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Restriction generated oligonucleotides utilizing the two base recognition endonuclease *Cvi*JI*

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ABSTRACT

The conversion of an anonymous DNA sample into numerous oligonucleotides is enzymatically feasible using an unusual restriction endonuclease, *Cvi*JI. Depending on reaction conditions, *Cvi*JI is capable of digesting DNA at a two or three base recognition sequence. *Cvi*JI normally cleaves RGCY sites between the G and C to leave blunt ends. Under 'relaxed' conditions *Cvi*JI* cleaves RGCY, and RGCR/YGCY, but not YGCR sites. In theory, *Cvi*JI* restriction of pUC19 (2686 bp) should produce 157 fragments, 75% of which are smaller than 20 bp. Instead, 96% of the *Cvi*JI* fragments were 18–56 bp long and none of the fragments were smaller than 18 bp. Thermal denaturation of these fragments generates sequence specific oligonucleotides homologous for the cognate template. The enzymatic conversion of anonymous DNA into sequence specific oligomers has implications for several conventional and novel molecular biology procedures.

INTRODUCTION

Oligonucleotides are essential tools in many molecular biology applications, including DNA sequencing, polymerase chain reaction (PCR) and other forms of nucleic acid amplification, mutagenesis, nucleic acid capture and enrichment techniques, as probes in the detection and isolation of genes, and as aids for cloning DNA. The development of chemical methods for synthesizing oligonucleotides 2–200 bases in length has accelerated the evolution of modern molecular genetics (1). Single oligonucleotides of defined sequence can be chemically synthesized and purified within a day. The synthesis of such oligomers obviously requires prior knowledge of the nucleic acid sequence.

The ability to enzymatically produce oligonucleotides directly from a DNA sample, without prior sequence information, would circumvent the need for chemically synthesized oligomers in certain applications. An important consideration with any enzymatic method for producing oligonucleotides is the length of such polymers. If for example, an oligonucleotide is to be used with a large genome, it has to be long enough so that the sequence has a probability of occurring only once in the genome. This minimum length has been calculated to be 17 nucleotides for the human genome (2). Oligonucleotides used for sequencing or PCR amplification are generally 18–30 bases long. Shorter oligonucleotides can bind at multiple positions, even with small genomes, and thus generate spurious extension products. An ideal enzymatic method for generating oligomers should thus produce polymers greater than 17 bases in length.

At present, there is no reliable method for the enzymatic conversion of DNA into oligonucleotides 18–60 bases long, a useful size range for most molecular biology applications. Complete digestion with known deoxyribonucleases reduce DNA to mononucleotides or short oligomers of 2–6 bases (3). Partial DNAse I digests are used for randomly fragmenting large DNAs into a size suitable for shotgun cloning (4). Precisely timed partial DNAse I digests produce fragments of the appropriate size range for generating oligonucleotides after a thermal denaturation step. However, DNAse I partial digests require large amounts of material to empirically determine the appropriate incubation time. In addition, DNAse I is variable in its digestion rate, is sensitive to trace contaminants, and requires recalibration with new batches of enzyme (3). Four base recognition endonucleases cleave DNA into fragments which are several hundred to several thousand base pairs (bp) long. The use of multiple four base recognition endonucleases infrequently produces oligonucleotide sized fragments.

As an alternative to these methods, we reasoned that complete digestion with a two or three base restriction endonuclease would cleave DNA frequently enough to generate oligonucleotides after a thermal denaturation step. *Chlorella* virus IL-3A encodes the restriction endonuclease *Cvi*JI, which is capable of cleaving DNA at a two or three base recognition sequence depending on the reaction conditions. *Cvi*JI normally recognizes RGCY (R, Purine; Y, Pyrimidine) sequences and cleaves between the G and C to leave blunt ends (5). In the presence of ATP the enzyme cleaves DNA more frequently than the conventional reaction, an activity refered to as *Cvi*JI* (5, 6). The work described here characterizes the recognition sequence and restriction generated oligonucleotides resulting from *Cvi*JI* digestion. In one

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application, end-labeled *Cvi*JI* restriction generated oligonucleotides are ten fold more sensitive in detection assays compared to a conventional oligonucleotide probe. Additional applications which exploit the two/three base restriction capabilities of *Cvi*JI are also mentioned.

MATERIALS AND METHODS

Reagents

Restriction and modification enzymes were obtained from Molecular Biology Resources (Milwaukee, WI). The Minute Miniprep DNA Purification System, Sequal DNA Sequencing System, and competent *E.coli* cells are products of CHIMERx (Madison, WI). The 100 bp and 10 bp ladder molecular weight markers and lambda DNA were obtained from Life Technologies (Bethesda, MD). Ampicillin, X-GAL, IPTG, XOMAT AR X-ray film, and genomic DNA from *Clostridium perfringens*, *Micrococcus luteus*, and human placenta were from Sigma (St. Louis, MO). Hybond-N nylon membranes were obtained from Amersham (Arlington Heights, IL). [γ-^{32}P]ATP was purchased from Du Pont (Boston, MA).

*Cvi*JI restriction of DNA

One μg of DNA was digested under *Cvi*JI conditions (10 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-HCl, pH 8.0) or *Cvi*JI* conditions (1 mM ATP, 20 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris−HCl, pH 8.0) at 37°C for 1 hr. High resolution analysis of these digests was achieved by electrophoresis on 2.0−2.8% agarose gels in 90 mM Tris−HCl, 90 mM boric acid, 2 mM EDTA, pH 8.0 (7).

Restriction site analysis of *Cvi*JI* digested pUC19

One μg of pUC19 DNA was digested to completion with *Cvi*JI* as specified above. The enzyme was heat denatured (65°C for 15 min) and the 5′ terminal phosphate groups were removed using calf intestinal alkaline phosphatase to prevent cloning multiple inserts. This material was combined with *Eco*RV digested M13Nano1 (an M13mp18 cloning derivative), ligated for 3 hr with T4 DNA ligase, and transformed into competent *E.coli* DH5α F' cells (8). The cells were plated on T top agar containing X-GAL and IPTG (9). White plaques were randomly picked and grown overnight in 2 ml of 2× TY broth. After centrifugation to remove cells, single stranded phage DNA was purified and sequenced by the dideoxy chain termination method (10) using a radiolabeled 'universal' primer. The entire insert was sequenced from 100 clones, yielding 200 *Cvi*JI* cleavage junctions and thus 200 recognition sites.

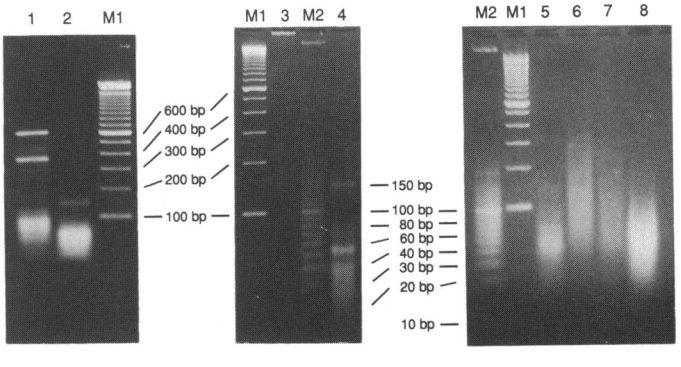

Figure 1. *Cvi*JI restriction digests of various DNAs. The first panel is a low resolution agarose gel (1% agarose and TAE buffer) whereas the last two panels are high resolution agarose gels (2.5% agarose and TBE buffer). Lane M1: 100 bp DNA ladder, lane M2: 10 bp DNA ladder, lane 1: *Cvi*JI digest of pUC19, lane 2: *Cvi*JI* digest of pUC19, lane 3: supercoiled pUC19 (0.25 μg), lane 4: *Cvi*JI* digest of pUC19, lane 5: *Cvi*JI* digest of bacteriophage lambda, lane 6: *Cvi*JI* digest of *Clostridium perfringens*, lane 7: *Cvi*JI* digest of human placenta DNA, lane 8: *Cvi*JI* digest of *Micrococcus luteus*. Except where indicated, the amount of DNA per sample is 1.0 μg.

*Cvi*JI* restriction generated oligonucleotide probes

pUC19 DNA was cleaved with *Cvi*JI* and dephosphorylated as described above. These fragments were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (3) and heat denatured at 100°C for 2 min. A 24mer sequencing primer (universal forward pUC/M13) was also end-labeled. The detection sensitivity of these probes were compared using duplicate Southern blots (11) containing known concentrations of *Eco*RI linearized pUC19 transferred to Hybond-N nylon membranes. The methods for preparing Southern blots, probe hybridization and washing can be found in (3).

RESULTS

CviJI* restriction digests

The restriction endonuclease *Cvi*JI recognizes the sequence RGCY and cleaves between the G and C to leave blunt ends (5). Under 'relaxed' conditions (in the presence of 1 mM ATP) *Cvi*JI cleaves DNA more frequently than the 'normal' activity. This relaxed activity is called *Cvi*JI*, for star or altered specificity. Previously we inferred that *Cvi*JI* might recognize the dinucleotide sequence GC (6). *Cvi*JI* restriction reduces most DNAs to small fragments 20−200 bp in length, regardless of the size or nucleotide composition of the sample. As seen in Fig. 1 (lanes 5−8), DNA isolated from bacteriophage lambda (48,514 bp, 48.6% GC), *Clostridium perfringens* (3.6 megabase pairs, 26.5% GC), human placenta (3.2 gigabase pairs, 42% GC), and *Micrococcus luteus* (3.2 megabase pairs, 72% GC), are all reduced to small fragments approximately 20−200 bp in size (lanes 5−8). Even small DNAs, such as pUC19 (2686 bp, 49.7% GC), are reduced to numerous small fragments by *Cvi*JI* cleavage (Fig.1, lanes 2, 4). As expected, *Cvi*JI* and *Cvi*JI* does not cleave DNA with certain modifications, such as the RG^{m5}CY modified genome of virus IL-3A (12) or the glycosylated genome of bacteriophage T4 (data not shown).

The small plasmid pUC19, which contains 45 RGCY sites and 205 GC sites (13), was chosen to analyze *Cvi*JI* cleavage sites in detail. The pUC19 DNA sequence predicts that *Cvi*JI* digestion should result in fragments primarily 40−120 bp in length; this prediction was confirmed experimentally (Fig. 1, lane 1). The size range of DNA fragments produced by *Cvi*JI* digestion of pUC19 is primarily 20−60 bp in length (Fig. 1, lanes 2 and 4) as assayed by agarose gel electrophoresis (longer incubation times or the addition of more enzyme did not alter the appearance of this banding pattern, data not shown). This size range is larger than expected, as some 70% of the pUC19 *Cvi*JI* fragments are predicted to fall in the 2−20 bp range (Fig. 2A). Sequence analysis of 100 cloned *Cvi*JI* fragments from pUC19 established that 96% of these fragments were 18−56 bp long (Fig. 2B). This result indicates that *Cvi*JI* either does not recognize all GC sites, does not restrict all GC sites, or both. A detailed analysis of *Cvi*JI* restriction follows.

*Cvi*JI* recognition sequence

The cleavage site for a restriction endonuclease is usually determined by the primed synthesis method (14), which compares an end-labeled primer sequencing ladder alongside the same end-labeled primer synthesis reaction which has been digested by the enzyme. Because there are 16 possible NGCN (N = A or C or G or T) recognition sites, this approach was not practical. Instead, the recognition sequence was deduced by cloning and sequencing the entire insert from 100 clones containing *Cvi*JI* restricted pUC19 DNA fragments. This effort produced 2 such sites per fragment, or 200 junctions, the data for which is compiled in Table 1.

The NGCN sites in pUC19 can be divided into four classes based on their flanking R/Y nucleotides, the normal recognition sequence and three potential classes of relaxed sites (R1 = YGCY, R2 = RGCR, R3 = YGCR). The fraction of NGCN sites which belong to each classification in pUC19 is roughly equal (22.0%–27.8%, Table 1). If *Cvi*JI* cleaves all NGCN sites without sequence or strand preferences, the fraction of each classification should be equally represented. Instead, the majority of sites cleaved are normal, or RGCY sites (47.5%). R1 and R2 cleavages occured at nearly the same frequencies (25.5% and 27.0%, respectively). Out of 200 *Cvi*JI* junctions, no R3 restricted sites were found. Thus, *Cvi*JI* is capable of cleaving all NGCN sites except for YGCR, for a total of 157 sites in pUC19. Since *Cvi*JI* only cleaves 12 out of the 16 possible NGCN sites it is not a true two base recognition endonuclease. The frequent cutting activity of *Cvi*JI* is referred to as 'two base' for descriptive purposes only.

A total of 116 internal non-cleaved NGCN sites were present in the 100 sequenced fragments examined (Table 1). YGCR sites, which are not a substrate for *Cvi*JI*, represented the largest class of non-cleaved sites (52.6%). Only two RGCY sites were not cleaved (1.7%), indicating a strong preference for the normal recognition site. An approximately equal fraction of R1 and R2 sites were not cleaved (22.4% versus 23.3%), which is also similar to the fraction of these sites which were cleaved (see above). This data indicates that *Cvi*JI* does not discriminate between R1 and R2 sites, which is not surprising as RGCR is the compliment of YGCY. As *Cvi*JI* cleavage does not appear to distinguish between a 'top' or 'bottom' strand the data from R1 and R2 sites can be combined into a single classification. Thus, R1 plus R2 cleavage occured in 52.5% of the cases, which is similar to the 47.5% normal site cleavage. Non-cleavage of R1 plus R2 sites occured 45.7% of the time, considerably more frequent than the non-cleavage of normal sites. Based on this frequency of cleavage, an efficiency of restriction under *Cvi*JI* conditions is evident, where RGCY > RGCR/YGCY. A numerical value for *Cvi*JI* restriction efficiency can be calulated by dividing the non-cleaved sites by the cleaved sites and subtracting from one. The efficiency of normal *Cvi*JI* cleavage is 97.9% ([1−2/95] ×100) and R1 plus R2 cleavage is 49.5% ([1−53/105] ×100).

CviJI* is capable of cleaving RGCY and RGCR/YGCY sites, but does not cleave all such sites (2 RGCY and 53 RGCR/YGCY internal sites were found intact). There are several possible

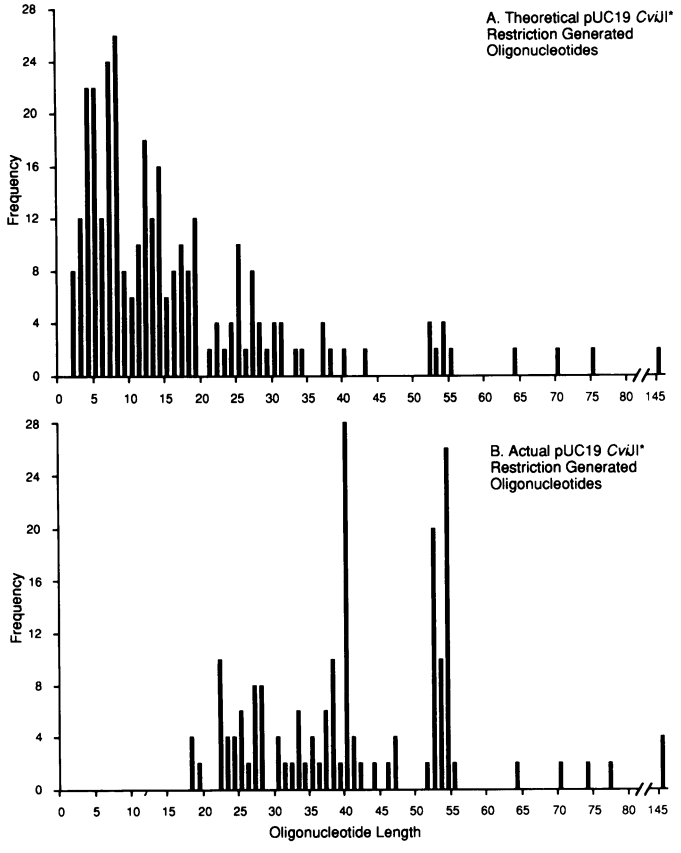

Figure 2. Frequency and distribution of *Cvi*JI* restriction generated oligomers. **A.** The theoretical distribution of *Cvi*JI* restriction generated oligomers was calculated from the expected number of RGCY, RGCR, and YGCY pUC19 DNA fragments multiplied by 2 (2 oligomers per fragment) for each size class. **B.** The experimentally determined distribution of pUC19 *Cvi*JI* restriction generated oligomers was calculated from the same data presented in Table 1, where the DNA fragments found by cloning and sequencing were multiplied by 2.

Table 1. Distribution of pUC19 *Cvi*JI* sites as determined by cloning and sequencing.

Classification Group	R/Y Structure	NGCN Sites Found in pUC19 (%)		CviJI* Sites Cleaved (%)	CviJI* Sites Not Cleaved (%)
Normal	RRYY	AGCC	9 (4.4)	23 (11.5)	1 (0.9)
		GGCC	11 (5.4)	24 (12.0)	1 (0.9)
		GGCT	10 (4.9)	13 (6.5)	0 (0.0)
		AGCT	15 (7.3)	35 (17.5)	0 (0.0)
			45 (22.0)	95 (47.5)	2 (1.7)
Relaxed (R1)	YRYY	CGCC	11 (5.4)	11 (5.5)	4 (3.5)
		TGCC	12 (5.9)	13 (6.5)	10 (8.6)
		TGCT	10 (4.9)	10 (5.0)	5 (4.3)
		CGCT	22 (10.7)	17 (8.5)	7 (6.0)
			55 (26.9)	51 (25.5)	26 (22.4)
Relaxed (R2)	RRYR	AGCA	16 (7.3)	13 (6.5)	5 (4.3)
		GGCA	8 (3.9)	11 (5.5)	3 (2.6)
		AGCG	11 (5.4)	12 (6.0)	11 (9.5)
		GGCG	22 (10.7)	18 (9.0)	8 (6.9)
			57 (27.8)	54 (27.0)	27 (23.3)
Relaxed (R3)	YRYR	CGCA	10 (4.9)	0	12 (10.4)
		TGCA	13 (6.3)	0	19 (16.4)
		CGCG	10 (4.9)	0	27 (23.3)
		TGCG	15 (7.3)	0	3 (2.6)
			48 (23.4)	0	61 (52.6)
		Total	205	200	116

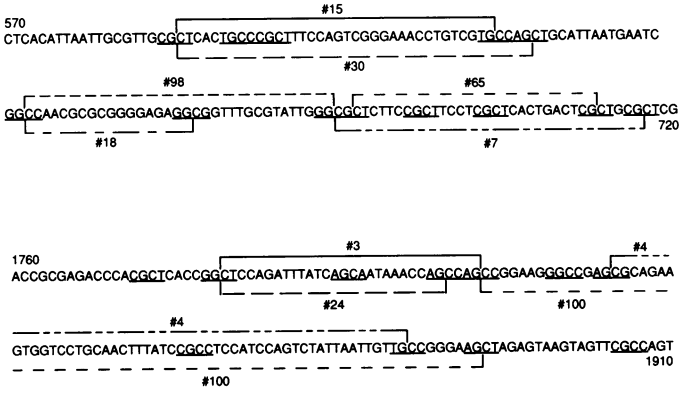

Figure 3. Sequence of 10 restriction fragments resulting from *Cvi*JI* cleavage. The sequence of pUC19 from two locations (bases 570–720 and 1760–1910) is shown. *Cvi*JI* sites are underlined. The numbered brackets indicate 10 DNA fragments out of the 100 determined by sequence analysis.

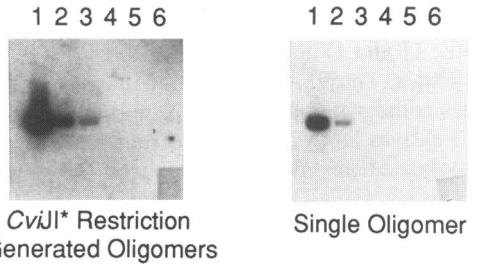

Figure 4. Detection sensitivity of *Cvi*JI* restriction generated oligomers compared to a single synthetic oligonucleotide. Known amounts of *Eco*RI linearized pUC19 were fractionated on a duplicate 0.7% agarose gel and transferred to a nylon membrane. ^{32}P end-labeled *Cvi*JI* oligonucleotides (0.2 pmoles) or a single synthetic oligonucleotide (10 pmoles of 24mer universal sequencing primer) were used to probe the Southern blots. Both Southern blots were exposed for two days on a single X-ray film. Amount of linearized pUC19/lane: Lane 1 = 500 attomoles (886 pg), Lane 2 = 50 attomoles (88.6 pg), Lane 3 = 5 attomoles (8.9 pg), Lane 4 = 1 attomole (1.8 pg), Lane 5 = 0.5 attomoles (0.9 pg), Lane 6 = 0.25 attomoles (0.4 pg).

explanations for this lack of complete restriction. The indigenous *E.coli* DNA methyl-transferase *dcm* could inhibit cleavage as the recognition sequence C^{m5}CWGG partially overlaps with that of *Cvi*JI* (15). However, this overlap could only affect two *Cvi*JI* sites in pUC19, at positions 544 and 832, one of which (832) was cleaved in the pool of 100 clones. One possibility is that *Cvi*JI* has an unusually large preferential cleavage rate such that some sites are never cleaved. *Nar*I is an enzyme that does not cleave all possible recognition sites (16). The simplest explanation for non-restriction at some *Cvi*JI* sites is that the sites are too near the ends of the fragment. Inspection of the DNA sequence from tn *Cvi*JI* cleaved fragments provides some evidence for this possibility (Fig. 3). Apparently, when two or more recognition sites are close together, hydrolysis of the first site precludes cleavage of its nearest neighbors. A number of restriction endonucleases fail to cleave recognition sites close to the ends of a fragment (17–19). Presumably, non-restriction at some *Cvi*JI* sites results from such 'end effects'. The length of the DNA fragment could also influence cleavage of internal sites, either directly or indirectly, by influencing the enzyme's ability to bind or bend such small fragments. Complete *Cvi*JI* restriction may depend on a combination of two factors, fragment length and distance from the end.

*Cvi*JI* restriction generated oligonucleotides

The high frequency of *Cvi*JI or *Cvi*JI* cleavage should generate numerous small oligonucleotides after a heat denaturation step. The theoretical number of pUC19 *Cvi*JI* restriction generated oligomers is 314 (157 *Cvi*JI* restriction fragments×2 oligomers/fragment), the size distribution of which is shown in Fig. 2A. Assuming that *Cvi*JI* is capable of cleaving DNA into fragments as small as 2 bp, most of the expected *Cvi*JI* restriction generated oligomers (75%) are 2–20 bases in length. In practice, 96% of the cloned *Cvi*JI* fragments were 18–56 bp in size, and none of the 100 fragments analyzed were smaller than 18 bp (Fig. 2B). Apparently *Cvi*JI* does not cleave DNA fragments below a certain threshold length. Since the smallest observed fragment was 18 bp, we assumed that this is the minimal size which can be cleaved. Whatever the reason for this phenomenon, *Cvi*JI* digestion of DNA produces a relatively small size range of oligomers (mostly 18–56 bases in length), the majority of which are a perfect size class for certain molecular biology applications. It should be noted that the size distribution of the cloned pUC19 *Cvi*JI* fragments reported in Fig. 2B agrees with the ethidium bromide staining intensities found on the agarose gel presented in Fig. 1 (lane 4), indicating that the data is not skewed by cloning or gel anomalies.

Detection sensitivity of *Cvi*JI* restriction generated oligonucleotide probes

*Cvi*JI* restriction generated oligonucleotides can be used as probes for nucleic acid hybridization assays. The release of tens to hundreds of oligomers per template DNA provides a form of signal amplification that should improve the detection sensitivity of hybridization assays when compared to a single synthetic oligonucleotide probe. The detection sensitivity of γ-^{32}P end-labeled *Cvi*JI* restriction generated oligomers prepared from pUC19 was compared to an end-labeled synthetic oligonucleotide (24mer universal sequencing primer). Both probes were used to detect identical Southern blots containing linearized pUC19 transferred onto a nylon membrane (Fig. 5). In autoradiograms exposed for 48 hr, the detection sensitivity of ^{32}P end-labeled *Cvi*JI* generated oligomers was 5 attomoles (8.8 pg, 3×10^7 molecules) of target (Fig. 4, lane 3). In contrast, the detection sensitivity of a single ^{32}P end-labeled synthetic oligonucleotide was 50 attomoles (88.8 pg, 3×10^8 molecules) of target (Fig. 4, lane 2), which is 10 fold less sensitive than the multiple oligomers resulting from *Cvi*JI* digestion. Repetition of this comparison confirmed that the ten fold difference was consistent (data not shown).

DISCUSSION

*Cvi*JI is unique among restriction endonucleases in that it cleaves DNA more frequently than other enzymes. Under normal conditions *Cvi*JI recognizes the sequence RGCY and cleaves between the G and C to leave blunt ends (5). The specificity of *Cvi*JI is 'relaxed' to cleave DNA even more frequently by including ATP in the reaction. Sequence analysis of cloned *Cvi*JI*

restricted fragments demonstrates that the enzyme cleaves between the G and C at RGCY, and RGCR/YGCY sites, but not YGCR sites. Thus the star condition allows one, but not two, mismatches in the outer positions of the normal site. Neither *Cvi*JI nor *Cvi*JI* cleaves DNA containing certain modified bases, such as the glycosylated genome of bacteriophage T4 or the methylated sequences (G/A)G^{m5}C(T/C/G) present in the IL-3A viral genome (12). The relaxation of endonuclease specificity, also known as 'star' (*) activity, is usually associated with modified solvent conditions, such as high glycerol concentration, low ionic strength, high pH, the presence of organic solvents, high ratio of enzyme to DNA, or the substitution of Mg^{2+} with other divalent cations (20, 21). *Cvi*JI* activity is not observed under these conditions (5). *Cvi*JI* activity requires Mg^{2+} and ATP and is stimulated by S-adenosylmethionine, a property which is characteristic of Type III restriction enzymes. A Type II restriction endonuclease with these properties has not been previously described.

Most dsDNAs are cleaved to small 20–200 bp fragments by *Cvi*JI*, regardless of the size or nucleotide composition of the DNA. In addition, *Cvi*JI* cleaves single stranded DNAs such as M13mp18, although the size distribution of these digests have not been characterized (data not shown). Detailed analysis of 100 cloned *Cvi*JI* cleaved fragments from pUC19 revealed that: (i) *Cvi*JI* cleavage is biased in that RGCY sites are almost always cleaved and RGCR/YGCY sites have an approximately equal chance of being cleaved or not cleaved. (ii) DNA fragments shorter than 18 bp are apparently not cleaved, even though they may contain restriction sensitive sites.

Depending on the application, there are advantages and disadvantages to this non-cleavage of restriction sites. A potential disadvantage is that certain RGCR/YGCY sites remain uncleaved. Thus, the use of the star reaction for high resolution restriction analysis of short DNA fragments, such as PCR products, will require an empirical approach to determine if a given site is or is not cleaved. Non-restriction at some *Cvi*JI* sites can also result in DNA fragments with overlapping nucleotides (see for example Fig. 3). Sixteen out of 100 fragments contained overlapping bases with another restriction fragment. The generation of overlapping fragments could make it difficult to predict which fragment contains the sequence of interest without additional analysis. Another consequence of non-restriction at some *Cvi*JI* sites is the inability to predict the exact number of fragments produced by *Cvi*JI* treatment. In theory, 157 pUC19 *Cvi*JI* restriction fragments are expected. The data indicate that approximately half of the RGCR/YGCY sites are not cleaved. Using these numbers as an approximate guideline, 102 pUC19 *Cvi*JI* restriction fragments are predicted. Even though *Cvi*JI* can recognize and cleave all NGCN sites except YGCR, its inability to restrict all such sites results in DNA fragments and oligomers of a size range which are useful in several molecular biology applications.

*Cvi*JI* digestion of anonymous DNA produces a large number of oligonucleotide sized polymers upon thermal denaturation, a method which is not practical using organic synthesis or other classes of endo- or exonucleases. The specificity of these oligomers can potentially be exploited in applications such as labeling, detection, amplification, cloning, and capture of nucleic acids. In general, oligonucleotides shorter than 17 bases are too small for applications such as sequencing, PCR, mutagenesis, and other primer extension events. *Cvi*JI* is an ideal enzyme for generating oligomers because it produces polymers greater than 17 bases long.

Several molecular biology applications which exploit the two/three base restriction activity of *Cvi*JI are feasible. The enzymatic conversion of long DNA sequences into tens or hundreds of sequence specific oligomers provides a form of signal amplification that improves the detection sensitivity of hybridization assays 10 fold over a single synthetic oligonucleotide probe. The detection sensitivity of these restriction generated fragments can be improved an additional 10 to 100 fold by labeling methods which attach multiple signals per oligonucleotide. Combining *Cvi*JI* restriction generated oligomers, a thermostable DNA polymerase, and labeled nucleotides in repeated cycles of denaturation, annealing, and extension, results in a very large amplification of labeled probes. This novel labeling scheme, termed thermal cycle labeling, permits detection sensitivities as low as 25 zeptomoles (manuscript in preparation).

*Cvi*JI* restriction generated oligomers can be used as anonymous primers for large scale mapping or sequencing projects. This strategy reduces large DNAs to small fragments, which are cloned in a special vector to permit their excision as single primers for sequencing or PCR (manuscript in preparation). This anonymous primer sequencing strategy can be useful in the initial stages of certain genomic sequencing schemes (22). Alternatively, the cloned anonymous primers represent a natural pool of genome specific DNA from which a particular function or structure could be enriched.

*Cvi*JI* partial digests can also be used to improve several molecular biology applications, such as shotgun cloning for sequencing (6), epitope mapping or panning, and construction of gene libraries. Quasi-random shotgun libraries were generated from nanogram amounts of DNA utilizing partial *Cvi*JI* reaction conditions. The two/three base restriction activity of *Cvi*JI* cleaves DNA frequently enough to be functionally random with respect to the rate at which sequence data can be accumulated from such a quasi-random approach (6). The size distribution of these quasi-random fragments can be controlled by the amount of enzyme added and the length of incubation, so that smaller or larger DNA fragments can be used for gene expression, epitope mapping or panning strategies.

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