Random oligonucleotide mutagenesis: application to a large protein coding sequence of a major histocompatibility complex class I gene, H-2DP

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ABSTRACT

We have used random oligonucleotide mutagenesis (or saturation mutagenesis) to create a library of point mutations in the α1 protein domain of a Major Histocompatibility Complex (MHC) molecule. This protein domain is critical for T cell and B cell recognition. We altered the MHC class I H-2DP gene sequence such that synthetic mutant α1 exons (270 bp of coding sequence), which contain mutations identified by sequence analysis, can replace the wild type α1 exon. The synthetic exons were constructed from twelve overlapping oligonucleotides which contained an average of 1.3 random point mutations per intact exon. DNA sequence analysis of mutant α1 exons has shown a point mutant distribution that fits a Poisson distribution, and thus emphasizes the utility of this mutagenesis technique to "scan" a large protein sequence for important mutations. We report our use of saturation mutagenesis to scan an entire exon of the H-2DP gene, a cassette strategy to replace the wild type α1 exon with individual mutant α1 exons, and analysis of mutant molecules expressed on the surface of transfected mouse L cells.

INTRODUCTION

The development of techniques to alter DNA sequences in vitro is a powerful tool in biological research. Coupled with gene transfer technology this approach has been useful for detailed structure/function analysis. Historically, chemical mutagenesis and selection of MHC mutants in somatic cells (1,2) or intact animals (3) has been difficult due to the low frequency of mutants recovered. The advent of site directed mutagenesis made possible the generation of a particular mutant at any region of a sequence. However, the caveat to this approach, if investigating a large protein domain, is that there must be an a priori reason to examine a particular mutant. Thus, for reasons of efficiency and economy, it is not practical to produce a large number of different mutants in a large protein domain by this method. The use of saturation mutagenesis overcomes this drawback.

Unbiased misincorporation of nucleotide precursors in oligonucleotide synthesis produces a complete spectrum of single base substitutions. The construction of mutant libraries that contain all possible single point substitutions represents an efficient way to "scan" a large protein domain for mutants which may affect function. This strategy has
been used successfully to study small target sequences (4,5,6). Here we report our adaptation of this technique to a large, mammalian protein domain (90 amino acids). We have generated a complete mutant library in the 270 bp α1 exon of a murine MHC class I gene.

The mouse MHC, H-2, is a polymorphic gene family that encodes a set of cell surface molecules critical for cell-cell recognition events during immune responses. MHC class I molecules are the recognition structures used by cytolytic T lymphocytes (CTL) (7). Class I gene sequences are unusual in their high degree of polymorphism, both in the sequence divergence between alleles, and in the number of alleles the mouse population. The functional analysis of these molecules has benefited from the large variety of monoclonal antibodies (mAb) and cellular analyses which are specific for individual alleles. Many class I genes have been sequenced (8). Although there is considerable sequence variation among alleles, a striking structural similarity was observed in the organization of exons in the genomic sequences (9,10). The class I genes contain eight exons: a leader sequence encoding amino acids processed off the mature molecule, three exons encoding extra-cellular protein domains (α1,α2,α3), an exon encoding a transmembrane sequence, and three small exons encoding a cytoplasmic tail.

A variety of in vitro mutation techniques have been used to map the CTL and mAb recognition determinants (epitopes) on class I molecules. These include the production of chimeric genes by switching of exons among alleles. The hybrid genes were introduced into cells and expressed at the cell surface. CTL specific for the parental molecules were then assayed to detect the presence or absence of CTL epitopes on the hybrid molecules. The consensus of these studies indicate that CTL epitopes are formed by conformational interaction of the α1 and α2 protein domains (11,12). Mutants affecting T cell recognition do not always affect mAb recognition, implying somewhat different molecular interactions between T cell receptors or mAb and class I molecules (11,13,14). Site directed mutagenesis studies were then used to examine polymorphic residues which contain allele specific residues (15,16). Unfortunately, the site directed approach is cumbersome as there are 180 amino acids in the two protein domains which contain the majority of CTL and B cell epitopes.

Using saturation mutagenesis we created a mutant library which contains a complete repertoire of single base substitutions in the α1 exon of the H-2DP gene. This approach is particularly well suited to class I genes. The polymorphic nature of the molecules (up to 20% at the amino acid level between alleles) makes choosing a candidate for site directed mutagenesis difficult. Therefore, we reasoned that saturation mutagenesis would be exceptionally useful as this removes the bottleneck of creating individual site directed mutants, and also allows us to sample residues at polymorphic and conserved positions throughout an entire protein domain, without any preconceived bias.
In this report we describe our method to adapt saturation mutagenesis to a large coding sequence of the H-2DP gene. We created mutant α1 exons by synthesizing a series of twelve overlapping randomly mutated oligonucleotides. These were assembled into synthetic restriction fragments and cloned into M13mp19. One hundred and twenty clones, each containing 340 bp of synthetic DNA, were sequenced. We constructed a "cassette" mutagenesis system where individual mutant α1 exons could be subcloned back into the remainder of the H-2DP gene sequence. Here, we show the transfection and initial expression analysis of five mutant products on the mouse L cell surface. We have recently reported on the CTL recognition of these same mutants (17), and here show a much more detailed description of our mutagenesis procedure and our analysis of the cell surface expression of mutant molecules with a DP specific mAb. We believe this method represents an important approach to analyze large protein domains.

MATERIALS AND METHODS

Enzymes. All restriction endonucleases and DNA ligase were purchased from New England Biolabs.

DNA Synthesis. Twelve oligonucleotides, ranging in size from 30 to 67 bases, were synthesized on an Applied Biosystems Model 380A DNA synthesizer and used to construct the randomly mutagenized synthetic α1 exon (fig. 1). Each of the 4 phosphoramidites was dissolved in dry acetonitrile to give a 0.05 M stock solution. (This is one half the concentration recommended by Applied Biosystems, and was done simply to save reagents. Usually our syntheses performed in this way are more efficient than the ones described in this paper.) A mixture of all 4 phosphoramidite monomers was made by mixing equal volumes of the 0.05 M stocks. This equimolar mix was then added to each of the pure solutions at a level of 1% by volume to produce the "doped" phosphoramidite solutions, which were then loaded onto the machine in the positions normally used for the pure phosphoramidites (doped A in the A bottle, etc.). The synthesizer was programmed with the wild-type sequence for each oligonucleotide, and the column was packed with controlled pore glass beads bearing 1 micromole of the 3' terminal nucleoside corresponding to the wild-type sequence at that position. The standard cycle supplied with the machine for a 1 micromole synthesis was used. Under these conditions there is approximately a 5-fold molar ratio of phosphoramidite to growing oligonucleotide chains during the coupling reaction. We believe that the level of mutagenesis should depend only on the composition of the doped phosphoramidite solutions. In this case the expected average number of mutations per clone is $270 \times 0.01 \times \frac{3}{4}$ (size of the mutagenic target in bp x fraction of equimolar mixture added x probability that incorporation of a residue from the doping mix will lead to a mutant sequence), which is approximately 2.

Purification. Each synthetic oligonucleotide was purified on a 40 cm long 8% acrylamide
gel (made in buffer containing 3.6M urea, 1X tris borate buffer (90 mM tris, 90mM boric acid, 2mM EDTA; TBE), and 25 % formamide) which was electrophoresed for 16 hours at 5 watts constant power in 1x TBE. Bands were visualized by ultraviolet light shadowing and excised with a razor blade. Resolution and excision of single base increments is critical, such that subsequent assembly of coding regions retain the correct reading frame.

Assembly of synthetic mutant restriction fragments. All oligonucleotides with an internal 5' end (in relation to the final synthetic structure) were phosphorylated with T4 polynucleotide kinase (New England Biolabs). Ten picomoles of each of the twelve oligonucleotides were then mixed in an Eppendorf tube and floated on a water bath at 95 C in 6mM tris, 3mM MgCl2, pH 8.5. This was allowed to cool to room temperature over a period of 3 hours. This reaction was ligated overnight at 4C with 400 units of T4 ligase (New England Biolabs). The DNA was then fractionated on an 8% acrylamide gel, and the appropriate sized (340 bp) fragment was excised with a razor blade following staining with ethidium bromide. The 5' end of the intact fragment contained a Kpnl restriction site overhang. The 3' end contained an Sphl restriction site overhang.

Cloning of synthetic exons. The synthetic fragments recovered from gel purification were cloned into Kpnl-Sphl digested M13mp19, using standard recombinant DNA methodology (18).

DNA sequencing. M13 clones containing the mutant α1 exons were sequenced by the dideoxy method (19).

Construction of unique restriction sites bordering the WT α1 exon. Using the DNA sequence of the H-2DP gene as a guide (9), we ligated a unique Kpnl linker into a Smal site 45 bp 5' of the WT α1 exon in the plasmid pRM15. A unique Sphl linker was ligated into a Smal site 13 bp 3' of the WT α1 exon. Thus a Kpnl-Sphl digest of pRM15 will remove the WT α1 exon, allowing for reconstruction of an intact gene by ligation with an individual α1 mutant exon that has been removed from M13 replicative forms by a Kpnl-Sphl digestion.

Transfection of mouse L cells. Mouse Ltk- cells were transfected by electroporation with a thymidine kinase (tk) gene and a ten fold molar excess of an individual mutant H-2DP plasmid, as described (17).

RIA Analysis of Transfectants. The presence of a mutant gene product at the L cell surface was determined by RIA using standard procedures. Briefly, 10^5 cells were stained with the primary step antibody, then washed three times. Cells were then incubated with 125I protein A, washed three times, and the cell bound radioactivity counted. Both primary step mAb have been previously described (20,21); mAb 7-16.10 is specific for the transfected H-2DP products, and mAb 16-1-2 is specific for the L cell endogenous H-2Kk product.
RESULTS

Our strategy was to construct a "cassette" mutagenesis system in which we could excise and then replace the wild type α1 exon of the H-2DP gene with a mutant exon. Both strands of the synthetic mutant exons were synthesized.

Synthesis of oligonucleotides

We designed twelve overlapping oligonucleotides ranging from 30 bases to 67 bases in size. The structure of the mutated overlapping oligonucleotides is shown in figure 1. We chose to make the oligonucleotides with approximately 30 bp overlaps in relation to the opposite strand, reasoning that this design would minimize any bias against mutations in the overlapping regions. For example, shorter overlaps might produce a severe bias against mutations in the overlap regions since they would have a higher destabilizing effect during the annealing process, prior to ligation.

Assembly of Synthetic Restriction Fragments

In our initial experiments we purified each oligonucleotide by polyacrylamide gel electrophoresis. Apparently homogeneous bands were cut out, the DNA eluted, and quantitated by A260. The fragments were then assembled as we describe, and cloned into M13mp19 for sequence analysis.

Sequence analysis of the first set of recombinant clones yielded an unanticipated problem in maintaining the proper 270 bp reading frame. Although we observed base substitutions as expected, the far most common mutations were insertions or deletions.

Figure 1. The structure of the synthetic α1 exon is shown. The nucleotide sequence of each of the twelve synthetic oligonucleotides is shown. Vertical bars show the end of each oligonucleotide. The 5' end of the intact synthetic structure retained a Kpnl restriction site overhang. The 3' end retained an Sphl restriction site overhang. The coding sequence starts at nucleotide 52, and ends at 322. The overall length of the synthetic DNA is 340 bp.
Figure 2. The arbitrary number of each of the twelve oligonucleotides is shown above each sample. The size range, in bases, is shown at the right side. The oligonucleotides between 60 and 67 bases showed substantial populations of insertions and deletions. The two smaller oligonucleotides (#1 and 12) showed very little heterogeneity. The correct size for all oligonucleotides corresponded to the most prominent band, except oligonucleotide #4. The correct size for this molecule was the faint bottom band, shown by the arrow (see text).

Particularly susceptible to this problem was a sequence from nucleotide positions 89 to 100 which contains a 12 bp stretch of G’s or C’s. In this site we found a large number of G or C insertions. Other sites of random deletions and insertions were also seen.

In order to determine if this was a property of the synthetic DNA, or its subsequent processing in E. coli we phosphorylated each of the oligonucleotides with 32P and loaded 10 femtamoles on a DNA sequencing gel. Following separation and autoradiography, we found that none of the oligonucleotides migrated as a homogeneous species (Figure 2). Rather, they all ran with at least three and some times as many as six discrete bands, indicating that each of the component oligonucleotides was heterogeneous in length. This suggested to us that the problem was not in processing or repair of unligated junctions by E. coli, but a problem with chemical synthesis and insufficient stringency in purification. The problem was compounded by the structure of the synthetic DNA design. All twelve oligonucleotides were heterogeneous, and therefore the joint probability of annealing a structure of the correct length was very low.

In order to circumvent this problem, 0.5% of the crude synthesis material was electrophoresed on a 40 cm denaturing polyacrylamide gel. Fractionation of such small amounts of crude synthesis material allows for single base increment resolution of large oligonucleotides. The oligonucleotide bands corresponding most closely to the appropriate lengths were excised, eluted from the gels and assembled and ligated as be-
The correct size for all the oligonucleotides, except #4, was the most prominent band. The correct size for oligonucleotide #4 was the faint bottom band shown in figure 2. This was deduced from the large number of insertions present in this region from the sequence analysis of the first set of recombinant clones. After assembly, these synthetic restriction fragments were separated on an 8% polyacrylamide gel and a band corresponding to 340 bp was excised. When these fragments were cloned into M13mp19 and sequenced a large number of clones corresponded to wild type length.

**Sequence Analysis of Synthetic Restriction Fragments**

We sequenced 120 independent M13 recombinants. Fifty-one of these clones retained the correct reading frame. The distribution of mutations in these 51 clones fits a Poisson distribution. Thirteen of these clones did not contain a point mutation. The mutations in the remaining 38 clones are collectively shown in figure 3. There were wild type (WT), single, double, triple, and quadruple mutations per coding sequence. The fit of the observed distribution is compared to the expected Poisson distribution in table 1. There is an average of 1.3 mutations per clone. We predicted an average of two mutations per clone, based on the "spiking" concentrations of the nucleotide precursors. This slight discrepancy may be explained by a selection for WT sequences in the annealing process, as the absence of mismatches would produce a more stable structure.

**Estimation of the Mutant Library Size**

Following cloning of the synthetic exons into M13mp19, we recovered approximately 10,000 independent recombinant phage. It is useful to estimate the completeness of the mutant library. Our sequence analysis of 120 clones shows 25% (30/120) of all the recombinant phage contain in frame, potentially useful mutant sequences. Therefore we estimate 2,500 independent, in frame mutant clones in the library. The target sequence was 270 bps of coding sequence and there are 810 (3 substitutions x 270 positions) total possible single base change mutants. In the library of 2,500 recombinants, we calculate a greater than 99% probability of having cloned each possible single point mutant at each position. This of course does not mean that every position is mutated to each amino acid, since single base changes can result in only a limited number of amino acid substitutions. However, we have identified up to four different amino acid substitutions at a single position (see figure 3).

Of particular interest was to determine if there was any bias against recovery of mutations at or near the junction sites. We have defined the junction fragments as the window of 5 bp of either side of every junction. When we examined these sequences (defined as 90 bp, that is, 9 internal junctions in the coding sequence x 10 bp,) we found 18 mutations. This is not significantly different from the expected 22 mutations ($\chi^2=0.73; 1$ dof). Thus an advantage of using large overlapping oligonucleotides is the ability to recover mutations near the sites where the oligonucleotides abut.
Figure 3. The collective sequence data from the first 38 in frame 340 bp mutant α1 fragments is shown. Also added to this figure are data from six additional mutant α1 fragments containing a total of 20 mutations. These additional mutants are not included in the calculations for mutant frequencies, as they were selected for multiple substitutions. The wildtype nucleotide sequence is shown in lower case letters. The amino acids encoded by the α1 exon are shown in the single letter code (above each codon). Mutants are shown below the wildtype sequence by the nucleotide substitution (lower case letters) above the amino acid encoded by that substitution (upper case letters). An X indicates a stop codon. An 0 indicates a silent substitution. A number below the amino acid indicates that the substitution is part of a multiple event. Each number corresponds to a different mutant clone. Thus the 1 below amino acid 4 (S>L) and 7 (Y>C) indicates that both substitutions were in the same clone. The ends of the DNA shown in this figure do not show the KpnI and SphI restriction site overhangs. Only coding sequence mutations were tabulated.

Reconstruction of Intact Mutant Genes

We engineered a genomic copy of the H-2DP gene to contain a KpnI restriction site 45 bp 5’ of the α1 exon, and an SphI restriction site 13 bp 3’ of the α1 exon. The strategy was to be able to efficiently remove the WT α1 exon and replace it with a identified mutant. An outline of the strategy of converting SmaI sites into KpnI and SphI sites is shown in figure 4. The WT exon can be removed and a mutant exon directionally ligated back into the rest of the WT gene for transfection.
The observed frequency of the distribution of clones containing 0, 1, 2, 3, or 4 mutations was not significantly different from a Poisson curve ($\chi^2 = 6.317; 4$ dof). The observed frequency was calculated from the 51 in frame clones described in the text.

**Cell Surface Expression of Mutant Products**

Mutant genes DP14(A11V;E32Q), DP20(Y27N), DP164(R14L), DP174(P57Q), and DP181(D37H) have been transfected into mouse L cells and their products expressed at the cell surface. Nomenclature for these mutants is an arbitrary DP number followed by, in the single letter code, the wild type amino acid, the amino acid position, and the mutant amino acid. These five mutants have been found to express relatively equivalent amounts of the mutant class I molecule as cells transfected with the WT DP gene (L12a). Figure 5 shows the expression analysis using mAb 7-16.10. DP174(P57Q) either expresses a slightly lower amount of the mutant molecule, or has a minor variation in the epitope for this mAb. We are currently investigating these possibilities. However, we have shown that DP174(P57Q) is recognized by self-restricted CTL at levels indistinguishable from the L12a cells (17). Although DP20(Y27N) is very similar to L12a for the binding of mAb 7-16.10, this mutant has been found to be altered in recognition by alloreactive CTL (17).

**Figure 4.** The general strategy for constructing unique restriction sites bordering the wildtype a1 exon in the H-2DP gene in pRM15 is shown. Existing Smal sites were converted to either a KpnI restriction site or a SphI restriction site by the insertion of linker molecules. These restriction sites are compatible with the ends of the synthetic mutant fragments. The black boxes represent exons, as described in the text. The scale of this map is approximate.
Figure 5. RIA analysis of five H-2DP mutants along with the positive control cell line expressing the WT DP molecule (L12a), and the negative control parental Ltk-cell line. Values are the mean of triplicate samples and expressed as cpm of $^{125}$I bound to the cells. The binding of mAb 16-1-2 which is specific for the Ltk-endogenous H-2Kk molecule has been normalized to 20,000 cpm bound (data not shown). This provides an internal control for each cell line tested to which the binding of the mAb 7-16.10, specific for the transfected H-2DP product, can be compared. ■ no mAb control, □ a one to twenty dilution of mAb 7-16.10, ■■■■■■■■ a one to two hundred dilution of mAb 7-16.10.

DISCUSSION

We have applied the technique of saturation mutagenesis to a large protein coding domain. The target sequence of 270 bp revealed a set of problems previously unanticipated in the synthesis and use of large synthetic oligonucleotides.

The design of the synthetic DNA we used in these experiments highlighted a problem which we believe to have occurred in automated chemical synthesis. The addition or deletion of extra nucleotides in the final synthetic structure correlated well with the observed heterogeneity of each oligonucleotide when separated at a resolution of single base increments. Therefore, we believe assembling large fragments of synthetic
DNA by our strategy is not a problem with subsequent processing or repair of the molecules in *E. coli*, but does require careful separation and purification of the oligonucleotide components from the crude stock material. Of note, the smaller oligonucleotides (approximately 30 bases) did not have appreciable insertions or deletions (see figure 2), whereas the larger oligonucleotides (60 to 67 bases) were subject to inappropriate additions or deletions during synthesis. We speculate that insertions could have resulted from the addition of two phosphoramidites in a single round of synthesis. Indeed, the most frequent error we found was the addition of a base identical to the previous base. This is consistent with the presence of detritylated phosphoramidites in the precursor population, allowing a double addition when an unblocked base is incorporated into a growing chain. The large number of insertions and deletions in the oligonucleotides used in this study may not be a typical problem for experiments of this nature, but simply a reflection of poorer than average quality in this particular automated chemistry. However, if relatively large coding sequences are to be assembled from numerous oligonucleotides, great care should be taken to purify and assay the sizes of each individual component. The joint probability of annealing a DNA structure which retains the correct reading frame is very low if numerous oligonucleotides have varying lengths.

The mutations described here occurred in a random manner, as would be expected by a Poisson distribution. This random distribution of mutants is an extremely powerful technique for surveying a large coding domain for structure/function studies. Even a relatively small number of mutants allow large regions of the molecule to be sampled. Indeed, the collective mutations we have described in figure 3 shows the largest gap of unmutated α1 protein sequence is only a stretch of four amino acids. We believe this approach is favorable for the analysis of large protein domains in comparison to other *in vitro* mutagenesis methods such as linker scanning (22), πVX recombination (23) and chemical mutagenesis (24). Linker scanning analysis results in the insertion of new amino acids at conveniently located restriction sites. This is limited by the availability of restriction sites, and also is more likely to disrupt the overall integrity of the molecule. The πVX system relies on frequent recombination events to generate hybrid sequences. Thus only changes at pre-existing polymorphic residues can be sampled. Although the polymorphism in MHC genes is high, this still only allows 10-20% of the residues to be analyzed. Indeed, our initial functional results have shown a conserved residue to be critical in allogeneic T cell recognition (17). Chemical mutagenesis requires the independent use of different chemicals on the same sequence to avoid bias. This requires multiple independent reactions and libraries. As our functional results have shown Tyr 27 to be important, we are now currently engaged in mutating the adjoining amino acids residues around this site. We have used saturation mutagenesis as...
an initial approach to scan regions of a large protein domain. We can now concentrate on a critical region in greater detail to determine if, for example, Tyr 27 is uniquely important or is part of a T cell epitope which extends into neighboring amino acids.

The functional analyses reported here show that the epitope for mAb 7-16.10 is not significantly altered. The crystal structure reported recently for HLA.A2 (25,26) allows for interpretation of the three dimensional structure with reference to functional or non-functional alterations in mutant molecules. We assume conservation between human and mouse molecules. Mutations Y27N, E32Q, and A11V would probably be inaccessible to direct interaction with antibodies. D37H, R14L, and P57Q would be more likely to encode changes detectable by antibodies. However, the mAb 7-16.10 has no substantial change in its ability to bind the D37H or R14L mutant molecules, but may bind the P57Q mutation at a slightly lower level. P57Q occurs at an interesting place in the molecule. This Pro residue is at a break in an α helix in a very accessible portion of the molecule, and is currently under further analysis.

We believe that the technique of saturation mutagenesis represents an important and useful approach towards understanding protein structure and function, and provides an important alternative to linker scanning mutations or the πVX system for examining large protein domains. This methodology allows for the generation of a spectrum of mutations in proteins domains which may have previously been unapproachable to detailed structure and function analysis. It can be coupled with the newer techniques of mutant recovery used in vectors which produce single strand DNA(27), and allow the recovery of mutants directly.

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