DNase I along with HP1:HP2 was sequenced **(section 2.3.14)** from pA15S(1)DNase I using primer MR01 **(section 2.1.2)**. The DNase I sequence showed no mutation and was in proper reading frame **[Fig. V(e)]**.

5.3 Transformation of *D. discoideum* (Ax-2) cells with pA15S(1)DNase I

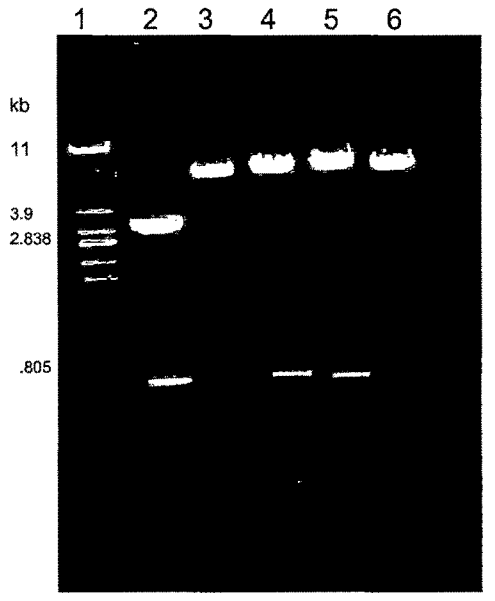
Cells of Ax-2 strain of *D. discoideum* in log phase (2 X 10^6/ml, 10 ml per plate) were transformed with pA15S(1)DNase I (7-8 μg) by $CaPO_4$ method **(Section 2.4)** along



5.2 Fig. V(b) - Schematic summary of construction of pA15S(1)DNase I

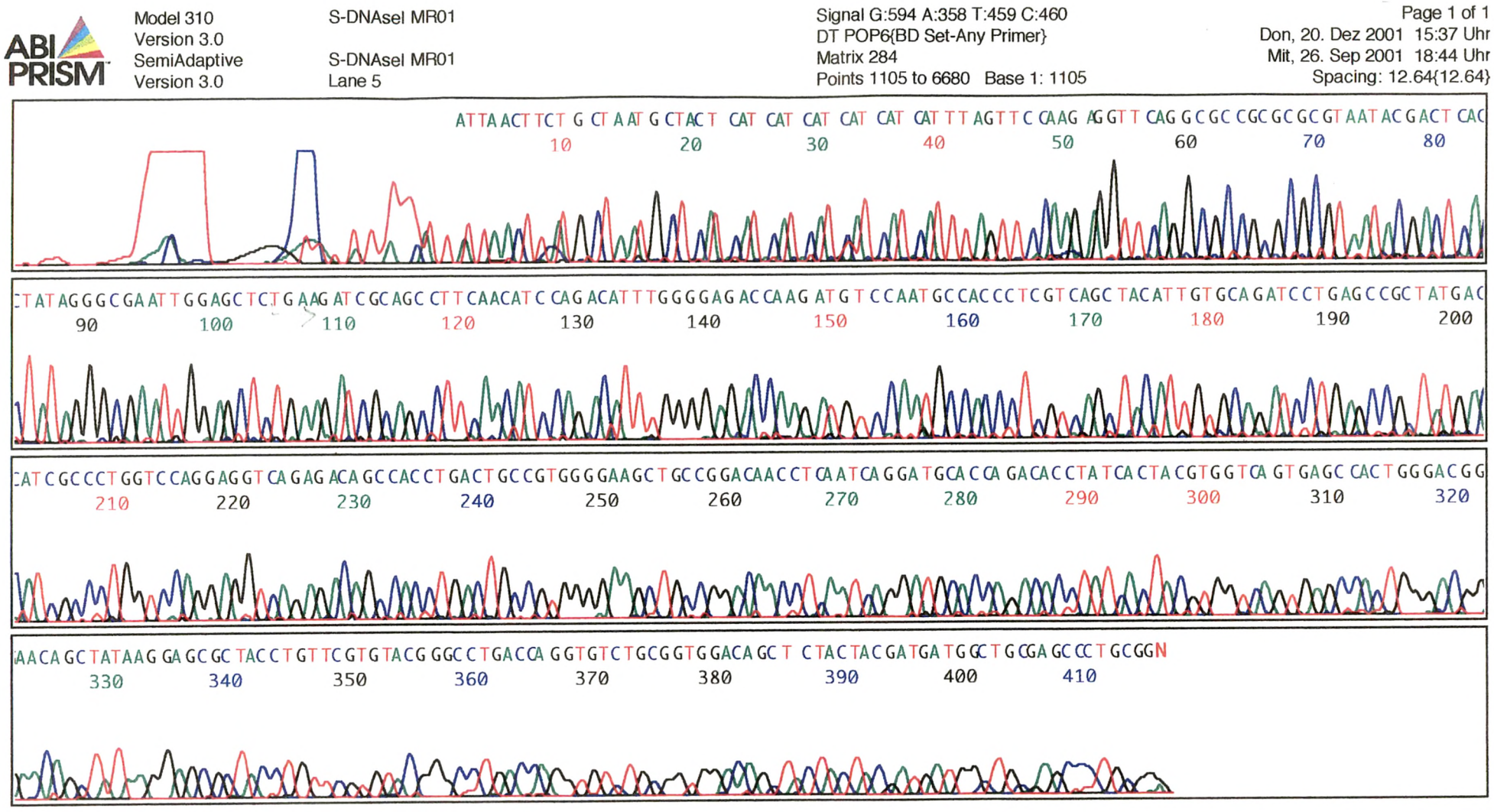


5.2 Fig. V(c) - Colony blot of *E.coli* cells transformed with pA15S(1)DNase I. DNase I gene radiolabelled with ^{32}P was used as a probe for detecting recombinants colonies



5.2 Fig. V(d) - Construction and RE digestion pattern of pA15S(1)DNase I on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pMR#7 digested with *SacI*
Lane 3: pA15S(1) digested with *SacI*
Lane 4: pA15S(1)DNase I digested with *SacI*
Lane 5: pA15S(1)DNase I digested with *NarI*
Lane 6: pA15S(1) digested with *NarI*



5.2 Fig. V(e) - Sequence of pA15S(1)DNase I : In pA15S(1)DNase I, DNase I gene has been cloned in the *SacI* site of pA15S(1) and was found to be in frame with the secretory sequence (105 onwards).

with proper controls. pA15GFP transformed cells were used as positive control and untransformed cells as negative control for antibiotic resistance. Transformants growing in media containing G418 at 10 μg/ml concentration were selected and subsequently maintained at 5 μg per ml. Clones were observed after 10-15 days and positive clones growing on plates containing G418 were transferred to 24 well plates for further growth and observation.

5.3.1 Morphology of cells transformed with pA15S(1)DNase I

Initially many small clones were observed but after some time most of them disappeared and few grew. As compared to positive control (pA15GFP), few transformants (2-10 clones/plate) were obtained with pA15S(1)DNase I. In 24 well plates, cells grew till 10^5/ml density and then growth was arrested. Under microscope transformants showed altered phenotype as compared to the positive control as well as untransformed cells. The cells were able to grow to a density of ~ 10^5 /ml and then they started getting detached from the plate, finally becoming round like beads - characteristic of dying cells **[Fig. V(f)]**. Cell death indirectly may indicate the lethal effect of DNase I expression.

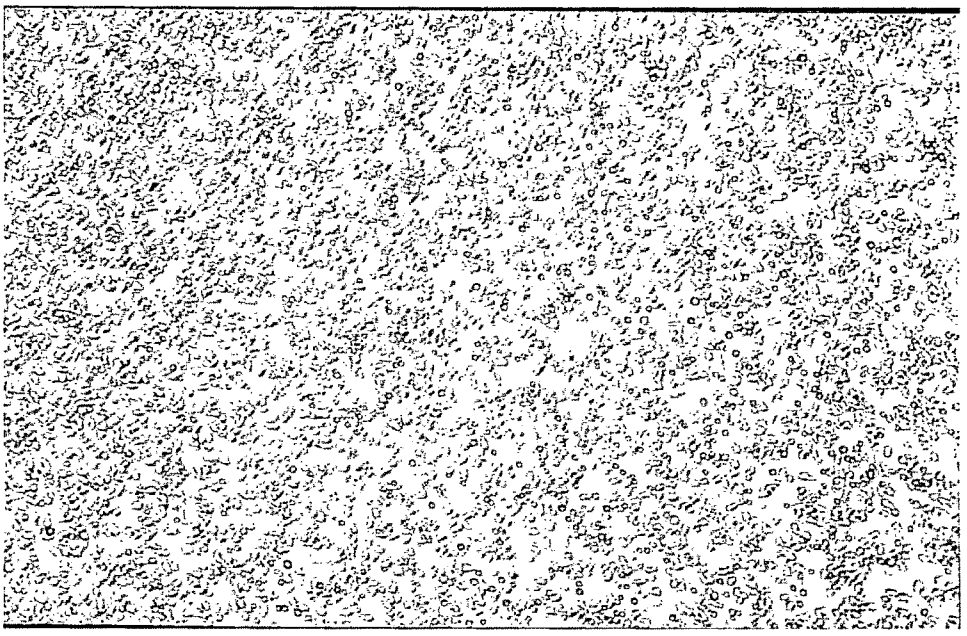
5.3.2 Drop test for viability and impaired development

To check for the altered growth pattern and morphology, drop test was done with the transformants along with control. 10^4 cells were dropped on *Klebsiella* lawn and grown at 21°C for four days **(section 2.5.6)**. Transformants showed delayed and less clearance of *Klebsiella* lawn lacking aggregation and fruiting bodies as compared to control **[Fig. V(g)]**. At this stage reason for lack of development was not clear i.e. whether it was because of a fraction of cells being dead or damaged which were unable to contribute to development or due to defect in developmental process by itself. Therefore vital staining of transformants was done.

5.3.3 Trypan blue staining for viability

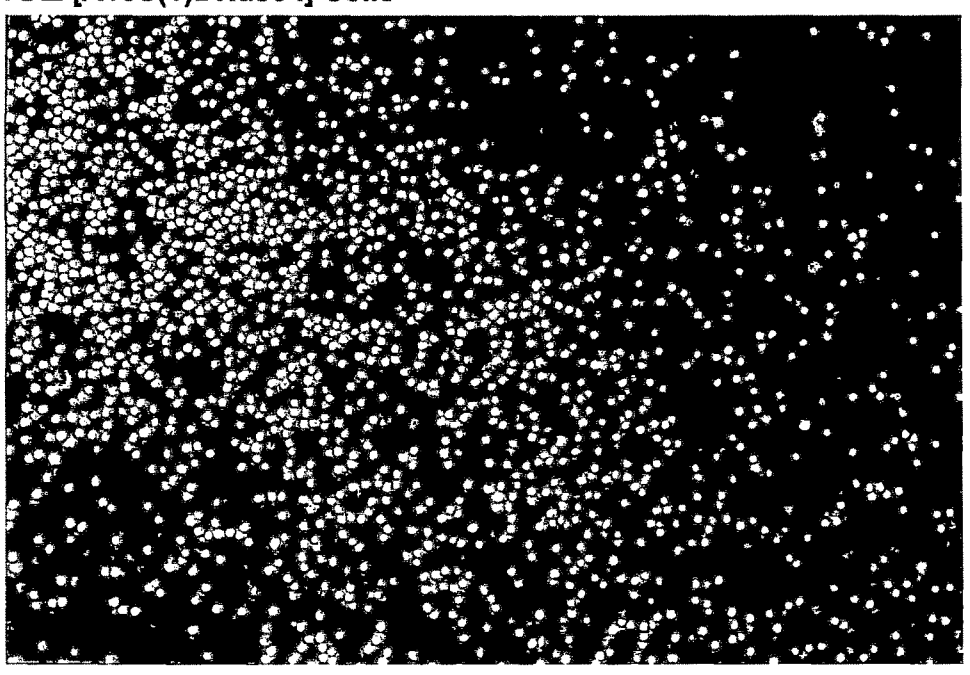
Trypan blue is a vital dye used to distinguish living cells from dead cells. DNase I transformants as well as control were mixed with Trypan blue solution (0.4% stock) in 1:1 ratio and observed under the microscope. Cell death due to DNase I

AX2 Cells



10X

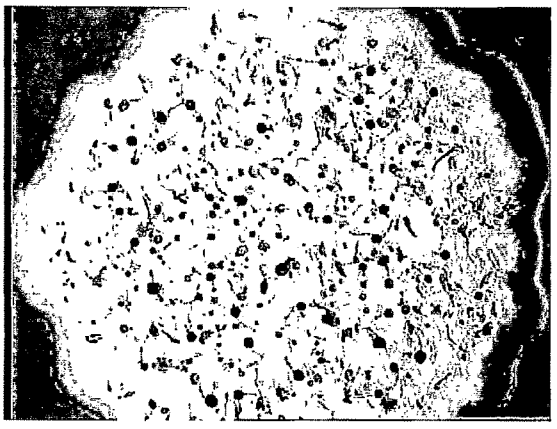
AX2 [A15S(1)DNase I] Cells



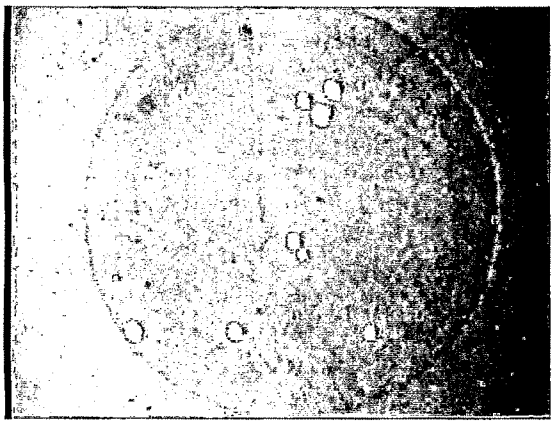
10X

5.3.1 Fig. V(f) - Morphology of Ax2 cells transformed with pA15S(1)DNase I

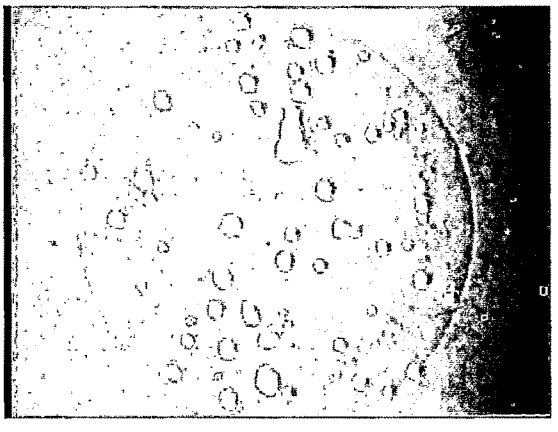
AX2 Cells



AX2 [A15S(1)DNase I] Cells
Clone # 1



AX2 [A15S(1)DNase I] Cells
Clone # 2



5.3.2 Fig. V(g) - Drop test for viability and impaired development of Ax2 cells transformed with pA15S(1)DNase I

expression was reconfirmed by majority of transformants taking up the stain and hence appearing blue under the microscope **[Fig. V(h)]**.

5.3.4 SDS-PAGE of cells transformed with pA15S(1)DNase I

Morphologial alteration and Trypan blue staining results indicated the expression of DNase I. Therefore it was decided to look for the expressed protein in the cells on SDS-PAGE. Due to insufficient number of cells in each clone (10^5 instead of 10^6), it was not possible to do proteomics of each clone individually. Therefore several clones were pooled up for the sample preparation. 100 µg of protein/well was loaded on 10% PAGE and the gel was run and finally stained with commassie blue **(section 2.5.7)**. PAGE protein profile of the transformants showed few alterations as compared to untransformed cells - appearance of new bands with disappearance of others **[Fig. V(i)]**. A band at 31.4 kD corresponding to DNase I was expected but because of the uncertainty of the extent of glycosylation and the possible degradation of the precursor, it was difficult to ascertain whether DNase I was being expressed or not.

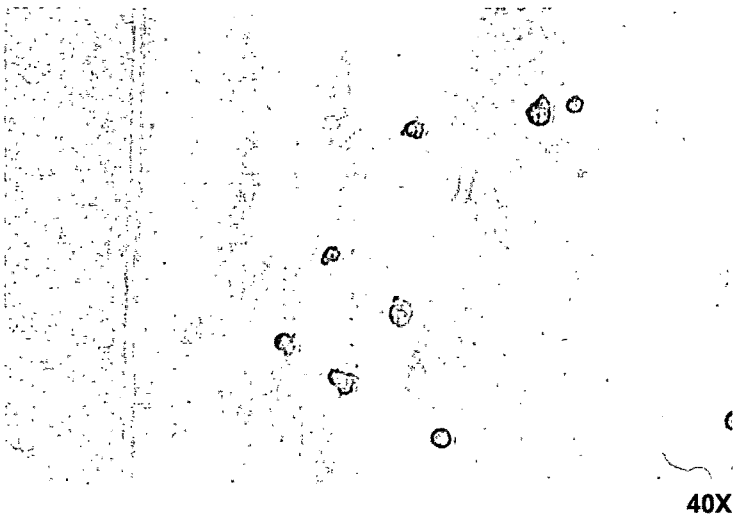
5.3.5 Detection of DNase I by Western blotting

Since no distinct polypeptide corresponding to 31.4 kD appeared in AX2 [A15S(1)DNase] SDS-PAGE, Western blotting was done with antibodies developed against DNase I **(section 2.5.8)**. Bacterial cells transformed with DNase I were used as positive control **[Fig. V(j)]**. No immunoreactive band was observed at 31.4 kD in transformed cells on western blot **[Fig. V(k)]**. It was difficult to ascertain whether DNase I was being expressed or not due to the uncertainty in the size of the recombinant protein undergoing varying extent of glycosylation or degradation.

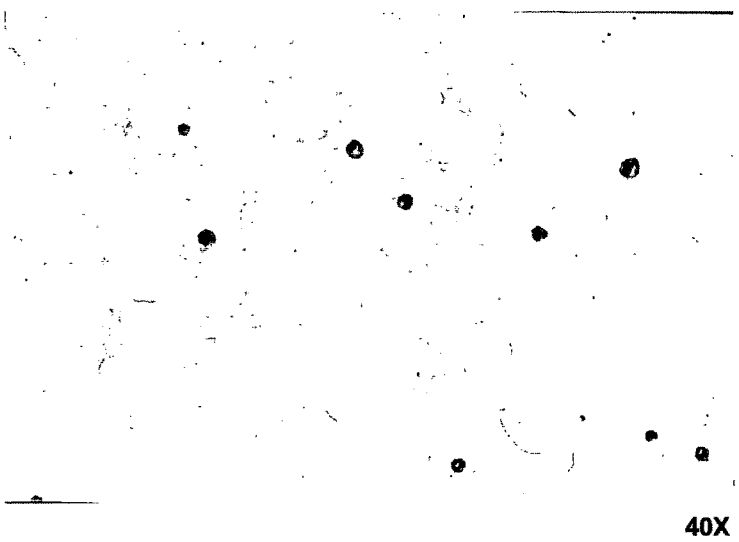
5.4 Construction of pERDNase I

No further confirmation of DNase I activity in media or cell was possible due to the apparent death of transformants at an early stage of growth. Therefore it was not possible to analyze the DNA or mRNA of transformants to directly confirm that cell death was occurring because of DNase I expression in the transformants. Therefore in order to confirm the direct correlation between cell death and DNase I expression, it was planned to express DNase I under the control of an inducible

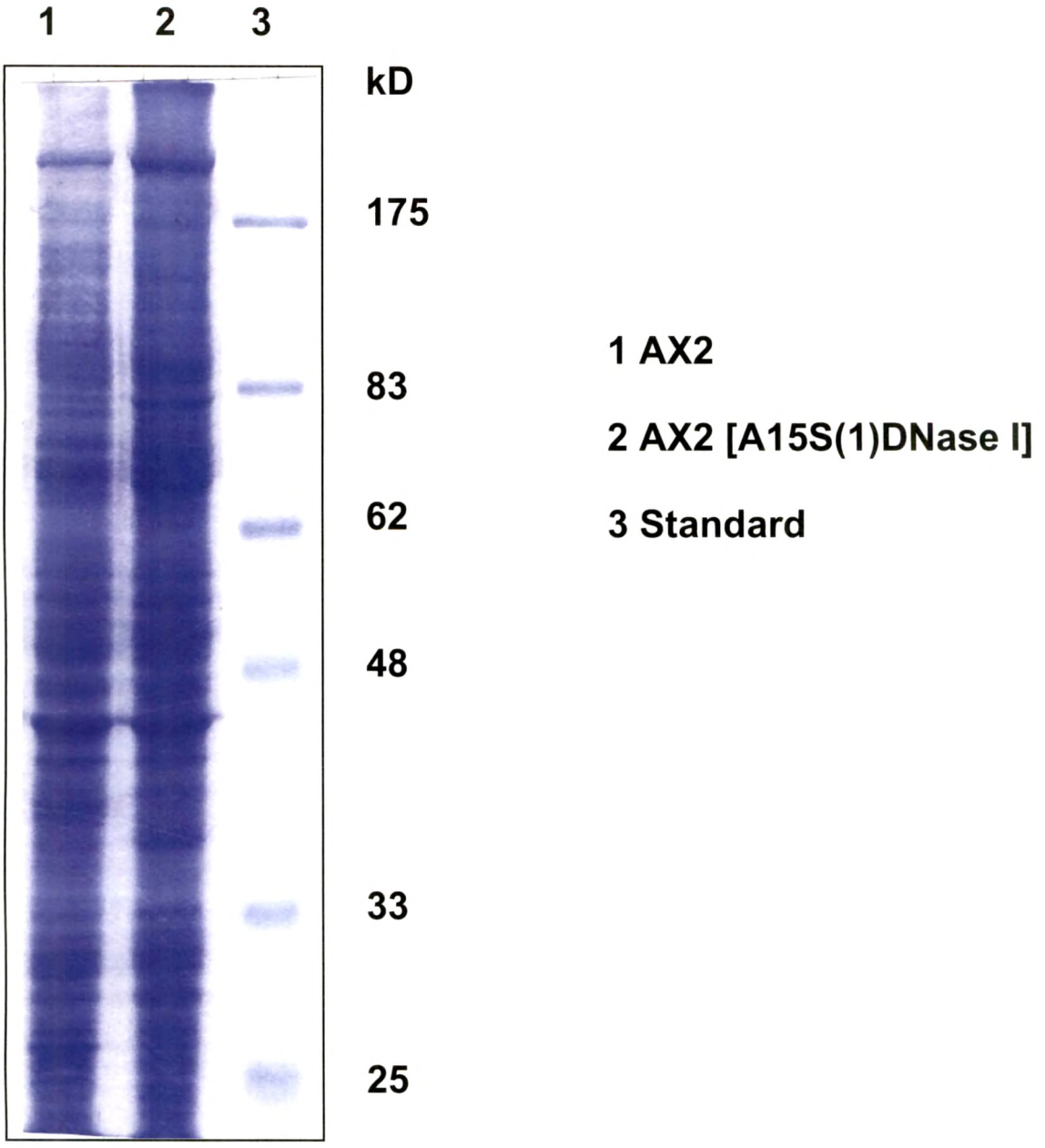
AX2 Cells



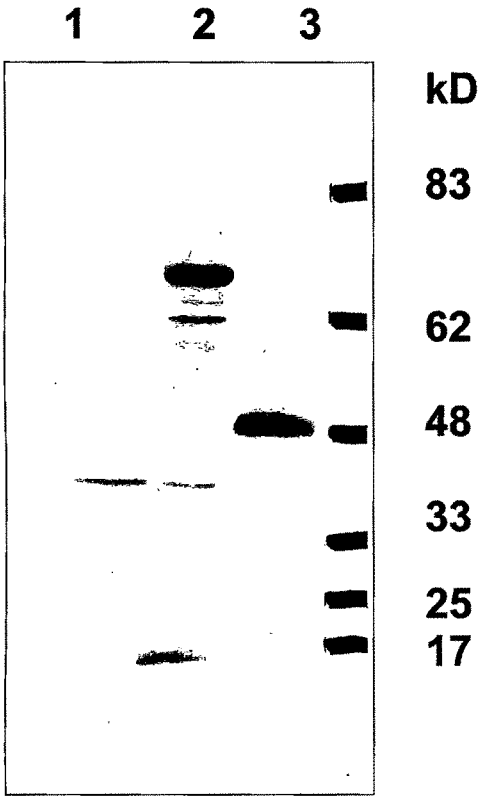
AX2 [A15S(1)DNase I] Cells



5.3.3 Fig. V(h) - Trypan blue staining of Ax2 cells transformed with pA15S(1)DNase I for viability

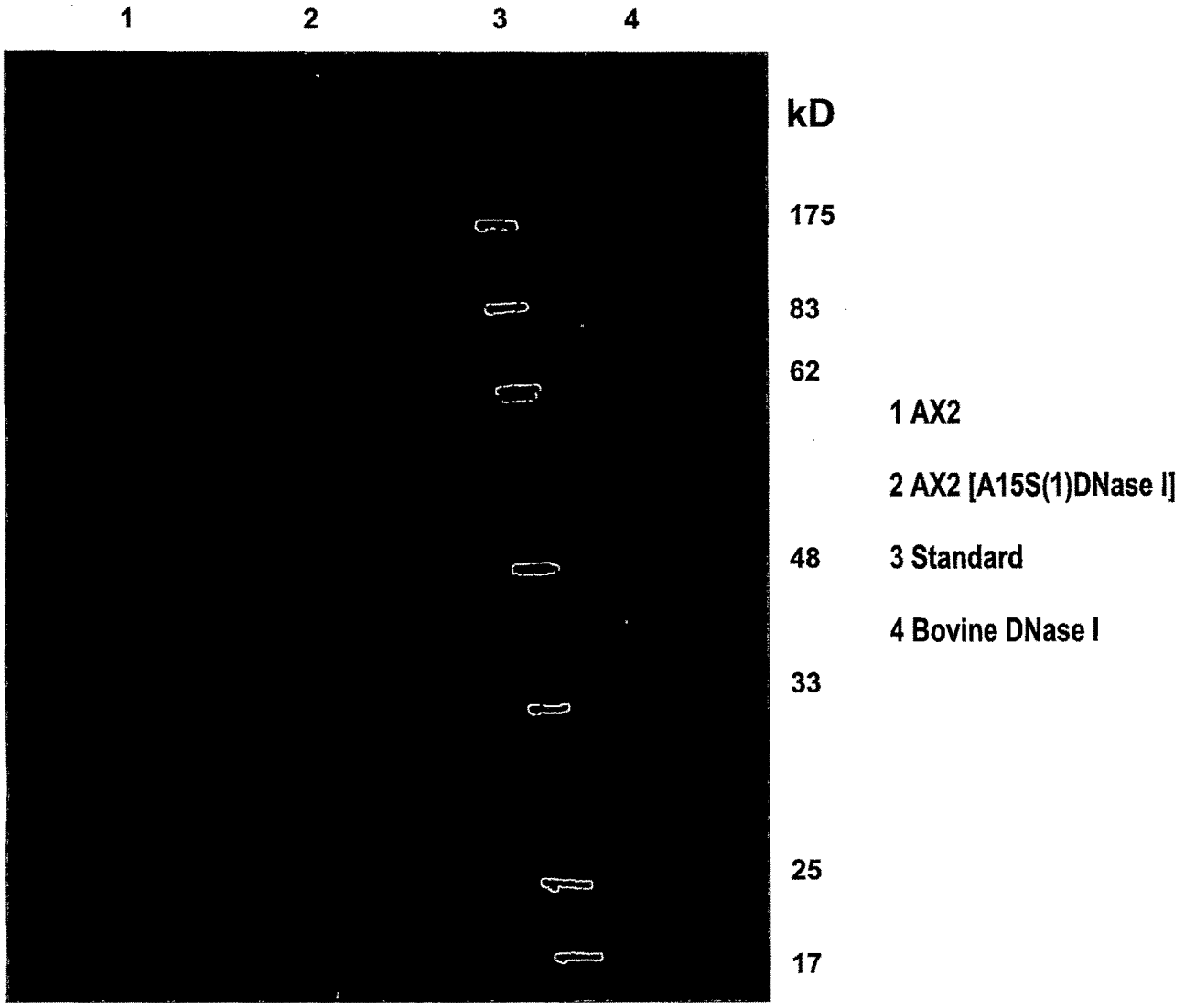


5.3.4 Fig. V(i) - 10% SDS-PAGE of Ax2 cells transformed with pA15S(1)DNase I



1 *E. coli* (pGEXTDNase I) - IPTG
2 *E. coli* (pGEXTDNase I) + IPTG
3 Bovine pancreas DNase I

5.3.5 Fig. V(j) - Western blotting of DNase I expressed in *E. Coli*



5.3.5 Fig. V(k) - Western blotting of DNase I expressed in *D.discoideum*

promoter. pER III is an extrachromosomal *D. discoideum* vector having *ras* promoter and csA (contact site A) leader sequence **(section 2.1.5.6)**. *ras* is a promoter inducible with cAMP. pER III was used for controlled expression of DNase I.

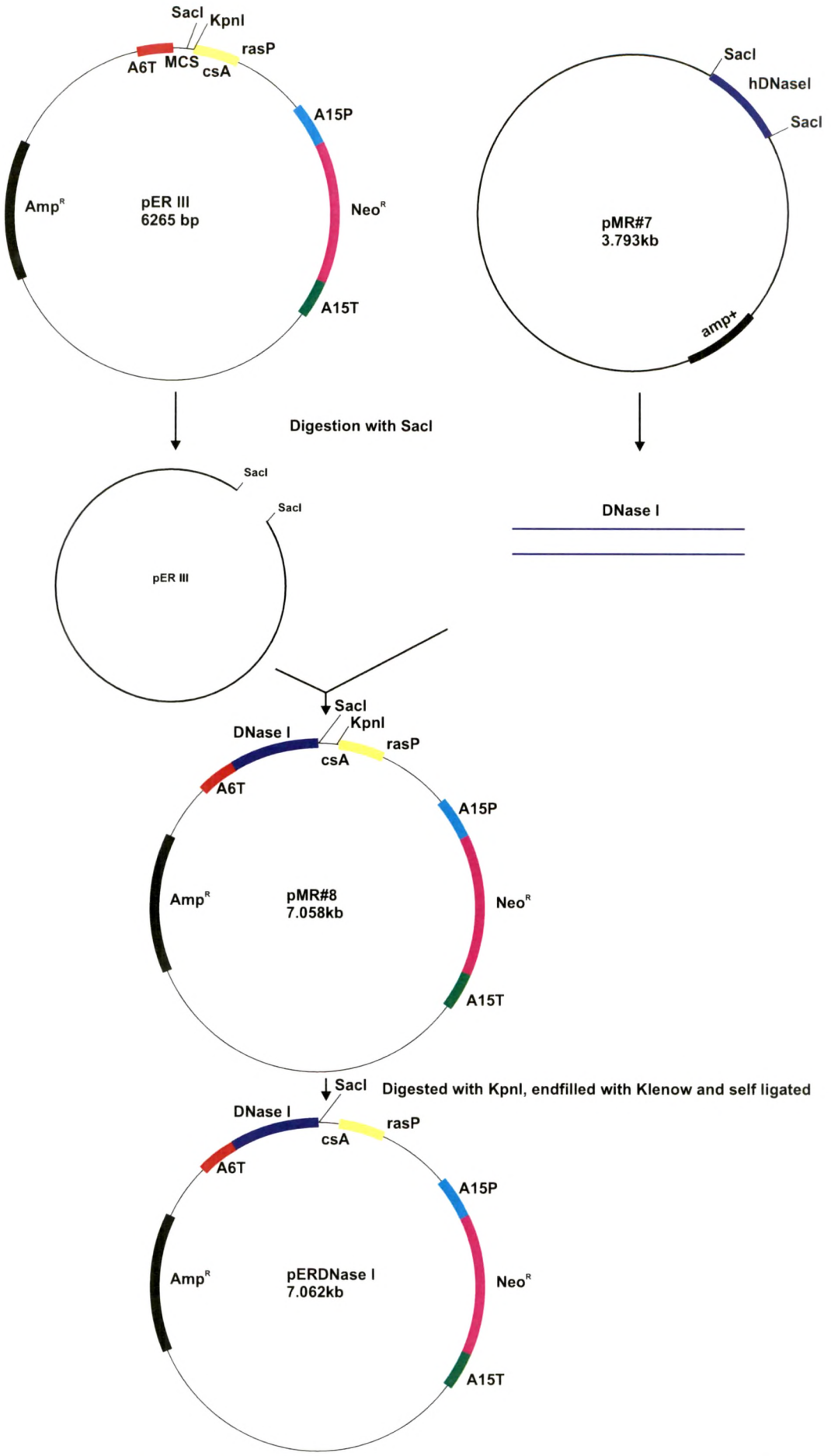
pMR#7 (PCR amplified DNase I cloned into pGEM-T **(section 2.1.5.5)** was digested with *SacI* to release the DNase I gene **[Fig. V(n), lane 2].** DNase I gene was gel eluted and purified using Quagene DNA elution kit. Purified DNA was ligated **(section 2.3.10)** to *SacI* digested and dephosphorylated pER III **[Fig. V(n), lane 3]**. The new vector formed was henceforth named as pMR#8 (7.058 kb). *E. coli* cells were transformed with pMR#8 by electroporation method **(section 2.3.1.2)**. Since there was no antibiotic selection for the recombinants, colonies obtained on ampicillin plates were replica plated for colony blot assay **(section 2.3.13)**. DNase I gene radiolabelled with ^{32}P by nick translation was used as a probe for detecting recombinants **[Fig. V(m)]**.

Positive clones containing DNase I insert in pMR#8 were further confirmed by RE digestion **(section 2.3.5) [Fig. V(n)]**:

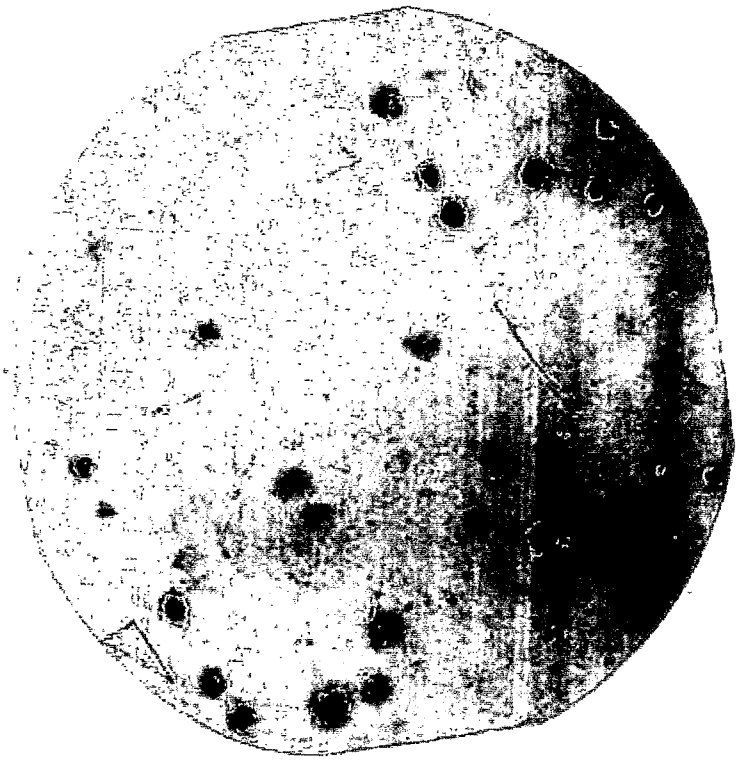
i) *SacI*: *SacI* was used for cloning of DNase I in pMR#8. Release of two fragments of 6.2 kb and 793 bp from pMR#8 on digestion with *SacI* confirmed the presence of DNase I in pMR#8 **[Fig. V(n), lane 4]**.

ii) *KpnI-NarI*: one *NarI* site is present in the DNase I sequence and one *KpnI* site is in the pER III backbone. Release of two fragments of 6.3 kb and 697 bp from pMR#8 on digestion with *KpnI-NarI* confirmed the correct orientation of insert in pMR#8 **[Fig. V(n), lane 5]**.

On sequencing, DNase I gene was found to be out of frame in pMR#8 by one nucleotide (data not shown). To make the gene in-frame, *KpnI* site (present 5' to *SacI*) was digested **[Fig. V(n), lane 6]** and 5' overhangs were end filled with *Klenow* enzyme **(Section 2.3.9.1)**. End-filled DNA was self-ligated (blunt end ligation). The new vector formed was henceforth named as pERDNase I (7.062 kb) **[Fig. V(l)]**. *E. coli* cells were transformed with pERDNase I by electroporation method **(section 2.3.1.2)** and transformants were screened for loss of *KpnI* site



5.4 Fig. V(I) - Schematic summary of construction of pERDNase I



5.4 Fig. V(m) - Colony blot of *E.coli* cells transformed with pMR#8. DNase I gene radiolabelled with ^{32}P was used as a probe for detecting recombinant colonies.

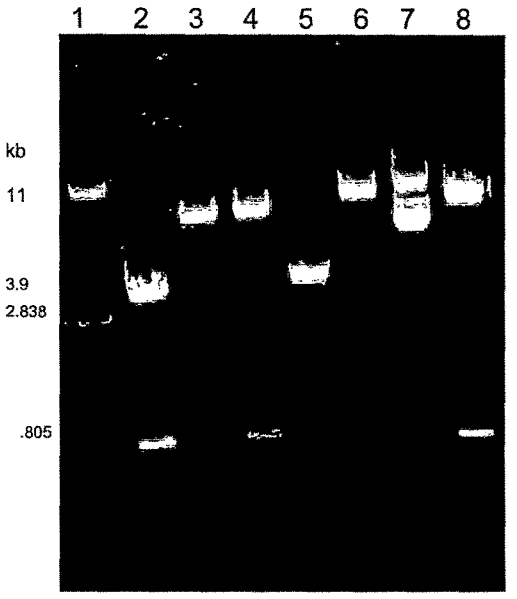
[Fig. V(n), lane 7]. Since *Klenow* also acts as an exonuclease, release of 750 bp fragment of DNase I from pERDNase I as confirmed by *SacI* digestion indicates no damage to the backbone of the plasmid **[Fig. V(n), lane 8]**.

To further confirm the DNase I sequence and its reading frame in pERDNase I, DNase I gene was sequenced **(section 2.3.14)** from pERDNase I using primer MR11 **(section 2.1.2)**. The DNase I sequence showed no mutation and was in proper reading frame **[Fig. V(o)]**.

5.6 Transformation of *D. discoideum* (Ax-2) cells with pERDNase I

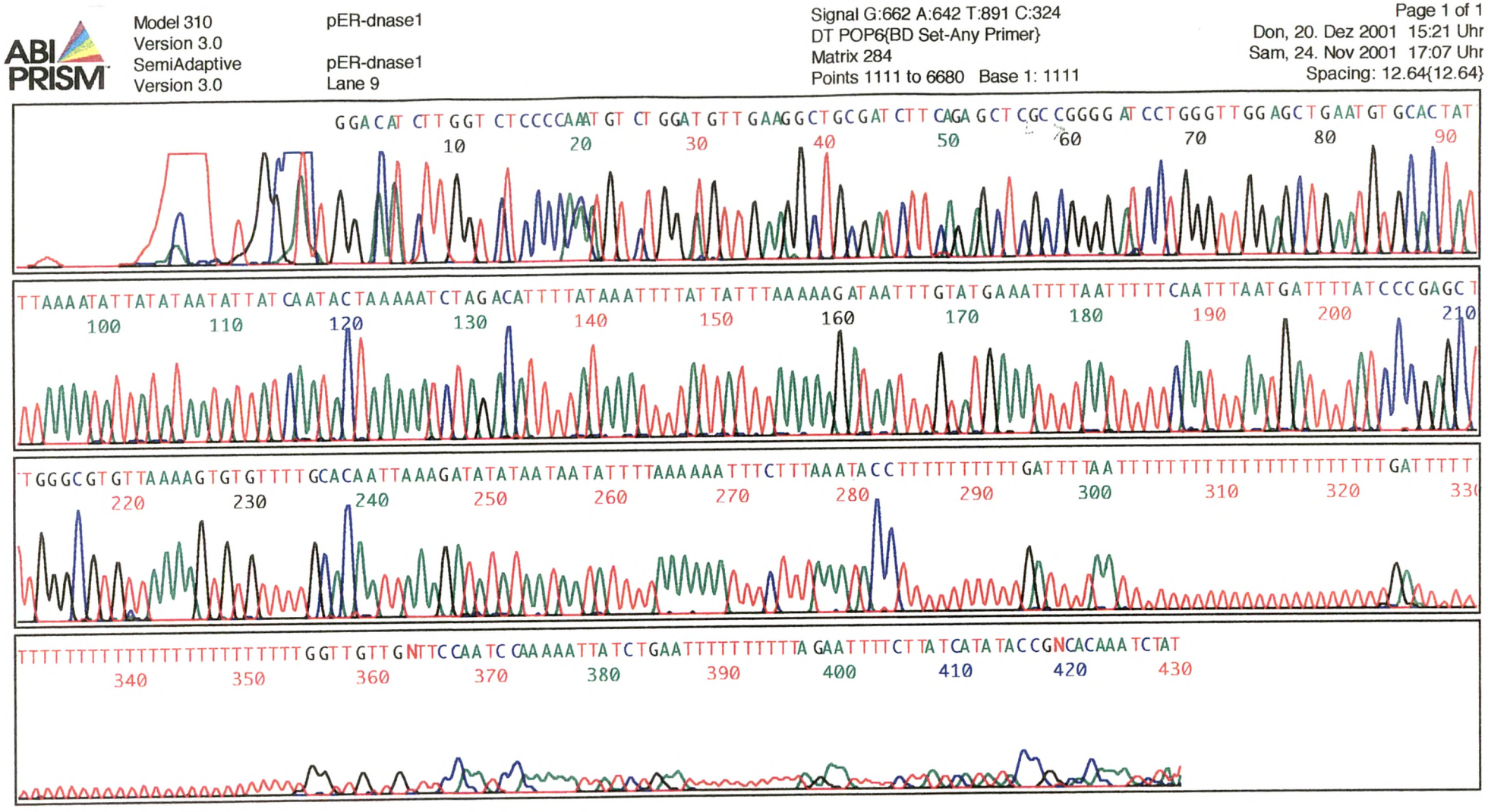
Cells of Ax-2 strain of *D. discoideum* in log phase (2 X 10^6/ml, 10 ml per plate) were transformed with pERDNase I (7-8 μg) by $CaPO_4$ method **(Section 2.4)** along with proper controls. pA15GFP transformed cells were used as positive control and untransformed cells as negative control for antibiotic resistance. Transformants growing in media containing G418 at 10 μg/ml concentration were selected and subsequently maintained at 5 μg per ml. Clones were observed after 10-15 days. Positive clones growing on plates containing G418 were transferred to 24 well plates for clonal selection. Once the wells were full with cells, petri-plates were inoculated and from fully grown plates, cells were inoculated into flasks for further growth and induction.

Cells transformed with pERDNase I grown in axenic medium were healthy and grew at rates comparable to AX2. When grown on *Klebsiella* lawns **(Section 2.5.6)** cells showed normal growth and development **[Fig. V(p)]**. Altered phenotype on phosphate-buffered agar plate was observed after induction with cAMP **(Section 2.5.9)** - reduced level of fruiting body formation with small spore sacs **[Fig. V(q)]**. However, no major protein band of 31.4 kD immunoreactive to polyclonal antibodies against bovine DNase I was observed either with cells or with ten fold concentrated starvation buffer (data not presented) reinforcing earlier observation that either DNase I is not a major protein in transformants or it is quickly degraded.

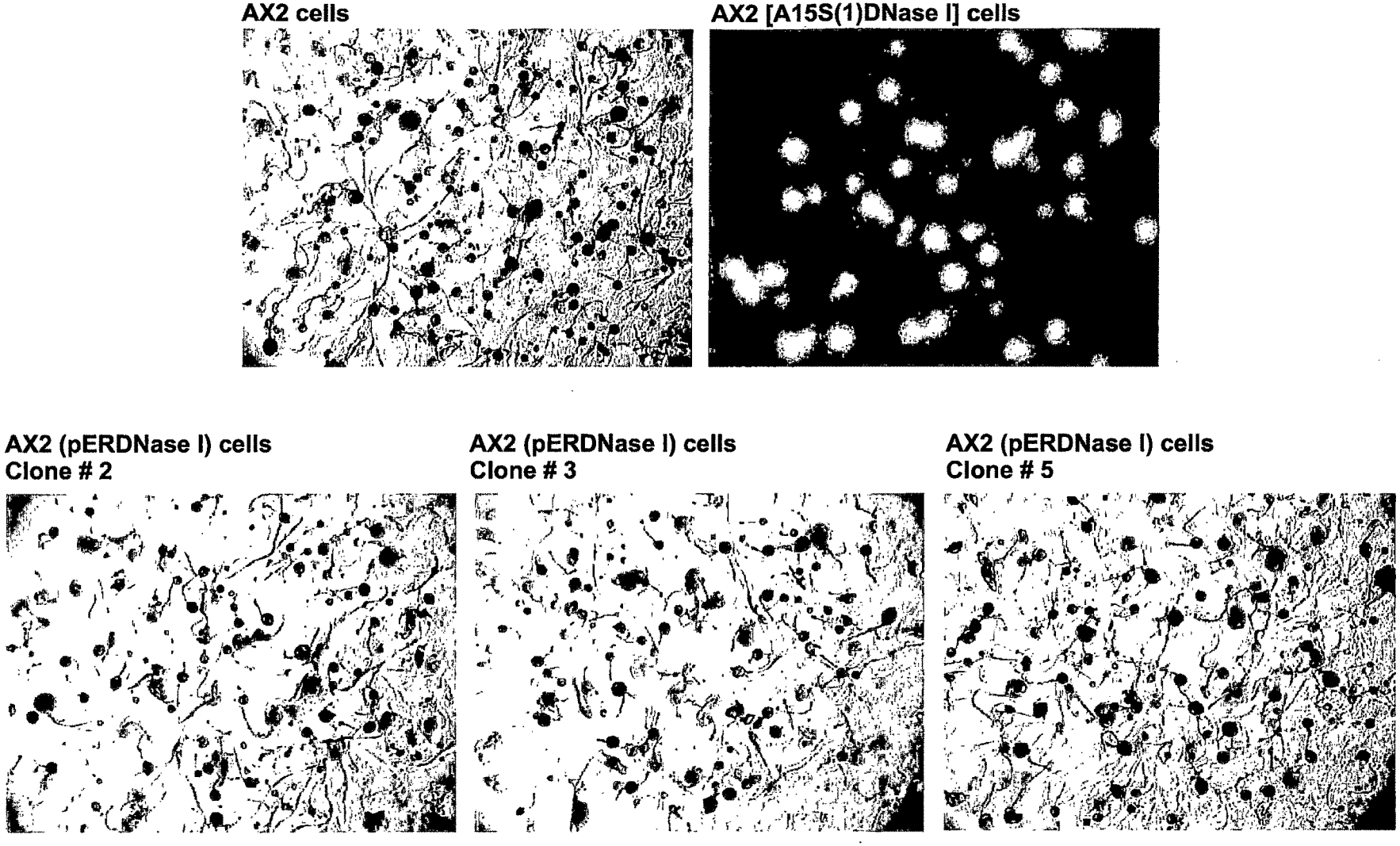


5.4 Fig. V(n) - Construction and RE digestion pattern of pERDNase I on 0.8% agarose gel

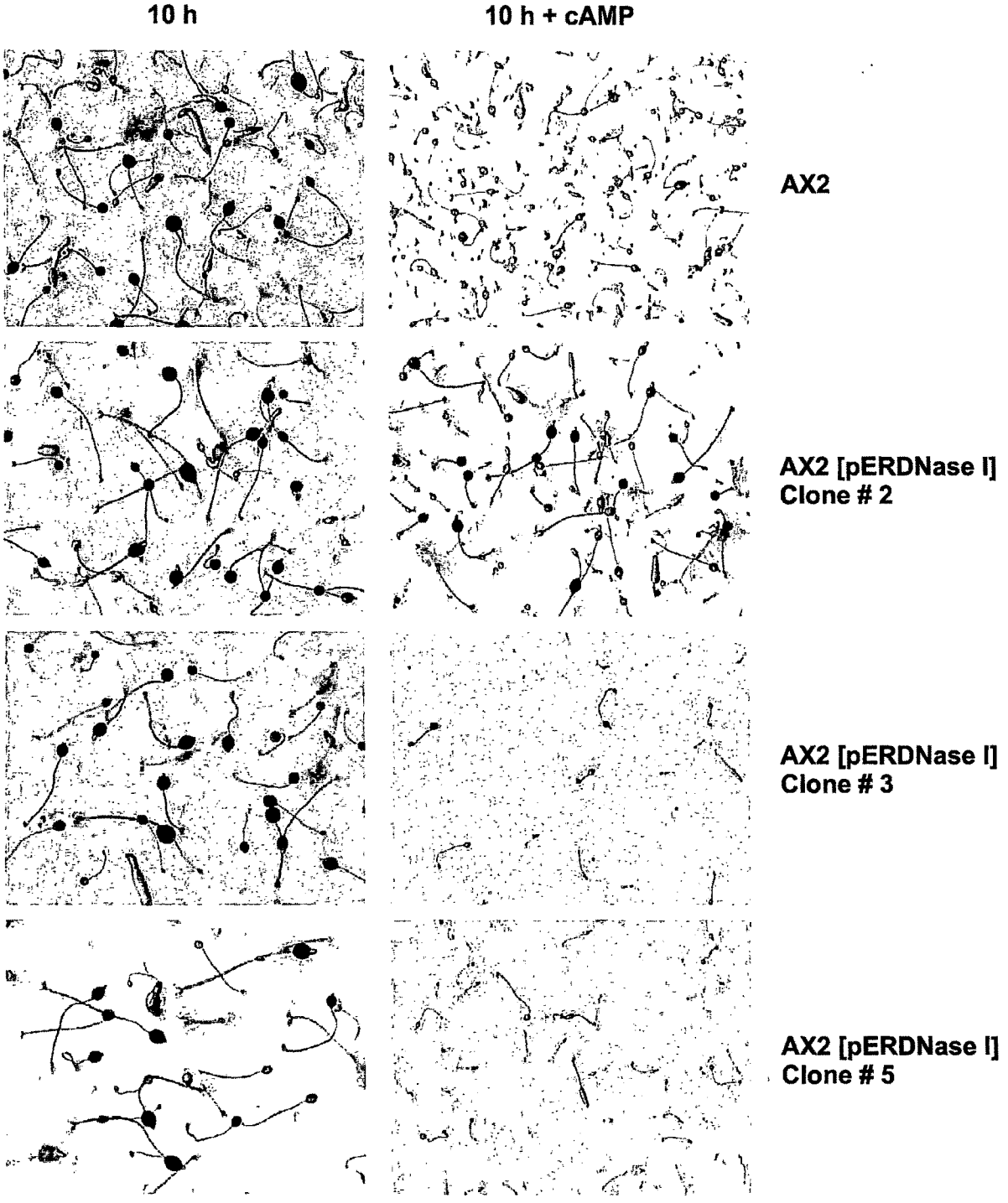
Lane 1: lambda DNA digested with *PstI*
Lane 2: pMR#7 digested with *SacI*
Lane 3: p ERIII digested with *SacI*
Lane 4: pMR#8 digested with *SacI*
Lane 5: pMR#8 digested with *KpnI-NarI*
Lane 6: pMR#8 digested with *KpnI*
Lane 7: pERDNase I digested with *KpnI*
Lane 8: pERDNase I digested with *SacI*



5.4 Fig. V(o) - Sequence of pERDNase I : In pERDNase I, DNase I gene has been cloned in the *SacI* site of pER III and was found to be in frame (57 onwards).



5.5 Fig. V(p) - Drop test for viability and development of Ax2 cells transformed with pERDNase I



5.5 Fig. V(q) - Growth of cAMP induced Ax2 cells transformed with pERDNase I on starvation plates

5.6 Discussion

Human DNase I was used as a test protein to demonstrate the expression capabilities of *D. discoideum* because it undergoes most of the post-translational modifications (di-sulfide linkages, N-glycosylation) and is also a clinically important drug being made in CHO cell line. While choosing DNase I as a test protein, toxicity due to its expression was considered not to be a hindrance since the gene was cloned in a secretory vector (pA15S(1)) having *D. discoideum* secretory signal (PsA signal sequence), which directs the active protein outside the cell, into the medium.

Change in phenotype (round and smaller cells) indicates cell death of the pA15S(1)DNase I transformants. The phenotypic changes were because of the expression of DNase I and not due to any other factor involved in transformation was confirmed by taking pA15GFP transformed cells as a control which appeared healthy and grew at rates comparable to AX2.

The two important intracellular activities of DNase I involves binding to G-actin which leads to reduced levels of F-actin hence affecting the cytoskeletal activities and degradation of genomic DNA through endonuclease activity, thereby causing cell death. Cell death at an early vegetative stage of the transformants was confirmed by Trypan blue staining. In drop test experiment, delayed clearance of *Klebsiella* lawn by transformants and fewer or no fruiting bodies showed cell death due to expression of DNase I under the consecutive Actin15 promoter which remains active throughout growth and early development. Cell death due to neutralization of G-actin by DNase I was ruled out by the fact that actin is the major protein made during growth of *D. discoideum* and the recombinant protein produced in the transformants was too less to neutralize the G-actin pool of the cells and hence affect the cytoskeleton causing cell death. Also SDS-PAGE of cytoskeletal proteins of transformants appeared similar to Ax-2 cells (data not presented). Therefore cause for cell death seemed to be the endonuclease activity of DNase I. Catalytic amount of enzyme is enough for DNA degradation and the protein sequence contains internal nuclear localization sequence (NLS). Genomic DNA of transformants could not be tested for DNA laddering due to insufficient number of cells (10^8 cell are required for genomic DNA preparation).

Actin 15 is a strong constitutive promoter remaining active throughout growth and hence expression of DNase I under it's contol caused cell death from the very beginning. This could be the reason for less number of clones in pA15S(1)DNase I.

A band at 31.4 kD corresponding to DNase I was expected on SDS-PAGE but because of the uncertainty of the extent of glycosylation and the possible degradation of the precursor, it was difficult to ascertain whether DNase I was being expressed or not.

Death of transformants indicated that the expressed protein was not being secreted out. Cellular retention of DNase I could be due to either an inappropriate signal used in mammalian cells to control secretion or perhaps inappropriate glycosylation.

No further confirmation of DNase I activity in media/cell was possible due to death of transformants at an early stage of growth. Because of the lack of direct evidence of DNase I expression in the transformants, it was expressed using an inducible promoter *ras* (pERDNase I). Phenotype observed on induction of the promoter of the concerned gene gives direct proof of the expression of gene.

Promoter Induction → **gene expression** → **phenotype**

pERDNase I transformed cells looked healthy in axenic medium and grew at rates comparable to AX2. Cells placed on *Klebsiella* lawns grew and developed normally, which argues that if the *ras* promoter would become active after the initiation of cell development in the middle of the plaques, the DNase I protein had no effect on developmental processes (growth is absent at this stage). Conversely, this argues that the DNase I affects synchronous development on phosphate-buffered agar plates only because it produced a fraction of dead or damaged cells that were unable to contribute to development. On phosphate-buffered agar there was a clear phenotype that occurred only after the *ras* promoter was induced with cAMP, which argues for the effect being due to the recombinant protein, which was not detectable by Western blotting. However we are not sure whether this was due

to low amount of protein or poor cross-reactivity between bovine antibody and human DNase I. It should be noted that a good response to the bacterially produced human DNase I may have been caused by the large amount of protein on the Western blot, and that the low amount produced in *D. discoideum* may have been overlooked due to low affinity of the anti-bovine DNase I antibody.

Expression of DNase I as indicated by death of pA15S(1)DNase I transformant suggests that *D. discoideum* is capable of making active recombinant protein. Activity of the expressed DNase I protein has been further confirmed by alterted phenotype of the cAMP induced pERDNase I transformant as compared to the nontransformant. Cellular retention of this protein could be due to either an inappropriate signal used in mammalian cells to control secretion or perhaps to inappropriate glycosylation. Biologically active protein indicates that it had been processed through part of the secretory pathway.