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Generation of a large library of point mutations in polyoma middle T antigen

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ABSTRACT

Polyoma middle T antigen (MTAg) transforms cells by associating with and activating a variety of intracellular proteins, including src family members and a phosphatidylinositol-3 kinase. In order to assist in the study of the relative importance of the various associated biochemical activities for transformation by polyomavirus MTAg, a library of MTAg mutants was constructed. Chemically mutagenized MTAg DNA was purified from wild-type DNA by separation on denaturing gradient gels and placed into a recombinant retrovirus vector. Utilizing the resultant library of MTAg-expressing retroviruses, fibroblast cell lines expressing individual MTAg mutants were generated and screened for a non-transformed morphology. Of the first seven non-transformed clones tested, all express the MTAg protein. We estimate that approximately 24% of the G418-resistant colonies contain a transformation-defective MTAg mutant. A more thorough evaluation of one such clone revealed four single base-pair changes as compared to wild-type. Further genetic dissection of this mutant reveals that substituting leucine for proline at amino acid 248 results in a completely transformation defective MTAg. The utility of this mutagenesis and screening procedure as well as the description of several new MTAg mutants is described. This library of mutations should be of general interest for studying the transforming ability of MTAg.

INTRODUCTION

Polyoma virus encodes three early gene products known as the T or tumor antigens which are referred to by their respective sizes as large, middle, and small T antigens (1). These T antigens are responsible for the transforming properties of the virus. Genetic analysis of polyoma virus has demonstrated the central role of middle T antigen (MTAg) to transformation which has been confirmed in studies using a cDNA clone which expresses only MTAg (2, 3, 4, 5). Thus, MTAg is sufficient to transform established cell lines to form foci in monolayer cultures, colonies in soft agar and tumors in syngeneic or nude mice (5, 6, 7, 8, 9, 10, 11, 12).

MTAg, a 421 amino acid protein, is expressed as 56 and 58 kilodalton (kd) species which differ primarily in their pattern of serine and threonine phosphorylation (13). MTAg is associated with the cytoplasmic face of membranes via a hydrophobic stretch of 22 amino acids at its carboxy terminus (14, 15). While MTAg has no known intrinsic biochemical activities, three associated biochemical activities have been reported. These include association with and activation of members of the src family of tyrosine kinases (src, yes, and fyn but not lck), association and activation of a phosphatidylinositol (PI)-3 kinase (16, 17, 18, 19, 20, 21, 22, 23, 24, 25) and association with protein phosphatase 2a (26). Genetic analysis of MTAg has revealed that association and activation of tyrosine kinase activity is necessary but not sufficient for transformation by MTAg (27, 28, 29, 30). That is, all mutations which lack this associated activity fail to transform but several mutants exist which are fully active when assayed in an in vitro immune complex kinase assay yet are transformation defective. Similarly, the association with protein phosphatase 2a is necessary but not sufficient for transformation by MTAg (31). However, the correlation between the presence of PI-3 kinase activity and transformation is better than that for tyrosine kinase activity or protein phosphatase 2a association (20, 27). That is, transformation defective mutants exist which are defective when assayed for PI-3 kinase activity yet associate with protein phosphatase 2a and are as active as wild-type in assays of tyrosine kinase activity in vitro. However, one mutant, dl1015, is severely transformation defective yet remains fully active when assayed in vitro for associated tyrosine kinase, PI-3 kinase activity, and association with protein phosphatase 2a (5, 8, 20). Thus, the dl1015 mutant raises the possibility that other proteins or activities may be necessary for transformation by MTAg.

Although many MTAg mutants have been reported, most of them are deletion mutants (30). In fact, the few transformation defective point mutants of MTAg which exist tend to have profound defects in association with cellular proteins suggesting that the mutation is grossly changing MTAg structure. In order to obtain additional information regarding the transformation functions necessary for MTAg as well as to begin a more precise analysis of the domains of MTAg which interact with various proteins we have undertaken a random mutational analysis of MTAg. The procedures we have used are a random saturation mutagenesis procedure as described by Myers, et. al. (32) combined with a non-selective screen for MTAg transformation function using a recombinant retrovirus vector. The mutagenesis procedure allows for the generation of large numbers of independent point mutations which can be separated from wild

type based on differences in denaturing properties while the use of retrovirus vectors facilitates the identification and recovery of interesting mutations. Here we detail the construction of this large library of point mutations in MTAg which appears to contain many transformation defective point mutants. We also describe the phenotype of several new mutations of MTAg. The associated biochemical properties of these mutants are described elsewhere (33).

METHODS

Construction of DNA vectors

All plasmid construction steps were carried out using standard molecular cloning techniques (34). Restriction enzymes were obtained from New England Biolabs. pUC8-MT and pUC8-NG59b were obtained from W. Morgan (5), pGC1, pGC2, and M13rv1 courtesy of R. Myers (32). The recombinant retrovirus vector pDOL- was a gift from R. Mulligan (35). pGC1 and pGC2 were modified by the addition of a synthetic oligonucleotide sequence (5′ CTAGAGCTCAGCATGC) at the Xho I site in the polylinker to yield pBD5 and pBD6, respectively. This results in the addition of a Sac I and Sph I site to the polylinker sequence. pBD15 was constructed by initially replacing the Xmn I-Pvu I fragment of pGC1 with the homologous fragment from pUC8 in order to remove an intervening Hinc II site present in the ampicillin gene of pGC1. The polylinker region of the resulting plasmid was modified by cleaving with EcoR I and Bgl II, filling in the ends with reverse transcriptase (Molecular Genetics) and religating the plasmid. This plasmid was cleaved with Hinc II and a Bgl II linker inserted to generate pBD15 with a polylinker as follows: 5′ BamH I, Xba I, Bgl II, Xho I, Cla I 3′ (Figure 1). pUC 8-MT was cleaved with Hind III, the ends filled in with reverse transcriptase, a Bgl II linker added and the entire cDNA inserted into pBD15 as a BamH I to Bgl II fragment.

In vitro mutagenesis and denaturing gradient gel electrophoresis

Chemical mutagenesis was performed as described (32). Times of exposure to the various chemicals were adjusted as described in the Results section. Perpendicular and parallel gradient gels were prepared and run as described (36). The apparatus for these gels was obtained from Green Mountain Laboratory Supply, Waltham, MA.

Cells and tissue culture

Balb/3T3 (clone A31) (from C.D. Scher) , psi-2 cells ((37) gift from C. Cepko), and Cos-1 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% donor calf serum (Gibco) at 37°C in 10% CO_2.

Retrovirus infections and transformation assays

Transfection of psi-2 cells and infection of Balb/3T3 cells were performed as previously described (6). 24 hours following infection with virus supernatants, cells were split into two 100 mm diameter dishes with half selected for G418 resistance (effective G418 concentration of 40 μg/ml) and the other half allowed to grow to confluence. Cells were fed every 3 to 4 days with DMEM supplemented with 10% donor calf serum. Desired clones were isolated, expanded into mass cultures and frozen in aliquots in liquid nitrogen.

Psi-2 and Cos-1 fusions

Balb/3T3 cells carrying clones of interest were grown to confluence in parallel with psi-2 or Cos-1 cultures. Cultures were split 1:2 and cocultivated until dense in 100 mm dishes. Cells were washed 3 times with warm (37°C) DMEM and fused in the presence of 2 ml of a 1:1 mixture of DMEM and polyethylene glycol 1000 (Baker) for 1 minute at room temperature. Plates were then washed 8 times with 4 ml of DMEM supplemented with 10% donor calf serum and then carried in culture. The following day, viral supernatant was harvested from the psi-2/Balb culture and used to infect a naive culture of Balb/3T3 fibroblasts. The Cos-1/Balb culture was fed daily for 3 days prior to preparation of a Hirt supernatant (38).

Immunoblotting

Immunoblotting was performed as previously described (39) with the exception that 2% gelatin (Bio-Rad) was used to block excess binding sites on the nitrocellulose. The rabbit anti-polyomavirus T-antigen serum used was provided by D. Pallas (40) and was diluted 1:1,200 for incubation with blots.

RESULTS

Construction and purification of polyoma middle T antigen mutants

Since little is known about the physical structure of polyoma middle T antigen (MTAg), our initial goal was to construct a large library of MTAg mutants which would later be characterized. We have chosen to use a random, saturation mutagenesis procedure as described by Myers, et. al. (32). This mutagenesis procedure takes advantage of the fact that single base substitutions alter the denaturation of a portion of the DNA molecule strongly enough to allow separation from wild type molecules (41). In order to allow detection of base pair substitutions in all melting domains of a DNA fragment Myers et. al. have engineered plasmids (pGC1 and 2) which allow a fragment of interest to be attached to a GC-rich DNA sequence ('GC-clamp'). This GC-rich region remains in the duplex configuration at all denaturant concentrations used for separation of wild-type DNA from mutant DNA fragments.

Construction of 'GC-clamp' plasmids

As fragments of 600 base pairs or less are preferred for separation by this technique, a MTAg cDNA (5) was divided into three fragments and placed in both orientations into appropriately modified 'GC-plasmids' (Figure 1). These constructions were designed so that all ligations would be two piece ligations for maximal efficiency and would preserve the sequence of each fragment. Following mutagenesis as described below, mutant fragments were cloned into pBD15-MT (Figure 1). This plasmid contains the entire MTAg coding region and has been designed so that all of the sites used to create the various MTAg fragments are unique sites in the resultant plasmid. In pBD15-MT, the MTAg gene is bounded by a 5′ BamH I site and 3′ Bgl II and Xho I sites so that the entire gene can be readily placed into BamH I or BamH I and Sal I sites of the retrovirus vectors used as the ultimate destination of the mutagenized gene. The latter construct (BamH I-Xho I) was preferred as this placed the gene of interest in the proper orientation with respect to the viral LTRs.

Chemical Mutagenesis

Single stranded DNA was obtained by infection of an F' bacterial host containing a GC-plasmid construct with a mutant M13 bacteriophage, M13rv1 using standard procedures (32). To maximize the chance of obtaining single-base pair substitutions in all positions of the target DNA, three different chemicals, nitrous acid, formic acid and hydrazine, were used to induce mutations. The chemical treatments and second strand DNA synthesis were performed as described by Myers, et. al. (32). However, as we found that obtaining double-stranded DNA was the most unreliable step in the mutagenesis procedure, it was modified slightly. In general, the ability to obtain double-stranded DNA was inversely related to the time of chemical exposure. Thus, time-course experiments were performed in which times of exposure to the various chemicals were varied from one quarter to one times the recommended time of exposure prior to second strand synthesis. Any double-stranded target fragments obtained were ligated into appropriately cleaved, unmutagenized GC-plasmids. Competent HB101 E. Coli cells (BRL) were transformed and the entire transformation mixture was plated on a 150 mm bacterial plate containing ampicillin. Colonies were pooled and plasmid DNA was analyzed on denaturing gradient gels as described (36). Using this modification, the percentage of mutants obtained is generally lower. Since the power of the denaturing gradient technique is the ability to separate mutant from wild-type, it has been exploited to purify mutants for analysis.

Denaturing gradient gel electrophoresis

Optimal conditions for separation of each fragment were first established. This was accomplished by determining the concentration of denaturant at which melting domains within the wild-type fragments undergo denaturation. Thus, each target fragment attached to the 'GC-clamp' was run on a polyacrylamide gel containing a concentration gradient of denaturant perpendicular to the direction of electrophoresis as described (36).

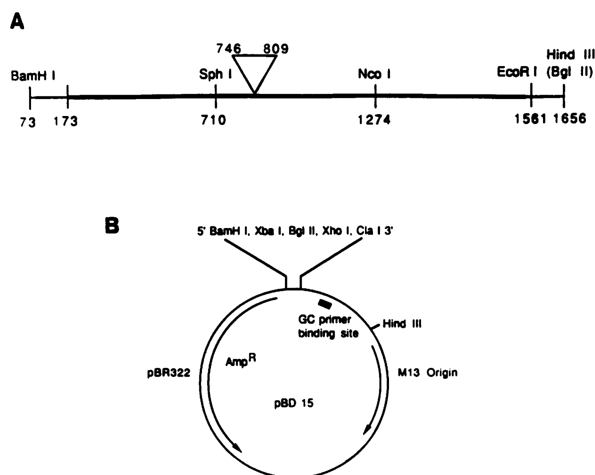

Figure 1. A. Schematic of MTAg cDNA. Wild-type MTAg, from W. Morgan (5) with convenient restriction sites is depicted. Symbols: ▬, MTAg open reading frame; ▽ MTAg intron. Nucleotide numbering as per Soeda et al (46). MTAg fragments placed into appropriate GC-plasmids were as follows: 1) BamH I-Sph I; 2) Sph I-Nco I; 3) Nco I-EcoR I. B. Schematic of GC plasmid. The GC plasmids as constructed by Myers, et. al. (32) contain the ampicillin resistance gene and colE1 origin of replication from pBR322; a bacteriophage M13 origin of replication; and a polylinker region. Upstream of the polylinker is a 19 bp primer sequence which is used for synthesis of a second strand of DNA and for dideoxy sequencing. Downstream from the polylinker is a GC-rich region which terminates in a Hind III site. pGC1 and pGC2 are identical plasmids except for the orientation of the polylinker (32). pGC1 was modified as described in the Materials and Methods section to yield pBD15.

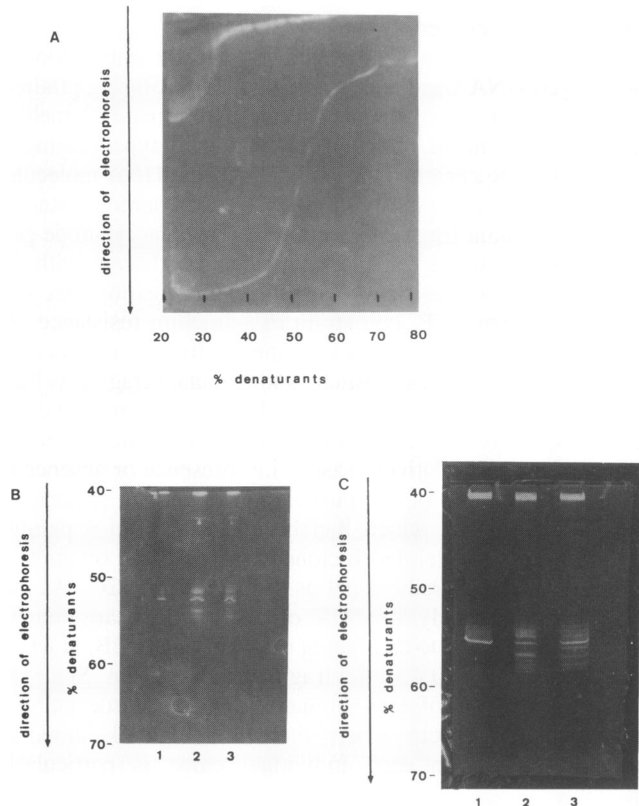

Figure 2. Denaturing gradient gels. A. Perpendicular denaturing gradient gel. A gel containing a linear 20 to 80% gradient of denaturants perpendicular to the direction of electrophoresis was prepared as described (36). pBD5 with the Sph I-Nco I fragment of MTAg was digested with Sph I and Hind III. This sample was layered across the top of the gel. Electrophoresis was carried out at150V for 8 hours at 60°C. DNA was visualized by ethidium bromide staining. The upper band corresponds to the backbone with the lower band representing the Sph I-Nco I fragment attached to a GC-rich region. The midpoint of the melting transition corresponds to the Tm of the single melting domain of the attached target DNA fragment. The percentage of denaturant corresponding to the Tm can be determined by measuring the position of the transition on the horizontal axis of the gel. In the example shown, the Tm corresponds to a denaturant concentration of approximately 52.5%. B and C. Parallel denaturing gradient gels. Single stranded DNA from pBD5 and pBD6 containing the Sph I-Nco I fragment of MTAg was treated with nitrous acid and a second strand synthesized as described (32). These fragments were then recloned into pBD5. The Sph I-Hind III fragment (target fragment plus 'GC-clamp') was excised from pooled DNA and subjected to electrophoresis on a gel containing a linear 40–70% concentration of denaturants parallel to the direction of electrophoresis. Electrophoresis was carried out at 150V for 6 hours at 60°C. B. Lane 1: Wild-type fragment. Lane 2: Nitrous acid treated DNA from pBD5-Sph I-Nco I-MTAg. Lane 3: Nitrous acid treated DNA from pBD6-Sph I-Nco I-MTAg. Mutant DNA running above and below wild-type was excised from this gel and recloned into pBD5. In Lane 2, pooled DNA was composed of approximately 30% mutant and 70% wild-type while in Lane 3, mutant fragments represent approximately 10% of the total. Pooled, mutant DNA was analyzed on a second parallel denaturing gradient gel as shown in 2C. C. Lane 1: Wild-type fragment. Lanes 2 and 3: Pooled, mutagenized DNA obtained from Lanes 2 and 3 of Figure 2B, respectively. These lanes contain approximately 90% and 50–60% mutant fragments, respectively. This same pooled, mutagenized DNA was used to replace the wild-type fragment in a MTAg containing retrovirus vector.

An example of this type of gel is presented in Figure 2A. Similar gels have been run for the remainder of the fragments (Table 1).

As can be seen from Table 1, the orientation of each fragment with respect to the GC-rich region can have little effect or dramatic effects on the denaturing properties of the attached fragments. In selecting the appropriate plasmid for recloning reverse transcribed, mutagenized DNA, the GC plasmid which yielded the easiest pattern of melting transitions, i.e. a single transition, was selected.

To separate mutant from wild type fragments, pooled, mutagenized DNA was then run on parallel denaturing gradient gels using a range of denaturant concentrations around the melting transition point noted above, such that wild type fragments denature near the center of the gel (41). Mutant DNA molecules run faster or slower than wild type DNA as demonstrated in Figure 2B. Mutant fragments were eluted from acrylamide gels (34) and ligated into a GC-plasmid backbone cleaved with the appropriate pair of restriction enzymes. The ligation mix was used to transform an E. coli strain to ampicillin resistance. At this point we had the option of screening individual colonies for the presence of mutations or batch cloning mutant fragments back into their proper sequence context. We decided to clone the mutant fragments in batches as we felt that we could screen for mutants of interest relatively easily, i.e. presence or absence of foci. As an example of the purification of mutant fragments, pooled mutant DNA which had been eluted from a parallel denaturing gradient gel and recloned was analyzed on another parallel denaturing gradient gel as seen in Figure 2C. As can be seen, approximately 80−90% of the fragments are mutant. This can be compared to their gel of origin in Figure 2B, in which approximately 10−20% of the fragments are mutant. Since the phenotypic analysis of these mutants involves tissue culture, purification to the point where there is a high likelihood of obtaining mutants in each individual clone is particularly important.

Also apparent from the gels in Figure 2B and C is another problem of this technique which was encountered, that is, smearing of the wild type fragment as seen best in Figure 2C, lane 1. This accounts for the amount of wild type fragment still seen in figure 2C, lanes 2 and 3. As smearing only occurs above the wild type band, one of the ways we have circumvented this problem is to elute DNA above and below wild type separately. Thus, the DNA obtained from below the wild type band is nearly 100% mutant (data not shown). The DNA obtained from above the wild type band could be subjected to another round of purification, however we anticipate that this would only be necessary if we wanted to screen for phenotypically silent mutants. A more bothersome problem has been that one of the fragments (Nco I-EcoR I fragment) smears to such a great extent that effective separation is virtually impossible. A lengthy search for the cause of this problem resulted in the conclusion that it was most likely the sequence of the specific fragments which resulted in denaturation over a rather broad range of denaturants. In order to overcome this problem we have denatured and reanealled pooled, mutagenized fragments prior to running them on a parallel denaturing gradient gel (42). In this manner, approximately 10% of the fragments are heteroduplexes of mutant and wild type DNA. These heteroduplexes denature at a much lower concentration of denaturant than wild type fragments and thus allow for effective separation (42). However, the maximum purification possible using this particular method was by necessity 50% as one of the strands contains a wild type fragment.

Recombinant retrovirus constructs

Mutants of MTAg constructed as described above were recloned into their proper context into pBD15-MT and placed into a recombinant retrovirus vector. The retroviral vector we have chosen to use for the screening of mutants is a modified version of DO1 (DOL-) made by A. Korman in collaboration with R. Mulligan (35). The salient features of this vector include: 1) Moloney murine leukemia (MoMuLV) long terminal repeats (LTR); 2) psi-2 packaging site; 3) unique BamH I and Sal I cloning sites downstream from the 5′ LTR; 4) SV40 origin of replication/early promoter; 5) neomycin resistance gene regulated by the SV40 early promoter; 6) pBR322 origin of replication; and 7) polyoma early region sequences to increase transient viral titers after transfection.

Our preference was to use retroviral infections as opposed to transfections to screen the mutant retrovirus library for a non-transforming phenotype. By using retrovirus infections, which should result in a single retrovirus per cell, as opposed to direct transfection of mutant retrovirus, we avoid the problem of obtaining multiple retroviruses and multiple mutants in stably transfected cell lines. One of our concerns in using the retrovirus vector described above was the presence of the polyoma early region sequences in the region flanking the retroviral portion of the vector. These sequences created the possibility of recombination with mutant MTAg placed into this vector. However, they also allow the transfected vector to replicate to high copy number resulting in a higher transient viral titer. To determine whether recombination would be a problem, the transformation-defective NG59 mutation of MTAg (a single base pair change and a one codon insertion) was inserted into this vector and transfected into psi-2 cells with the transient viral supernatant being used to infect Balb/3T3 cells. Under these circumstances, no foci were observed on monolayers, with 100−200 G418-resistant colonies obtained. Metabolic labelling of G418-resistant clones with ^{35}S-methionine revealed that NG59-MTAg was being expressed in all clones tested (6/6).

Table 1. Denaturating properties of Middle T antigen DNA fragments

Fragment	Melting Transition (% denaturant)*
pBD5-BamH I-Sph I	45%
pBD6-BamH I-Sph I	Not done
pBD5-Sph I-Nco I	52.5%
pBD6-Sph I-Nco I	52.5%
pGC1-Nco I-EcoR I	34%, 48%
pGC2-Nco I-EcoR I	45%

* 100% denaturant = 7M Urea, 40% formamide

Table 2. Focus forming assay of Middle T Antigen mutants

Construct	G418-resistant colonies	Foci	%[a]
pDOL	~200	0	0
MTAg-wild type	65	50	77
NG59 mutation of MTAg	102	0	0
Sph I-Nco I mutations of MTAg[b]	141	75	53

[a] Foci/G418-resistant colonies
[b] Represents a combination of two separate experiments

Thus, we feel confident that if recombination does occur, it will be an infrequent enough event so that it should not present a problem.

Transformation Assays

The retrovirus, pDOL-, containing Sph I-Nco I mutations of MTAg, was transfected into psi-2 cells by calcium phosphate precipitation (43). Wild type MTAg and the transformation defective NG59 mutant of MTAg as well as pDOL- without insert were used as controls. Transiently expressed virus obtained 18 hours after transfection was used to infect Balb-3T3 cells as described (37). 24 hours following infection cells were split with half of the cells selected for G418 resistance while the other half were examined for focus forming ability on a monolayer. A tabulation of G418-resistant colonies versus foci is reported in Table 2. The ratio of foci to G418-resistant colonies was 0.77 for wild-type MTAg as compared to 0.53 for the MTAg mutant pool. These data suggest that approximately 24% of the retroviruses transduce nontransforming alleles of MTAg. Put another way, this suggests that of the approximately 65 non-transformed G418-resistant colonies obtained from these two retroviral infections, 50 might be expected to contain transformation defective MTAg mutants.

Although infection with the MTAg mutant pool resulted in a lower frequency of focus formation on cell monolayers it was possible that lack of transformation simply represented gross deletions in MTAg or complete absence of MTAg expression. Therefore, we characterized several tentative non-transformed G418-resistant colonies. Seven apparently flat G418-resistant colonies were selected at random for further analysis. While three of these colonies appeared morphologically transformed when G418 selection was removed, the remaining four colonies maintained their non-transformed appearance. Whole cell lysates of each colony were analyzed by immunoblotting (Figure 3A). All seven colonies were expressing a protein which comigrated with wild-type MTAg with one exception. This one colony, which by morphology was not transformed, appeared to be expressing a MTAg with a slightly altered mobility. Further analysis of this colony will be presented. However, it should be noted that the remaining six colonies plus many others from these two transient retroviral supernatants containing mutations in the Sph I-Nco I remain to be characterized. There are also similar experiments to be performed with the mutations in the remaining two MTAg fragments.

The G418-resistant, non-transformed colony, expressing a MTAg with a slightly altered mobility was tested to ensure that transformation-defective phenotype was conferred by the retroviral vector rather than a secondary cellular mutation. This was accomplished by fusing the cloned culture with psi-2 packaging cells (M. Corbley, T. Roberts, unpublished). The culture supernatant was then used to infect a naive 3T3 cell line. In this experiment, 87 G418-resistant colonies were obtained and no foci were observed. Six of these secondary G418-resistant colonies were selected and an immunoblot performed on cell lysates. All six colonies were expressing a MTAg with the same alteration in mobility as had been observed in the original immunoblot described above (Figure 3B). At the same time, the mutant virus was rescued for sequence analysis. This was accomplished by fusing cells expressing the mutant retrovirus with Cos-1 cells (44) using polyethylene glycol (45) and preparing Hirt supernatants (38). The Hirt supernatants obtained were used to transform an E. coli strain to kanamycin resistance. DNA from an unrearranged clone was subcloned into pBD15 for sequencing. DNA sequence analysis of the entire MTAg coding region revealed that there were four single base pair substitutions in the Sph I-Nco I fragment of MTAg (the fragment which had been originally mutagenized) with no other changes observed.

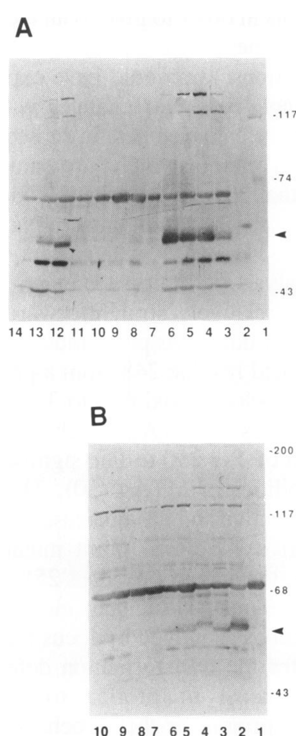

Figure 3. Immunoblot analysis of MTAg mutants. A. Primary infection of Balb/3T3 fibroblasts with pooled MTAg mutants. NP40 lysates of several G418-resistant colonies expressing control retrovirus, wild type MTAg, or various MTAg mutants were separated on a 7.5% SDS-polyacrylamide gel and electrophoretically transfered to nitrocellulose. Blots were probed with rabbit polyclonal anti-T antisera (40) and developed using an alkaline phosphatase conjugated anti-rabbit second antibody. Lanes: 1, Molecular weight markers (migration in kilodaltons indicated on the right of the figure); 2, Control retrovirus in Balb/3T3 fibroblasts; 3–6, Wild-type MTAg; 7–8, NG59 mutant of MTAg; 9–14, Non-transformed G418-resistant colonies obtained from infection with pooled MTAg mutants. The migration of MTAg is indicated with an arrow. B. Secondary infection. The cells expressing the MTAg from Lane 12 in panel A were fused with psi-2 cells and the transient viral supernatant was used to infect naive Balb/3T3 fibroblasts. Several G418-resistant colonies were selected and analysis above. Lanes: 1, Control retrovirus in Balb/3T3 fibroblasts; 2, 4: Wild-type MTAg; 3, Parental colony of MTAg mutant from Lane 12, above; 5–10, Individual colonies from the secondary infection with the MTAg mutant from Lane 12 in panel A. The migration of MTAg is indicated with an arrow.

Table 3. Focus forming assay of reconstructed MTAg mutants

Construct	G418-resistant colonies	Foci	%*
pDOL-	~200	0	0
pDOL-MT	65	52	80
pDOL-MTm	78	0	0
pDOL-248m	86	0	0
pDOL-190m	34	24	71
pDOL-337/343m	77	52	68

*Foci/G418-resistant colonies

The four observed changes are an A to G change at nucleotide 740 resulting in the substitution of aspartic acid for asparagine at amino acid 190, a C to T change at position 977 which changes a proline at codon 248 to a leucine, a C to T change at nucleotide 1244 which results in a proline to leucine substitution at amino acid 337 and a G to A change at nucleotide 1261 yielding an alanine to threonine change at codon 343, respectively.

In order to determine which one of the four changes was responsible for the transformation-defective phenotype, MTAg cDNAs containing single mutations at codons 190 and 248 and a double mutant at amino acids 337 and 343 were constructed by the appropriate substitutions (33). These MTAg mutants were sequenced to confirm the presence of these specific changes. Each of these three MTAg mutants were placed into the retrovirus pDOL-, transfected into psi-2 cells with transiently expressed virus used to infect Balb/3T3 cells. These mutants will be referred to by respective amino acid changes, i.e. 190m, 248m, 337/343m. The MTAg cDNA containing all four mutations was also recloned into pDOL- and will be referred to as MTm. Results of a focus forming assay are presented in Table 3.

As can be seen, the mutation at amino acid 248 was responsible for the transformation defect. In other experiments, the mutant 248m-expressing cells were maintained for 7 weeks at a monolayer without any foci resulting. Seventy three G418-resistant colonies were obtained in parallel cultures at 3 weeks post infection. It should be noted that by microscopic examination, cells expressing the 337/343m double mutant were virtually indistinguishable from wild type MT containing cells. However, foci obtained from cells expressing mutant 190m were not as large and the cells did not appear quite as morphologically altered as cells expressing wild type MTAg. Soft agar assays of pooled G418-resistant colonies demonstrate that both wild type MTAg and 337/343m double mutant expressing cells are capable of growth in soft agar. However, cells containing retrovirus vector alone or retrovirus with either the 190m or 248m point mutants are incapable of growth in soft agar.

DISCUSSION

We have described an approach to the genetic study of the functions of MTAg. This approach takes advantage of several existing techniques used for the study of MTAg and extends them by applying several novel techniques. The advantages of using cDNAs separately encoding each of the T antigens as well as the use of the retrovirus system has been previously described. (5, 6) Here we extend the use of this system by applying a random mutagenesis procedure to MTAg in order to study the effect of mutations on a variety of MTAg functions. In this paper, we describe the successful use of this random mutagenesis procedure combined with the use of the recombinant retroviral system to generate a large library of mutations in MTAg and describe several new mutations in MTAg.

In attempting these experiments, we had to address several concerns to optimize the methodology. First, the recombinant retrovirus vector was chosen to maximize the transient viral titer in order to increase the number of independent colonies obtained from a retroviral infection. Had we instead selected stable colonies producing mutant retrovirus, the likelihood of obtaining multiple mutant clones which were siblings would have been quite high. However, this vector contains the entire coding region of polyoma virus raising the possibility of internal recombination. In numerous control infections, the number of foci obtained using transformation defective point mutations has not been above background levels, Thus, if recombination within the vector occurs, it occurs at a low enough frequency so as not to present a problem. The second concern was whether non-transformed G418-resistant colonies could be recognized easily enough to be used as our phenotypic screen. Thus far, our success rate has been about 50–60% (4/7). Since our library of MTAg mutations remaining to be screened is quite large it will probably be to our benefit to subject pooled G418-resistant colonies to BuDR selection to enrich for non-transformed clones.

The first MTAg mutant characterized contained four single base pair alterations, all within the fragment mutagenized. This number of changes was surprising since the mutagenesis protocol was designed to mutagenize the target DNA lightly so that mostly single-base pair mutations would predominate. However, the original pool of MTAg mutants which was selected for analysis of our phenotypic screen (focus forming assay) was a relatively heavily mutagenized stock. From the original denaturing gel, approximately 50% of the DNA was mutant with 50% wild-type. While most of our mutagenized stocks contain a significantly lower percentage of mutants, this population was selected for the initial experiments in order to give us an idea of the likelihood of success of our phenotypic screen. Assuming a Poisson distribution of mutations, we would have expected 50% of the mutations to be single-base pair changes with the other 50% containing two or more base changes. In subsequent experiments, mutants have been made which are much more lightly mutagenized such that 80–90% of the mutations will be single base-pair substitutions. These mutants are currently being used in similar experiments. However, with current recombinant DNA techniques, the analysis of multiple mutants, such as the one presented above, is relatively straightforward.

The transformation defective point mutation obtained was a mutation of amino acid residue 248 from a proline to a leucine. This mutation lies in close proximity to Tyr 250 which is one of the tyrosine residues of MTAg which is phosphorylated in vivo (13). Mutation of Tyr 250 to Phe significantly impairs the transforming capabilities of MTAg (30). The Tyr 250 mutant is transformation defective and has decreased levels of associated tyrosine kinase activity. Several point mutations around this residue, specifically, mutations at residues 251, 252, and a double mutant of residues 249 and 251 maintain close to wild-type levels of transforming abilities as judged by focus forming capabilities (30). Possibilities for the transformation defect of this mutant include that the change of proline to leucine alters the configuration of the protein or that it behaves similarly to the Tyr 250 mutation by disturbing the sequence of a site of MTAg tyrosine phosphorylation. These possibilities are not mutually exclusive. However, biochemical data have shown that 248m is similar to wild-type MTAg when assayed for sites of in vivo phosphorylation and associated kinase activities in vitro, including associated tyrosine and phosphatidylinositol-3 kinase activities in vitro (33). Thus, it appears that 248m has a more subtle defect resulting in its transformation defective phenotype.

It is clear that the mutagenesis technique used in these studies is relatively tedious and has not been widely used. Despite this, we now have a large library of MTAg mutants which can be characterized. Because of the manner in which the library was constructed, the likelihood of individual G418-resistant colonies containing mutants is quite high. Further, virtually all of the G418-resistant colonies obtained express MTAg. Additionally, this procedure has already yielded an extremely interesting MTAg

mutant(248m). This mutant lies in a region of MTAg which has largely been ignored in the study of MTAg function. The precise nature of the defect in this mutant has been the focus of our recent studies. However, we are confident that other mutants of interest will be obtained from this MTAg mutant library.

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