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Production and *In Vitro* Characterization of Altered Tag Transformed Murine Fibroblasts

Kevin Driver and Lawrence Mylin

Honors Research Report

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INTRODUCTION

CD8⁺ Cytotoxic T lymphocytes (CTL) have been known to play a significant role in tumor suppression through their role in adaptive cell-mediated immunity. CTL operate in conjunction with the Major Histocompatibility Complex Class I (MHC I) mediated presentation of intracellular proteins by all nucleated somatic cells. To be effective, the CD8⁺ T cell receptor (TCR) must be able to detect specific amino acids of intracellular processed peptides bound and presented by MHC I molecules. MHC I function in binding small polypeptide segments of 8-10 amino acids in length in the endoplasmic reticulum and transporting them to the cell surface to be observed by sentry CD 8⁺ Lymphocytes. The MHC I binding peptides are produced by proteolytic processing of the transient pool of proteins actively translated on cellular ribosomes. Biochemically MHC I-mediated peptide presentation to CD8⁺ T killer cells (CTL) requires efficient binding of peptide anchor residues (single amino acids with extruding side chains) to specific sites on the external face of the MHC I, spatially located between two α -helices. Additional amino acid side chains require projection up from the MHC I face to be available for TCR interaction. Each different allelic MHC I molecule cannot bind all possible peptides; thus, to present the broadest spectrum there must be a balance between high binding affinity and a wide specificity (Madden 1995). When bound, the MHC I complex containing the bound peptide is targeted for localization to the cell membrane, where the MHC I/bound peptide complex is extruded on the cell surface. In the presence of epitope-specific CTL, the bound peptide should trigger recognition by CTL via the physicochemical interaction of the TCR with spatially available amino acid side chains of the presented MHC bound peptide and the MHC molecule itself. Upon CTL recognition of foreign antigen, intracellular signal transduction events are initiated terminating with the observer activated CTL effecting a directly targeted cytolytic response against the peptide presenting target cell (Janeway and Travers 1994).

Simian Virus 40 represents a useful experimental model to study CTL/MHC I restricted immunity. Simian Virus 40 has been shown to induce immortalization in mammalian cells through large tumor antigen (Tag) mediated functional abrogation of contact inhibition of cellular growth. SV40 has also been used as a vector for genes coding for large tumor (T) antigen (Tag Butel & Lednicky 1999). This antigen contains four epitopes that are presented by H2^b MHC I and immunogenic to murine CTL.

Multiple studies beginning with Tanaka *et al.* 1988 and Lill *et al.* 1992 have demonstrated the ability of SV40 Tag-expressing immortalized fibroblasts to successfully escape and survive CTL-mediated, specific Tag epitope targeted cytotoxicity. Molecular analyses of escape variants selected for by CTL coculture have consistently revealed epitope-dependent specific mutations or deletions: all observed escape facilitating mutations have been epitope-specific, primarily influencing the physicochemical MHC I bound epitope interaction with T Cell Receptors (Lill *et al.* 1992). However, the repertoire of observed escape variants has varied with regard to the Tag epitope targeted by CTL clone: published results of coculture with epitope I specific CTL clone Y-1 yield only large deletions and two different substitution point mutations at a single TCR contact residue codon, while coculture with epitope V specific CTL clones Y-5 and H-1 produced novel nucleotide point mutations at 5 codons each with mutants for 4 positions shared by both populations (Mylin & Tevethia, unpublished). Such results point to an as yet undetermined cause for the observed conservation of epitope I mutations compared to the diversity of epitope V escape variants.

There are several interesting biological features of epitope V which may elucidate its distinct variety of mutational escape patterns. First, epitope V is immunorecessive, i.e. *in vivo* proliferation of epitope V specific CTL is only observed in the absence of all other immunogenic Tag epitopes (Mylin *et al.* 1995). Additionally epitope V/MHC complexes exhibited the lowest

cell surface stability of the four T antigen epitopes (I, II/III, IV, and V), indicating a temporally limited presentation of epitope V at the cell surface (Mylin unpublished). Previous studies of *in vivo* immunogenicity have also revealed a decreased efficiency of intracellular processing of epitope V (Mylin 1999). Finally, when epitope V is translocated to replace epitope I at residues 207-215, epitope V specific CTL exhibit a significantly increased cytolytic efficiency *in vitro* (Mylin et al. 1995).

In contrast to the epitope-dependent *in vitro* mechanisms of escape, studies by Mylin *et al.* (1995) and others have shown that *in vivo* immunogenicity, the ability to isolate activated and proliferating epitope-specific CTL from immunized animals, can be influenced by epitope context, mediated both regionally and by immediate epitope flanking residues. However, it remains to be shown whether patterns of favored epitope specific mutations achieved by *in vitro* selected escape variants are also influenced by epitope context; understanding any context-effect on CTL escape mechanisms is the broad subject under which this investigation falls.

HYPOTHESIS AND STUDY AIMS

From the previously documented experimental evidence it seems likely that translocated epitope V targeted escape variants will manifest a more limited diversity of escape mechanisms (a more conserved mutational repertoire, i.e. fewer different mutations) than previous selection studies with Tag epitope V in its native context.

There are two compelling reasons in support of this hypothesis: first, altered Tag expressing cells will experience an increased selective pressure exerted by epitope V specific CD8⁺ T lymphocytes, as CTL showed an enhanced recognition and higher cytolytic efficiency with epitope V positioned at Tag residues 207-215 (Mylin *et al.* 1995). This increased selective pressure should result from an increased amount of epitope V presented at the cell surface by virtue of a putative enhancement of cellular processing of epitope V within presenting targets.

Second, the diversity of escape variants will likely be reduced by selection from a homogeneous starting population of CTL targets. Previous escape variant selections which characterized escape mutations selected from the outgrown K-0 cell line, which may have already contained novel mutations. The proposed study will select escape variants from a single cell derived clonal population.

The goal of this study was, therefore, to produce murine fibroblast cell lines with a single copy, epitopes I and V switched (translocated into the other epitope's native context), Tag gene integrated into the cellular genome (comparison of p323 altered Tag and wild type Tag shown in Figure 1). In addition to coding for an altered Tag, successful cell lines must actively express the desired Tag product and successfully process and present immunogenic Tag epitopes, recognized by CD8⁺ T lymphocytes and provoking a specific, target-directed lytic response. Such cells would be used for *in vitro* epitope specific CTL coculture selection for Tag escape variants.

EXPERIMENTAL DESIGN

Cell lines will be produced by *in vitro* transfection of primary murine fibroblast tissue cultures with Calcium-precipitated, high quality, purified p323 plasmid DNA coding the altered (epitopes I and V) switched Tag gene sequence. Multiple primary cell tissue culture flasks were transfected in parallel, generating putative novel transfection events and multiple single cell transformant-derived clonal lines as candidates.

Tag transformed cells are conveniently selected by virtue of their continued survival and proliferation *in vitro* compared to primary fibroblasts. This selection effect is mediated by Tag's oncogenic function of abrogating normal mechanisms of cell-cell contact inhibition (Butel & Lednický 1999). Selected tissue colonies will be identified by their dense outgrowth *in vitro*; selected colonies will be isolated and physically removed from primary cell cultures for clonal

expansion. Successfully selected candidate tissue cultures will be frozen and stored for molecular analysis and CTL selection.

Candidate cell lines will be screened for a) successful Tag expression by specific antibody staining detected by indirect immunofluorescence, b) successful recognition by epitope-specific CTL and ability to induce a productive immune response, and c) the presence of a single copy altered Tag integrant gene within the host genome.

MATERIALS

The specific materials to note are the different culture mediums used during this experiment.

1. RPMI 1640 (Gibco), supplemented with 10% by volume heat-inactivated Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U of penicillin, 100 U of streptomycin, 12.5 mg pyruvic acid, 5×10^{-5} M β -mercaptoethanol, and 5 ml HEPES buffer (1M) to a final volume of 536 ml.
2. Dulbeccos modified Eagle media (DMEM), supplemented with 5 or 10% heat-inactivated FBS by volume, 2 mM L-glutamine, 100 U of penicillin, 100 U of streptomycin, 100 U of kanamycin, 10 ml HEPES (1M), and 0.015% sodium bicarbonate to a final volume of 474.

METHODS

Harvesting Primary Kidney Cells

Primary fibroblast cultures were obtained by immediate surgical removal of kidneys from euthanized B6 mice. Kidneys were maintained in 10 % FBS DMEM on ice, until each individual kidney was minced to physically dissociate cellular tissue. Kidney chunks were then treated with Trypsin in trypsin diluent, mixed, and centrifuged to separate single cells. Trypsin supernatants (unsettled suspended cells) along with trypsin treated chunks were incubated in tissue culture flasks and expanded to primary fibroblast monolayers.

Plasmid Purification, Calcium Precipitation, and *In Vitro* Transfection of Primary Cell Cultures

Plasmid constructs containing an altered Tag gene sequence with epitopes I and V switched (with epitope I inserted into the epitope V coding region as well as epitope V inserted into the epitope I coding region) were obtained from Mylin in the form of bacterial samples containing the p323 plasmid. Bacterial cultures were expanded, and high quality plasmid DNA was extracted using Qiagen columns.

Plasmid DNA was then used for Calcium precipitation transfection. p323 test DNA (10 μ l at 0.17 μ g/ μ l) was dissolved aseptically into 888 μ l filter sterilized glass distilled water, 2 μ l salmon sperm (carrier) DNA (10 μ g/ μ l), and 100 μ l filter sterilized 2.5 M CaCl₂ solution (no test DNA controls were prepared with test DNA volume replaced with corresponding volume FS GDW). This calcium-DNA solution was then bubbled dropwise into an equal volume of 2x HeBS (8% NaCl, 0.32% KCl, 0.32% Na₂HPO₄x2H₂O, 1% Dextrose, and 5% HEPES all per volume, at 7.10 pH). One ml of calcium precipitated test DNA was then added directly to each primary cell culture flask, all seeded one day prior with 4x10⁵ cells/flask, for 24 hour incubation at humidified 37°C 5% CO₂. Transfection media was then replaced with 10% FBS DMEM, and flasks maintained, observing for spots of dense outgrowth. Spots were observed in 4 of 6 transfection flasks, and were physically removed using flame shaped glass Pasteur pipettes. Spots of dense growth were viewed under a light phase contrast microscope, and pipette “colony pickers” were used first to physically detach spots from the surrounding cell monolayer, followed by partial aspiration into the pipette for transfer to 12 well tissue culture plates. Several spots required multiple repeated pickings to ensure adequate samples. All resulting 12 well plate colonies were observed for continued proliferation *in vitro*, those successfully surviving were expanded into culture flasks for permanent storage in liquid nitrogen.

In Vitro Characterization of Immortalized Candidate Cell Lines

Following revival of frozen storage samples, each cell line was maintained in 10% FBS DMEM, passing as needed, usually 1:6 or 1:10 every 3-5 days. All cell lines (KD1, KD2, KD3A, KD3B0, and KD3B2), including spontaneously immortalized (Tag⁻) B6/SCL7, were expanded multiple times into T180 culture flasks for harvesting genomic DNA. Cells were harvested from each flask at saturation using Trypsin in Versene, then quenched with 10% FBS DMEM and centrifuged to pellet at 1000 rpm, 4° C (10 minutes). Cell yields were calculated for each cell line using hemacytometer based quantitation, and each cell pellet was resuspended in Phosphate Buffered Saline to a final concentration of 1x10⁷ cells/ml. From this cell suspension, 0.50 ml aliquots were transferred to microfuge tubes, pelleted; supernatant PBS was removed and pellets were stored semi-permanently at -80° C. High quality genomic DNA was purified from harvested cell pellets using DNeasy columns, reagents, and protocol (QIAGEN). Genomic DNA yield was verified using agarose gel electrophoresis and measuring Absorbance₂₆₀ with a UV spectrometer.

Additionally, the transfected Tag gene sequence coded for by p323 plasmid DNA was excised from its plasmid context for use as sequence specific probe for DNA hybridization analysis of candidate cell lines. p323 plasmid DNA was digested using BamHI and KpnI, releasing a double stranded Tag fragment of 3010 base pairs of length. Digested p323 DNA was electrophoresed, with the 3 kbp fragment physically excised from the gel for DNA extraction. Tag DNA was gel extracted using QIAEX II reagents and protocol, eluting nucleic acid from

resin beads twice, and immediately EtOH precipitating final DNA elutions overnight at -20° C. Final residual DNA for each gel extraction was dissolved in 10 μ l sterile GDW. To verify successful agarose gel extraction one μ l of each extraction sample was electrophoresed in parallel with varying amounts of p323 BamHI/KpnI digest DNA to roughly estimate yield.

Cell lines were next tested for functional Tag expression using Tag specific antibody staining detected by FITC labeled indirect immunofluorescence. Cell samples representing all five KD cell lines as well as B6/SCL7 and WT19 cells (Tag negative and positive controls respectively) were passed into 60 mm containing 18 mm glass coverslips for cell monolayer adherence. Cell cultures were incubated overnight at 37° C, 5% CO₂ (humidified) to produce confluent adherent monolayers; following incubation, media supernatant was aspirated, tissue monolayers washed three times with PBS then fixed with ice cold 95% EtOH for 45 minutes at -70° C. Alcohol supernatant was removed, and fixed cells were dried and stored at 4 C prior to antibody staining.

Glass coverslips were removed and separately treated and incubated with either primary Mab 901, 419 (Tag specific antibodies binding to N and C termini of Tag, dissolved in PBS), or PBS negative control (no primary antibody). Following primary antibody addition and PBS rinsing, all coverslips were separately treated and incubated with 401 goat anti-mouse FITC-labeled secondary antibody. All coverslips were rinsed with PBS, dried and mounted on microscope slides for fluorescent microscopy. Digital photos were taken of each cell line sample to show Tag cellular localization as well as qualitative observation of Tag levels (indirectly correlated to primary antibody binding).

Finally, all KD cell lines, as well as K-0 (wild type Tag) and K-1/4/5 (all Tag epitopes deleted) controls were used to detect K-11 (epitope I specific) CTL recognition and cytotoxicity. Cells were seeded to confluence in T75 culture flasks for CTL addition. To each culture flask containing approximately 1×10^7 cells, 2×10^6 CTL were added, harvested with Versene from 12 well culture plates. CTL co-cultures were incubated for three days, with a 10% FBS media replacement at 24 hours post CTL addition. Co-culture flasks were observed macro and microscopically for cytotoxic effects at one day and three days post CTL addition. Digital photos of cytotoxicity assay flasks under 10x light microscopy were taken at each observation.

RESULTS

Transfection and Selection

Following transfection, spots of dense outgrowth compared to a stable primary cell monolayers were noted in several p323 transfected culture flasks. Such spots of continued proliferation *in vitro* represent the positive selection, as Tag expression of successfully transformed cells confers an immortalized phenotype and indefinite growth in culture. After spot picking and small scale culturing samples in 12 well dishes, 5 unique cell lines were expanded

and frozen for long term storage. These five KD cell lines, 1, 2, 3A, 3B0, and 3B2, represent clonal expansion from four putative independent transfection events. The initial number designated the flask from which the spot derived, thus all cell lines designated 3 derived from a single transfection flask. The letter designation refers to multiple growth spots within the same flask. Thus 3A represents a separate event and from clonally identical 3B0 and 3B2, resulting from multiple sequential picking of a single spot of outgrowth. In addition to selection by immortalization, all five cell line monolayers showed a more dense, rounded morphology compared to well-spaced, angular primary cells. This immortalized phenotype is evident in microscopic photos of confluent monolayers shown in Figure 5.

Candidate Cell Line Screening

The results of Tag specific antibody staining followed by indirect immunofluorescence are shown in Figures 3 and 4. Note the brightly staining, rounded nuclei compared to weakly fluorescing cytoplasm for all five KD cell lines. Tag should be localized to the nuclei of Tag expressing cells; this localization is evident in all images of KD cell lines. Additionally, the nuclei staining appears dramatically different than the weak nonspecific cytoplasmic staining of both primary antibody/no Tag control B6/SCL7 cells as well as no primary antibody (PBS) control for nonspecific binding levels of the FITC-linked secondary antibody.

All five KD cell lines also show evidence of *in vitro* recognition and cytolysis by K-11 epitope I specific CTL. Confluent monolayers for all five cell lines as well as K-0 and K-1/4/5 controls immediately prior to CTL addition are shown by Figure 5. Note the densely packed surface of fibroblasts. One day following CTL addition, before the first media replacement, extensive reduction in the fibroblast monolayer was already evident for KD 1 and 2, as well as moderate reduction for KD3A (Figure 6). KD3B0 and KD3B2 flasks exhibited some cellular debris, however their monolayers were largely intact. In contrast, both K-0 and K-1/4/5 cells

showed little to no cytolytic activity from the added CTL. This would be expected for K-1/4/5 cells which present no CTL recognition epitopes (the only killing would be non-specific), however, this result was unexpected for K-0 cells expressing the wild-type Tag.

Three days following CTL addition, the cytotoxicity results are even more dramatic (Figure 7). Few, if any, viable fibroblasts are visible in flasks KD1, KD2, and KD3A. Note the small round CTL present on these photos. Additionally, KD3B0 and KD3B2 had developed larger holes in their fibroblast monolayers, however, this could also be due to overgrowth and peeling back of the monolayers evident both macroscopically and microscopically. Once again, K-0 and K-1/4/5 flasks showed little evidence of an effective cytolytic response from the K-11 CTL.

Production of DNA Elements for Southern Blot Hybridization Analysis

Already produced p323 plasmid DNA was used to derive a Tag sequence probe for hybridization analysis. After digesting p323 DNA simultaneously with BamHI and KpnI to free the Tag gene sequence, the digest mixtures were electrophoresed in large quantities on 0.6% agarose prep gels. The large 3 kb bands, corresponding to the Tag gene fragment, were then removed from the prep gel. A sample photograph of a band-removed prep gel is shown in Figure 8. Note the light bands immediately above and below the cut site; these were unexpected, likely due to alternate conformations of the plasmid DNA or inefficient enzyme cleavage, a strong possibility considering that both enzyme digests were performed in a less than optimal buffer.

Extraction of the gel-bound DNA yields highly pure concentrations of DNA below the range of UV absorbance detection, thus amounts have to be estimated by electrophoresis of a small sample along with known amounts of p323 KpnI/BamHI digest DNA. A sample electrophoresis of this type of comparative analysis is shown in Figure 10. Lanes 5-10 were loaded with decreasing amounts of KpnI/BamHI digest DNA (undiluted, 2:3 diluted with 1x

loading buffer, 1:2 diln., 1:3 diln., 1:6 diln., and 1:12 diln.) while lanes 1-3, 11, and 12 were loaded with gel extract samples. Note that the very faint bands at 3 kb in the gel extraction sample lanes are not more intense than the faintest band of KpnI/BamHI digest. Assuming that the intensity of the sample lanes approximates that of the 1:12 diln., the concentration and amount of gel extracted DNA can be roughly estimated.

Estimation of Tag gel extraction yield:

$$0.126 \mu\text{g}/\mu\text{l} (\text{p323 DNA}) \times 130 \mu\text{l} (\text{p323 DNA}) \text{ digested} = 16.4 \mu\text{g}$$

$16.4 \mu\text{g} / 1210 \mu\text{l} (\text{total volume of p323 digest}) = 0.0135 \mu\text{g}/\mu\text{l} \times (3000 \text{ bp Tag}/8700 \text{ bp p323 plasmid}) = 0.0047 \mu\text{g}/\mu\text{l}$, this represents concentration of electrophoresed gel extracted double-stranded Tag DNA equivalent in band intensity to 1 μl (1:12 diln.) of electrophoresed p323 KpnI/BamHI digest

Estimating the concentration of all gel extract samples similar to the described method and factoring the volumes of gel extracted DNA solution produced it was estimated that approximately 500 ng of high quality, Tag sequence specific probe DNA was produced and purified.

Also necessary for molecular analysis is genomic DNA samples of the candidate cell lines. After cell pellet samples were harvested and genomic DNA extracted using DNeasy columns and reagents (QIAGEN), samples were electrophoresed to verify yields. A sample gel photo taken of a genomic DNA electrophoresis is shown in Figure 9. Note the single heavy band present for each sample lane (from left 2-6 and 8-13). The significance of this data lies in the absence of low molecular weight bands indicative of polluting RNA or physically dissociated DNA fragments. Genomic DNA samples were then individually measured for absorbance at 260 nm UV. Measured Abs_{260} was converted to concentration via the following sample calculation:

$$0.036 \text{ Abs}_{260} \times 10 (\text{dilution factor}) \times 0.05 ((\mu\text{g}/\mu\text{l})/\text{Abs}_{260}) = 0.018 \mu\text{g}/\mu\text{l} \times 400 \mu\text{l} = 7.2 \mu\text{g}$$

Ultimately genomic DNA preparations for each sample cell line were pooled and remeasured for UV absorbance. This determined the amount of genomic DNA purified for each cell line: data shown in Table 1.

Table 1: Amount of Genomic DNA purified for each cell line.

KD1	KD2	KD3A	KD3B0	KD3B2	SCL7
104 μg	76 μg	60 μg	92 μg	97 μg	130 μg

This data then was used to estimate the efficiency of the DNeasy plasmid system. Altogether 42 columns were used, each eluted twice for a total of 84 elutions. Additionally each pellet processed on an individual column contained 5×10^6 cells, so the total DNA was extracted from 2.1×10^8 cells. Calculation of DNeasy column yield is shown below.

$559 \mu\text{g}$ (total DNA) / 42 (total columns or cell pellets) = 13.3 μg (DNA yield per 5×10^6 cells or DNeasy column) / 2 (elutions per column) = 6.7 μg genomic DNA per elution (the second elution of each column was actually much less than this, approximately $\frac{1}{2}$ the amount of DNA eluted from the column first)

DISCUSSION

Initial testing on cell lines derived from transfection has indicated a largely successful transfection. Not only were four novel, clonally-derived cell lines produced, but all altered Tag transformed cell lines: 1, 2, 3A, 3B0, and 3B2 have demonstrated Tag expression and functional localization to cellular nuclei as detected by Tag specific antibody staining coupled with FITC-linked indirect immunofluorescence. Qualitative levels of immunofluorescence indicate possibly lower levels of Tag expression by KD2 cells, however, this slight decrease could be due to integration at a locus with a generally lower transcription efficiency as well as lower Tag copy number.

Additionally, all cell lines stimulated cytotoxic effector functions from K-11 epitope I specific CTL *in vitro*, demonstrating not only CTL recognition, but also, by inference, efficient intracellular processing and presentation of Tag epitope I. Interestingly, all cell lines produced in this study elicited a more efficient immune response than wild type (native epitopes) Tag expressing K-0 cells. The more extensive cytolytic reduction of KD1, 2, and 3A monolayers may be indicative of lower Tag expression levels and thus Tag copy number, however this inference is not conclusive as the transcriptional efficiency, as mediated by the regional context of the Tag integration site could also produce comparable effects on Tag expression levels.

Unfortunately, Southern blot DNA hybridization analysis and molecular verification of single copy Tag integration was not completed due to time and methodological considerations, however, genomic DNA samples and putative Tag probe DNA were produced for future analyses, along with a plan for future molecular analyses (Figures 1 and 2). Enzymatically fragmenting genomic DNA for all cell lines samples with restriction enzymes, cleaving at known locations relative to the Tag sequence, should produce a somewhat predictable banding pattern after hybridization with a labeled probe. The intensity, migration distance (size) of the fragments, as well as number of bands hybridized for each restriction assay can be interpreted to determine which cell lines actually contain an genome-integrated single copy p323 Tag gene.

A mock Southern blot is shown in Figure 2 to illustrate the interpretation of such an assay. The second column from the left depicts the banding pattern of uncut genomic DNA (also refer to actual gel photo in Figure 9). Restriction nucleases should cleave at random (previously unknown, random is expressed here epistemologically) points throughout the genome. When electrophoresed, these smaller fragments (average fragment size approximately 4 kb based on 6 base pair recognition site of most restriction enzymes) will migrate into a broad spectrum pattern similar to that depicted in the third column of Figure 2. After denaturing these double stranded

fragments and transferring and immobilizing them on a solid support (usually a nylon membrane), labelled probe DNA is added to hybridize with complementary sequences of base pairs present on the blotted membrane. After washing away excess probe, detection of emission from the bound, labelled probe will show only several bands representing fragments with sequence complementarity to the single stranded probe.

The fourth column of Figure 2 shows the expected high molecular weight single band, of a single copy Tag integrant after restriction digest with an enzyme which does not cleave within the Tag sequence (for Tag, BglIII is a good candidate), in black; any additional bands (blue) present would indicate multiple Tag integrations. The fifth column of Figure 2 shows the predicted pattern of bands after restriction cleavage by an enzyme with a single cut site within Tag (PstI). Two bands would be expected, at relatively high molecular weights. More than 2 bands (blue) would indicate multiple integrations and the presence of a 3 kb band (in red), in particular, would indicate multiple Tag integrations in tandem, i.e. more than one Tag gene located together. Finally, the sixth column of Figure 2 represents an expected pattern for a multiple cut enzyme digest, in this case HindIII which cleaves at 3 sites within the Tag gene. In particular, HindIII should release 2 internal fragments of known length, at approximately 500 bp and 1200 bp. Taken together, the banding patterns of all restriction digests for each genomic DNA sample should provide a definitive answer as to whether or not each clonal population contains an integrated single copy of the p323 altered Tag gene sequence.

While the molecular screening of the five produced cell lines has not been completed, any cell lines containing a single copy integrant should represent a significant target for escape variant selection. The altered Tag cell lines should further our current understanding about the biological basis of tumor cell escape, by increasing the selective pressure of epitope V specific CTL against epitope V presenting targets. David Olsen has described the relationship between

selective pressure and mutational frequency or range in terms of a bell-shaped curve, such as one illustrated by Figure 11 below. At low selective pressure, relatively few novel mutation events occur, as they offer no significant survival advantage. However, with an increase in selective pressure to more moderate levels the survival advantage of productive mutations takes on a greater importance, yielding an increased mutational frequency. High selective pressure is able to, in a sense, overwhelm the target system, as mutations must be sufficient to survive overwhelming pressure; as a result most novel mutants are suppressed by the selecting force.

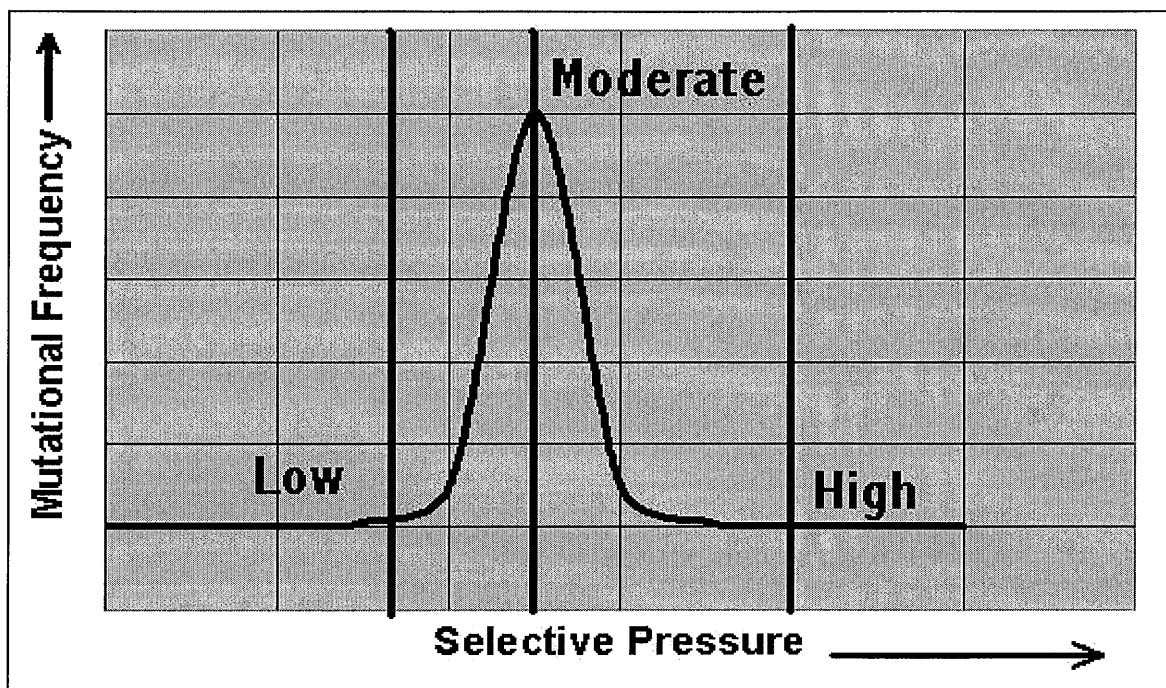


Figure 11: Theoretical Graph of Selective Pressure vs. Mutational Frequency for Tag Escape Variants Selected for by Epitope-Specific CTL

This effect has primarily been described in relation to a system of HIV protein inhibition; there are significant distinctions between the Tag escape variant selection system and HIV inhibition and suppression. Most notably, selective pressure on HIV replication and proteolytic processing is externally induced, i.e. unidirectional. In contrast the Tag escape variant system involves selective pressure in two directions, both externally from CTL, and internally from

target cell mediated processing and presentation of epitope peptides on MHC I. However, I feel that the proposed theoretical selective pressure model is relevant to the Tag escape variant selection methodology for several reasons.

First, previous experimental work in Tag escape variant selection has demonstrated a relationship between externally mediated pressuring factors on the mutational frequency and range of escape variants similar to the one proposed above. In unpublished further selections of K-0 cell lines with epitope I specific CTL, Mylin observed a dramatically increased spectrum of mutations within epitope I. The CTL clone used had been maintained in culture for an extended time and was showing effects of culture fatigue. Thus CTL activity may influence the frequency or range of escape mutations. Additionally, previous work using a more avid K-11 CTL clone showed that coculture media also has an effect on selective pressure. Addition of CTL to flasks containing DMEM, the standard fibroblast culture medium containing 21 mM HEPES buffer, yielded escape variants, while parallel CTL addition to flasks containing RPMI 1640, the standard CTL culture medium containing only 9.3 mM HEPES, yielded no actual fibroblast escape variants. In this case, media favoring the fibroblasts seemed to facilitate mutant survival; this effect is likely based on the increased buffering capacity of the supplemented DMEM used. CTL activity is likely increased in RPMI media tending toward more acidic conditions. It is also likely that the TCR avidity to the targeted epitope influences selective pressure in an analogous way; this represents a current topic of study.

Secondly, the selective pressure model outlined above has, I believe, heuristic value in this experimental system. It has already been established that tumor cells can achieve novel mutations allowing escape from CTL effected cytolysis. However, the goal of selecting and identifying escape variants is to demonstrate the biological significance of certain types of mutations; such an approach will not only yield new knowledge about possible mutants, but

about the biological dynamics of the TCR/MHC I bound peptide interaction. The goal, then, is to understand which mutations are the most critical in escaping immune evasion. While dramatically increasing selective pressure on Tag presenting fibroblasts will likely yield fewer novel escape variants, those that do survive should represent changes at residues most significant to either peptide processing, presentation, or CTL recognition. To this end, producing cell lines more efficient in epitope V processing and more efficiently lysed by epitope V specific CTL should allow more focused study of the epitope V biology.

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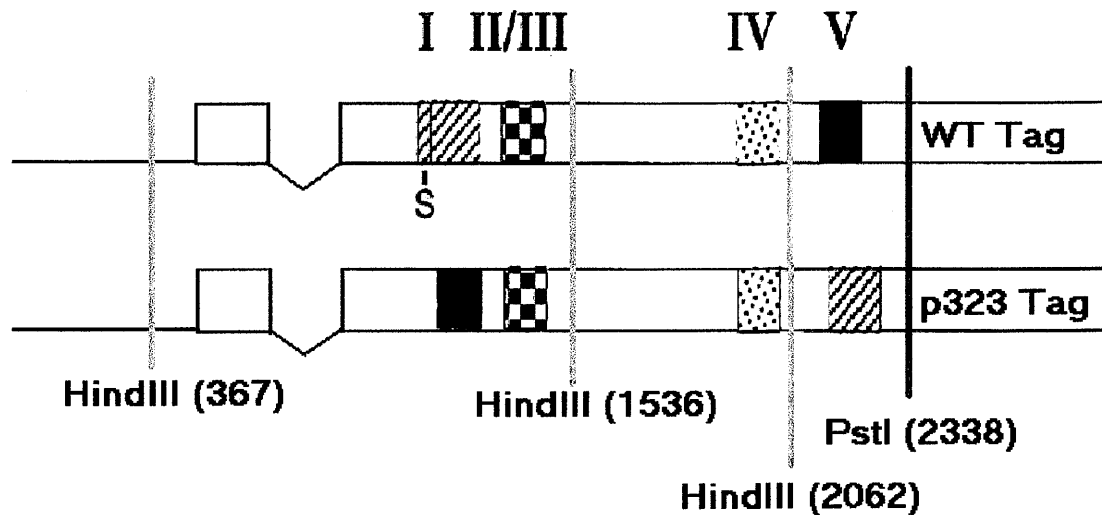


Figure 1: Transfected Tag gene construct with epitopes I and V switched (at bottom) with restriction endonuclease cut sites listed for Southern Blot Hybridization Assay

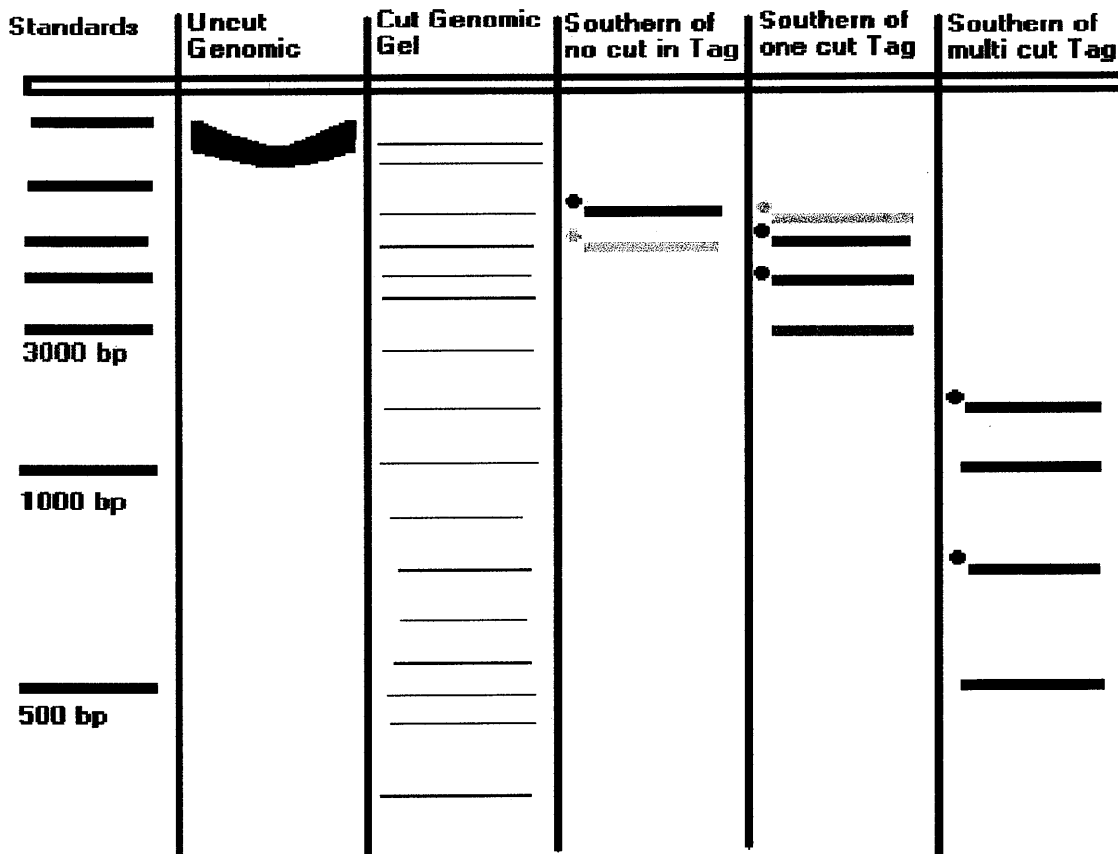


Figure 2: Possible Southern Blot hybridization analysis of altered Tag transformed cell lines. Black lines represent probe marked hybridized bands for desired products (single copy Tag integrants), colored (gray) bands represent multiple copy integrant bands, bulleted bands are expected, but of a priori unknown size relative to actual Tag genomic integration site.

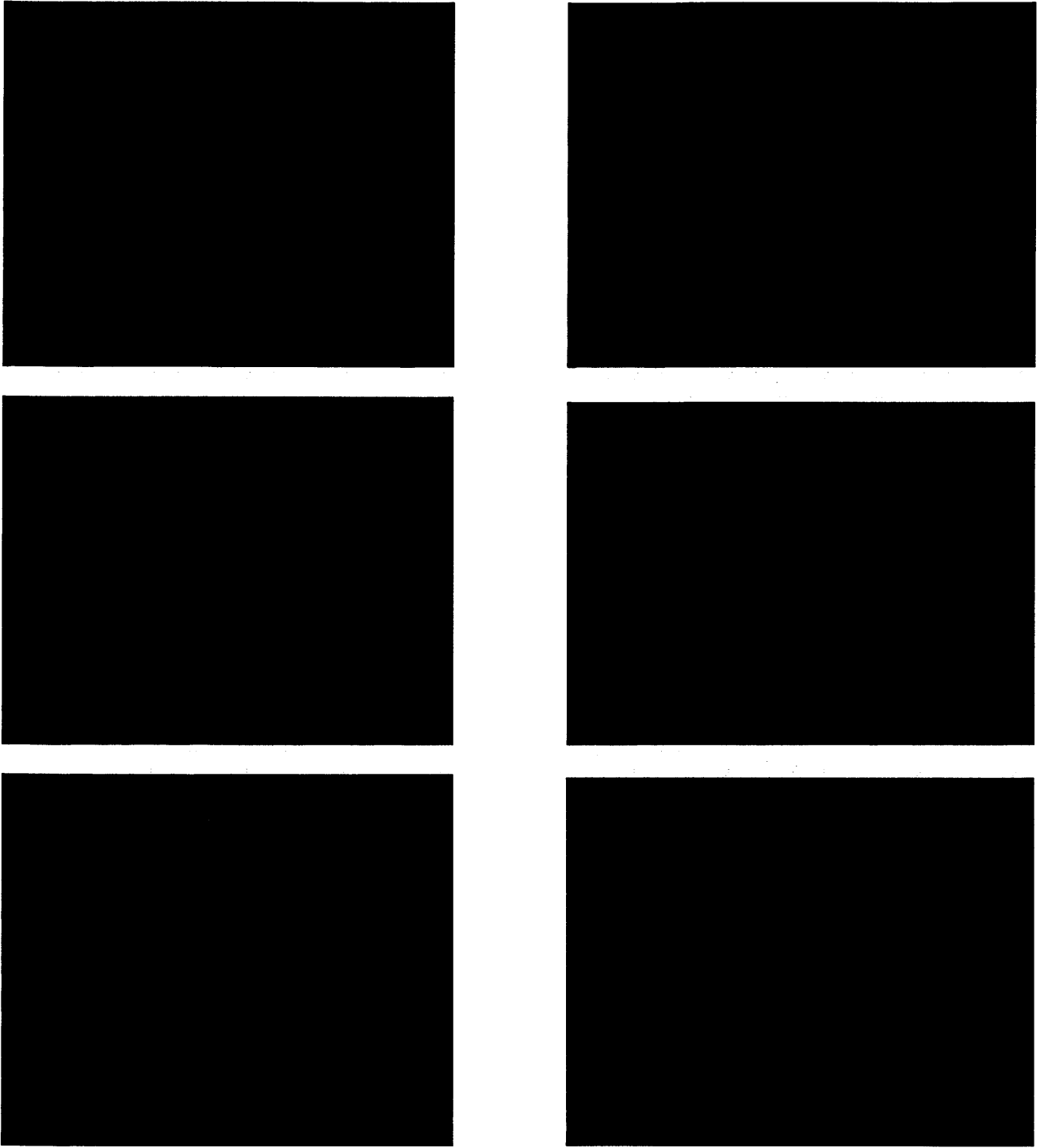


Figure 3: Tag Mab staining of fixed cell monolayers detected by FITC-linked immunofluorescence. Clock wise from top left: 901 (Tag specific 1° Mab) stained KD1, 419 KD1, 419 KD2, PBS KD1 (no 1° ab control), 901 KD3A, 901 KD2.

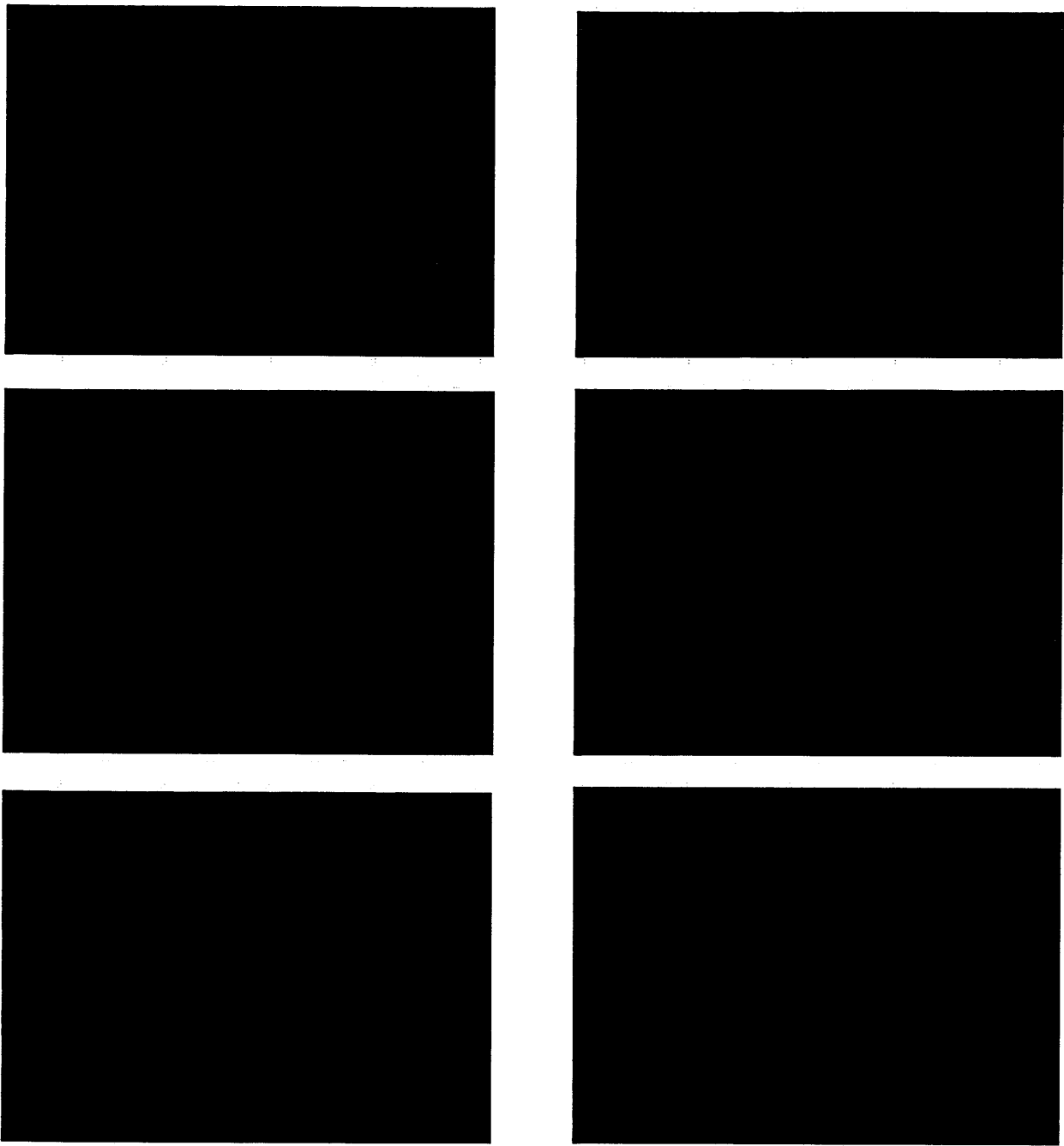


Figure 4: Tag Mab staining of fixed cell monolayers detected by FITC-linked immunofluorescence. Clock wise from top left: 901 (Tag specific 1° Mab) stained KD3B0, 419 KD3B0, 419 KD3B2, 901 B6/SCL7, 901 B6/WT19, 901 KD3B2.

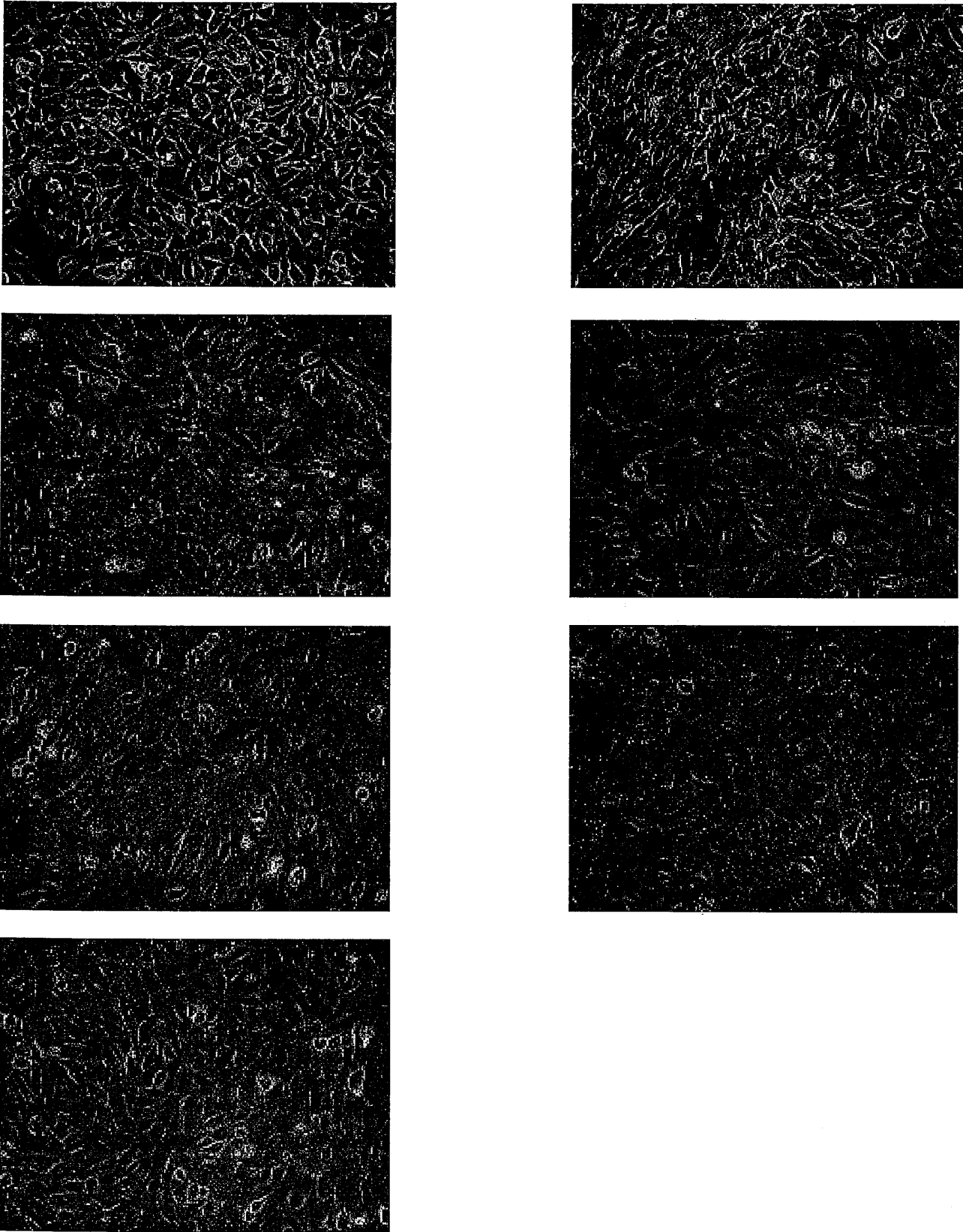


Figure 5: Photos of confluent tissue monolayers taken using phase-contrast light microscope (10x magnification). From top (left to right): K-0, K-1/4/5, KD1, KD2, KD3A, KD3B0, KD3B2.

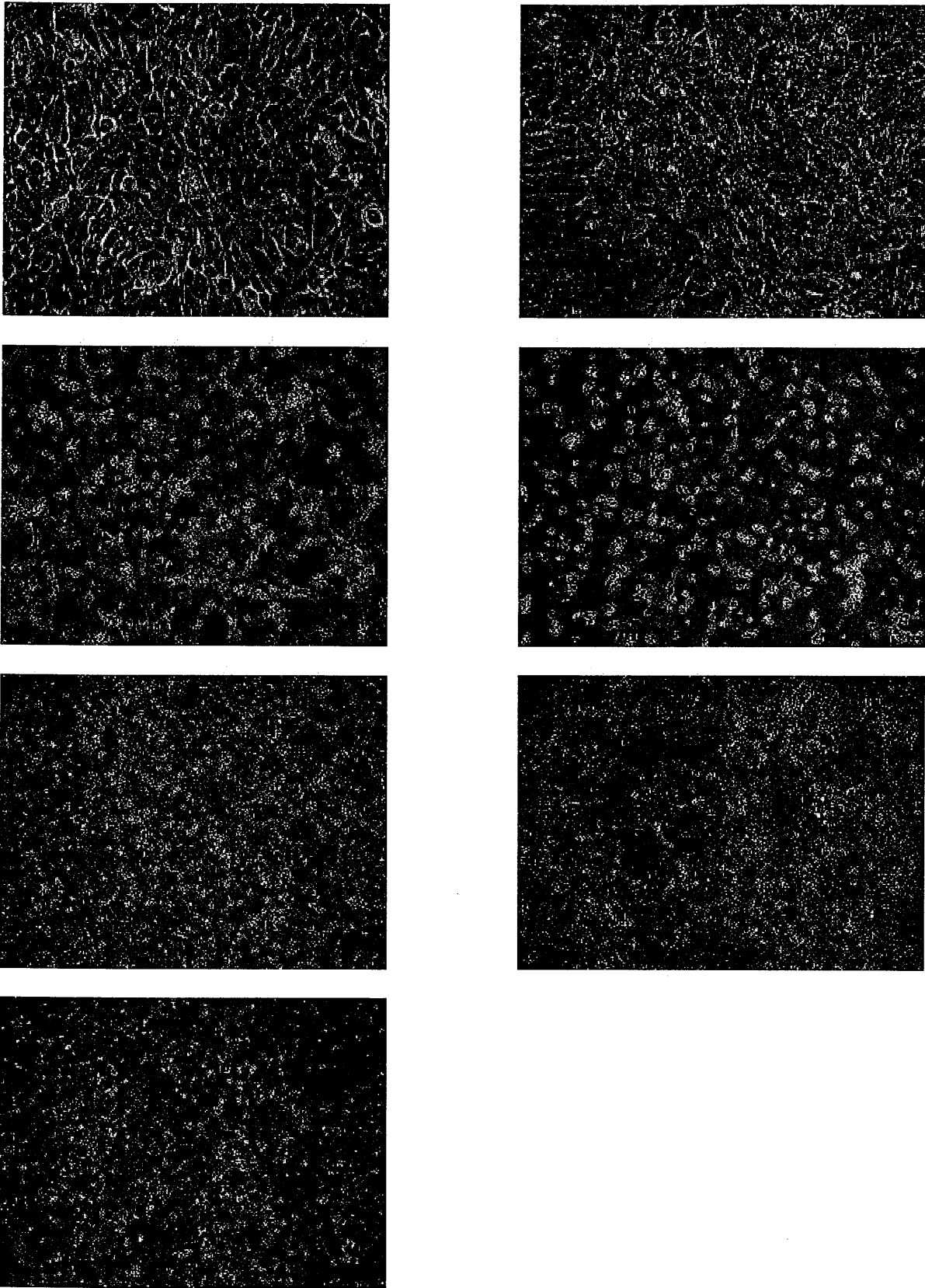


Figure 6: Photos of tissue monolayers taken one day post K-11 (epitope I specific) CTL addition using phase-contrast light microscope (10x magnification). From top (left to right): K-0, K-1/4/5, KD1, KD2, KD3A, KD3B0, KD3B2.

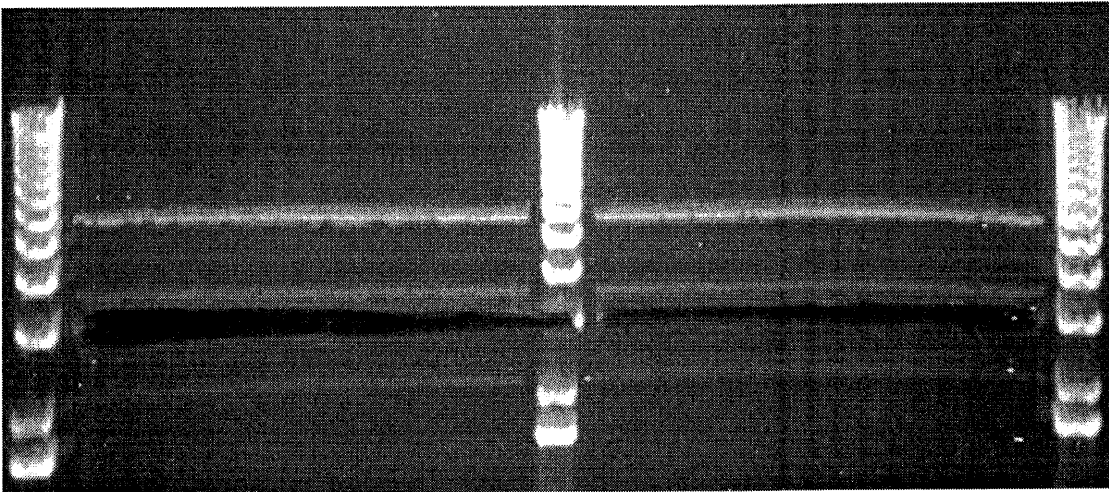


Figure 8: 0.8% Agarose prep gel with 3 kb fragment from BamHI/KpnI digest of p323 (altered Tag) removed for gel extraction

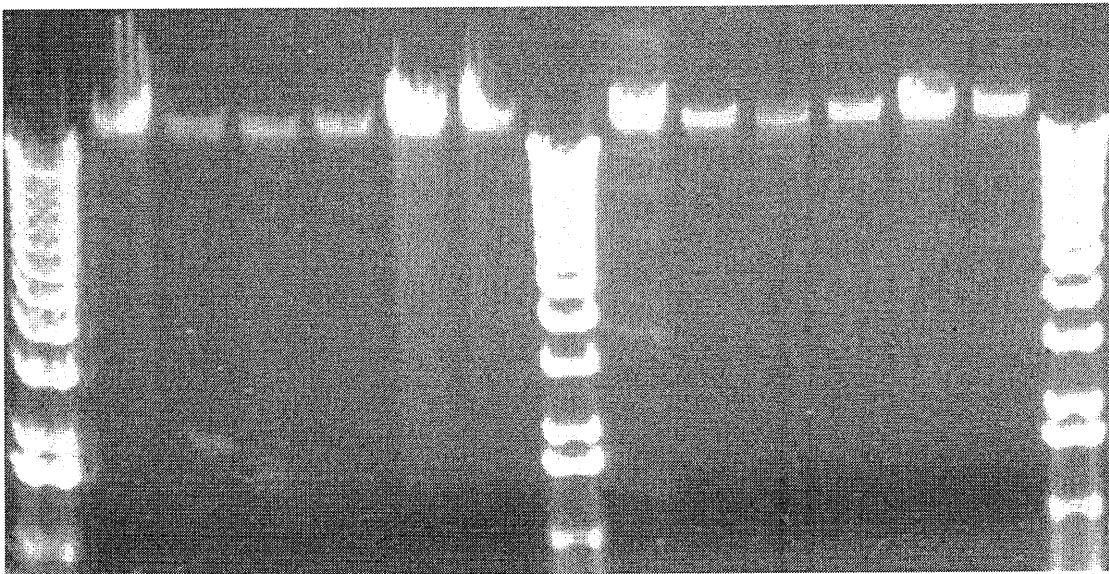


Figure 9: 0.6% Agarose Gel from electrophoresis of genomic DNA extracted from KD1-3B0 candidate cell lines



Figure 10: 0.8% Agarose gel from electrophoresis of gel extracted p323 Tag DNA