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Investigating Whether NdeI-Cut Ends are Available to Incorporate Deoxynucleotides

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Numerous experiments have indicated that *Nde*I-digested fragments have low ligation efficiency. A proposed explanation for this behaviour was that the 3' hydroxyl end is unavailable to incorporate additional nucleotides. That experiment labeled the ends of *Nde*I-digested fragments with biotin; however, the results raised the question of whether the procedure was size-dependent or if it provided support for the hypothesis. To try and answer the question, two ladders were created from λ DNA – one cleaved with *Hind*III and the other with *Vsp*I. Both ladders were subsequently labeled with biotin. pBR322 was also used to generate larger fragments if the process was indeed size-dependent. The plasmid was digested with both *Hind*III and *Nde*I, labeled with biotin, and then the fragments were digested again with *Vsp*I and *Sal*I before it was run on a 2% agarose gel. Western blot analysis was conducted after transferring segmented DNA bands to a nitrocellulose membrane by Southern blotting. The agarose gel showed that there were equal amounts of *Nde*I-digested fragments and *Hind*III-digested fragments; however, the IDV values indicated that there were more *Hind*III-digested fragments labeled with biotin than *Nde*I-cut segments. This pattern supports the hypothesis that *Nde*I-cut ends were unavailable to incorporate deoxynucleotides. Additionally, the autoradiograph film indicated that only the higher molecular weight bands of the two ladders were successfully labeled with biotin. As the lower molecular weight bands of the double digest were seen in the film, it may be possible that a limited amount of biotin was present during the labeling process. Another possibility for the absence of the lower bands on the film may be that the biotin had detached from the fragment ends during the Southern blot.

Cloning is used to generate information about an organism's genome. To study a particular gene, restriction enzymes cleave the DNA and vector at specific sites. Ligase then adheres the complementary ends of the fragment containing the gene of interest and the open ends of the vector by creating a phosphodiester bond between the 3' hydroxyl and the 5' phosphate end (3). In this process, choosing the right restriction enzyme is vital for obtaining the correct fragment of interest. Each restriction enzyme recognizes a short unique sequence in the DNA, and makes either staggered or blunt cuts at the site. Ligation of these fragments into vectors has been shown to vary depending on the type of end the enzyme creates. According to Bola (1), longer overhangs were observed to have better ligation efficiency than blunt ends. Furthermore, it was found that G, C-rich overhangs have better ligation efficiency than those that were A, T-rich (1).

Type II restriction endonucleases are enzymes that require Mg^{2+} to cleave specific DNA sequences that may span 4-8 base pairs long (4). Their chief biological role in the cell is to protect the host from foreign DNA, especially bacteriophage (4). In previous experiments, *Nde*I-digested fragments have shown to have low ligation efficiency compared to the *Hind*III restriction enzyme. *Nde*I, a type II restriction enzyme isolated from *Neisseria denitrificans*, cleaves and creates an overhang that is A, T-rich (Fig. 1). However, this enzyme showed lower ligation efficiency than a restriction enzyme that created A, T-rich blunt ends (1). The 3' end was thought to possibly have been distorted after cleavage, rendering the ends of the fragment unavailable for the ligation step (2). Thus, in a previous experiment, the recessed 3' ends of the digested fragments were labeled with biotinylated-14-dATP, and then a western blot was conducted to see if the biotin marker was efficiently incorporated (2). However, the procedure showed an inconsistent intensity between the different bands visible in the autoradiograph film,

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5′ – C A•T A T G – 3′
3′ – G T A T•A C – 5′
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FIG. 1 The restriction site *Nde*I recognizes and cleaves in the genome, leaving A, T-rich overhangs.

raising the question whether limited labeling was due to differences in size-dependent binding or if the *Nde*I ends were distorted by cleavage.

Here, the project determined whether the binding of the biotin-labeled fragments was size-dependent by creating two self-made ladders with λ DNA: one was cleaved with *Hind*III and one with *Vsp*I. The overhangs were subsequently labeled with biotinylated-14-dATP. Furthermore, pBR322 plasmid was used as another assessment of size limitation as cleavage of pBR322 with *Hind*III and *Nde*I generated larger fragments than cleavage of pUC19 with the same two enzymes. If the smaller fragments of the ladder could be labeled and detected on the autoradiography film, size limitation would not be a problem in the procedure and Choi's results (2) would support the hypothesis that *Nde*I distorts the DNA when it cleaves at the restriction site.

MATERIALS AND METHODS

Digestion of λ DNA by restriction enzymes to produce two self-made ladders. Two microfuge tubes consisting of 5 ug of λ DNA (Invitrogen, Cat. 25250-010) each were digested for 2 hours at 37ºC in a reaction volume of 30 ul, one with 5 units (U) of *Vsp*I (Fermentas, Cat. #ER0911) and the other with 5 U of *Hind*III (Fermentas, Cat. #ER0501). Afterwards, the self-made ladders were run on a 2% agarose (Amresco Agarose 3:1, Cat. E776-250G) gel to ensure that complete digestion occurred. Then, the reaction was heat inactivated at 65ºC for 20 minutes.

Labeling λ DNA fragments. 2 ug of digested λ DNA was then labeled with biotin-14-dATP (Invitrogen, Cat. 19524-016). The reaction used 100 uM of each biotin-14-dATP, dGTP, dTTP, and dCTP (Invitrogen), and 2 U of Klenow large fragment DNA polymerase (Invitrogen, Cat. 18012-021) in 1X React2 Buffer (Invitrogen, Cat. Y92500) in a total volume of 30 ul. Samples were left at room temperature for 15 minutes. The reaction was then stopped with phenol extraction. For phenol extraction, 100 ul of water and 130 ul of phenol were added to 30 ul of biotinylated sample. The sample was mixed, and centrifuged at 7500 x g (Eppendorf Centrifuge 5415D) for 1 minute. The aqueous layer was extracted for ethanol precipitation. 8 uL of 3 M sodium acetate and 150 ul of cold 100% ethanol was added to the sample, mixed, and left at -80ºC for 1 hour. Afterwards, the sample was spun at 16,000 x g for 15 minutes, air dried, and resuspended in 20 ul of sterile distilled water.

Creating Fragments from pBR322. 5 ug of pBR322 (Fermentas, Cat. #SD0041) was digested with 5 units of *Hind*III (Fermentas) and 5 units of *Nde*I (Fermentas) each in 2x Tango buffer (Fermentas, Cat. #BY5) in a reaction volume of 30 ul. Sample was incubated at 37ºC for 2 hours and heat inactivated by heating to 65ºC for 20 minutes. Products were run on a 2 % agarose (Amresco) gel to ensure that complete digestion occurred.

Labeling *Nde*I/*Hind*III fragments. 2 ug of pBR322 (Fermentas) that was digested with *Nde*I (Fermentas) and *Hind*III (Fermentas) as described above was labeled and purified in the same manner as dictated for the λ DNA fragments above. Afterwards, the fragments were digested with 5 U of *Vsp*I (Fermentas) for 2 hours at 37ºC in Orange (O) buffer (Fermentas, Cat. #BO5), and then inactivated at 65ºC for 20 minutes. 50 mM of Tris-HCl and 50 mM of NaCl were added to the products to change the buffer salt concentration, and then the fragments underwent a digestion with *Sal*I (Gibco BRL, Cat. 5217SA) for 2 hours at 37ºC. The sample was heat inactivated at 65ºC for 20 minutes. Figure 2 shows the fragments the restriction enzymes would produce on pBR322.

Blotting. All the samples and a GeneRuler 100 bp Plus DNA ladder (Fermentas, Cat. #SM0321) were then run in a 50 ml 2% agarose (Amresco agarose 3:1, 9012-36-6) gel. The gel was run at 47 V and 20 mA for 4 hours. The gel was subsequently stained by soaking for 60 minutes in 1 ug/mL of ethidium bromide and visualized. The gel was soaked in a denaturing solution of 1.5 M NaCl and 0.5 M NaOH for 30 minutes at room temperature, and then soaked in neutralizing solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5) for 30 minutes. It was then blotted overnight to transfer the fragments from the gel to nitrocellulose membrane (BioRad 162-0232) in 20X SSC (3.0 M NaCl, 6.3 M sodium citrate, pH 7.0). The membrane was allowed to air dry at room temperature for 5 minutes. Then 1.5 ul of the positive control (biotinylated primer: Eub338B, #24018466) was spotted onto the membrane. The membrane was then baked at 80ºC for 2 hours.

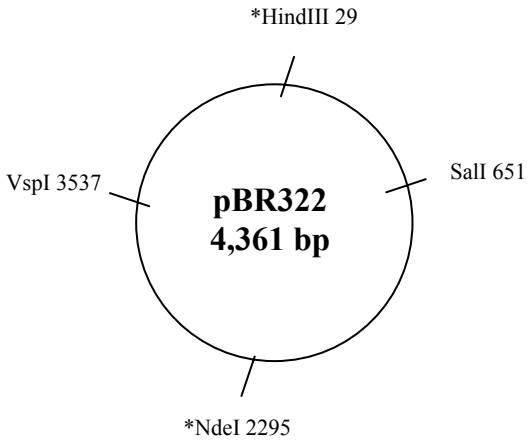

FIG. 2 The sites where the restriction enzymes used in the experiment cleaved on pBR322. (*) indicates the sites that were biotin-labeled.

Detection. The membrane was blocked in 1X blocking solution (Roche, Cat. 11921673001) diluted in 1X TBS-T, which contains 7.88 g of TrisHCl, 8.76 g NaCl and 0.05% Tween 20 in 1 L total volume at pH 7.5 for 1 hour. Then the membrane was incubated at room temperature with streptavidin-HRP (BioRad) at 1:2000 dilution in 0.5X blocking solution. The membrane was then washed with 1X TBS-T three times at 10 minute intervals. The lumilight plus substrate (Roche, Cat. 12015200001) was added to the membrane and incubated for 1 minute and then exposed to film for 15 seconds.

Quantitation with the AlphaImager System. Please refer to 1.4 AlphaImager System Quick Guide of *Alpha Innotech User's Manual*, version 4, pages 1-8 to 1-9. Integrated density values (IDV) were used to quantify the pixels in the bands of the gel and autoradiograph film.

RESULTS

Biotin-labeled ladders. Two ladders were made from λ DNA, one cleaved with *Hind*III and one with *Vsp*I. *Hind*III acted as the positive control as it was previously shown that its ends can be labeled with biotin. *Vsp*I generated a wide range of fragment sizes that was necessary to test if the biotin-labeling method was size-dependent. Table 1 shows the fragment sizes of the two ladders in descending order and the integrated density value (IDV) of the fragments that showed up on the film. As can be seen, very few fragments of each ladder were successfully labeled with biotin. Most of the bands labeled in the experiment were high molecular weight fragments and there was a

Table 1. Correlation of size and biotin-labeling efficiency for DNA fragments generated from λ DNA by *Hind*III and *Vsp*I.

HindIII		VspI	
Size (bp)	**Labeling (10^6 IDV)**	**Size (bp)**	**Labeling (10^6 IDV)**
23,130	15.7	16,063	12.7
9,416	15.7	7,135	12.7
6,557		3,602	
4,361		3,419	12.4
2,322		3,417	12.4
2,027		2,998	4.15
564		2,602	1.44
125		1,776	
		1,588	
		1,525	
		1,294	
		886	
		609	
		514	
		445	
		440	
		124	
		65	

decreasing efficiency of labeling as the size decreased but some larger bands were not effectively labeled.

Labeling NdeI/HindIII fragments. The plasmid, pBR322, underwent a double-digest with *Hind*III and *Nde*I, producing two fragments that were 2095 and 2266 base pairs long. Afterwards, the fragments were labeled with biotin, purified, and cleaved with *Vsp*I and *Sal*I to create four distinguished fragments. Table 2 shows the fragment sizes of the four bands and their IDV if the band appeared on the film (Figure 3B), as well as the samples that were loaded into each lane of the gel. It can be seen that the 3' L *Hind*III fragment exhibits a higher IDV value than the 3' L *Nde*I fragment.

Figure 3A shows the separated fragments of the samples while Figure 3B shows the fragments that were successfully labeled with biotin and detected by the film after exposure to streptavidin-HRP and substrate. In lane 5 of Figure 3B, three bands can be clearly seen on the film; however, Table 2 only shows that two of the four bands were observed on the film. The top-most band in lane 5 is the 2266 bp fragment that resulted when pBR322 was subjected to only *Hind*III and *Nde*I digests. It has an IDV of 43.9 x 10^6 that is twice the

The membrane was subsequently analyzed under UV to determine if the three segments of pBR322 would indicate any difference in intensity. The IDV values of the bands (data not shown) indicated that the 2266 bp fragment was still twice the value for the 3' L *Hind*III fragment, but the *Hind*III band was not significantly more intense than the 3' L *Nde*I fragment.

Analyzing the agarose gel picture, it was found that the uncut 2266 bp band was two times brighter than both the 3' L *Nde*I and 3' L *Hind*III bands, indicating that there was an equal amount of *Nde*I and *Hind*III fragments (data not shown). Furthermore, when the IDV of these two bands were added together, the sum was approximately the same as the 2266 bp fragment's value (data not shown).

DISCUSSION

The autoradiograph film (Fig. 3B) showed that the *Hind*III and *Vsp*I ladders were only partially labeled with biotin (Table 1). As there were 18 fragments and at least 32 ends to be labeled in the *Vsp*I-digested λ ladder, there may not have been enough biotinylated-14-dATP in the solution to label all ends, which would be required to determine the boundary at which size-dependent labeling may occur. Furthermore, there may not have been enough Klenow to anneal the deoxynucleotides to the overhang, or enough time with the amount of Klenow instilled in each reaction. The concentration and time required of Klenow was considered because although the higher molecular weight bands were labeled, one of the lower molecular weight bands from the double digest got labeled with biotin (Fig. 3B) as well. This shows that with the ideal conditions, concentrations and sufficient amount of time, there is a possibility to label the other bands of the ladder besides the uppermost fragments.

Although the ladders did not incorporate all its ends with biotin, there were enough bands labeled to indicate the sizes of the fragments. By analyzing the film and the agarose gel simultaneously, it was found that the pBR322 segments with biotin incorporated onto its ends were both on the left side of the plasmid. Further analysis showed that the 2266 bp produced by the primary double digest with *Hind*III and *Nde*I fragment

Table 2. Summary of the intensity of biotin-labeled bands detected by western blotting in lane 5 in Figure 3 and the theoretical fragment lengths of the corresponding fragments formed by double digestion with *Hind*III and *Nde*I.

Sample in lane 5	Labeled Fragments							
	(L) NdeI		(R) NdeI		(L) Hind III		(R) HindIII	
	length	10^6 IDV	length	10^6 IDV	length	10^6 IDV	length	10^6 IDV
HindIII and NdeI digest	1242	8.40	1644	n/a	853	25.9	622	n/a

(L) Left side of the restriction cut
(R) Right side of the restriction cut

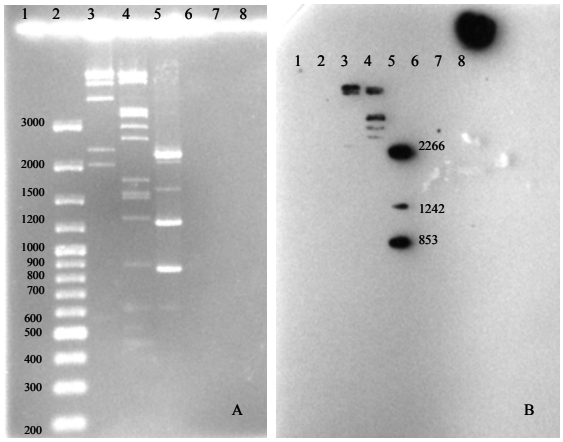

FIG. 3 Detection of biotin-labeled ladders and pBR322 fragments. (A)The agarose gel that was used to prepare the nitrocellulose blot. Lane 2 shows the 100 bp Plus DNA ladder; lane 3 shows the HindIII-cut λ ladder; lane 4 shows the VspI-cut λ ladder; and lane 5 shows the HindIII and NdeI double digest bands. (B) The autoradiograph film that detected the luminescence of the fragments on nitrocellulose membrane after it was exposed to streptavidin-HRP and to substrate. The lengths of the three fragments observed in lane 5 are labeled.

if it could be labeled. A reason why the 2266 bp fragment was minimally digested could be because conditions had to be changed between the use of *Vsp*I and *Sal*I, as the latter was from a different company and thus required the use of a different buffer. An alternate buffer was found to work well with *Vsp*I, and by adding a few salts, the environment could be changed to be optimal for *Sal*I. Possibly the environment was sub-optimal for *Sal*I to work in, thus it may be the reason why the enzyme was unable to cleave the fragments into their respective parts.

The IDV values of the fragments (Table 2) did show some interesting results though. The 2095 bp and 2266 bp fragments were originally generated from the double digest, and they corresponded to the left and right half of pBR322, respectively. An equal number of either fragment should be present, so the IDV of the 2266 bp fragment was expected to be similar to the IDV of the 2095 bp fragment. However, when the IDV values of the two fragments on the left side of the plasmid were summed, they were not close to the value obtained for the 2266 bp fragment. A previous analysis of the agarose gel had showed that there was an equal amount of left (L) end of *Nde*I fragments as there were of (L) *Hind*III fragments, but the IDV values on the film dictated that there were three-fold more of *Hind*III segments than there were of *Nde*I. This observation provides support that the (L) end of *Nde*I fragments do not incorporate biotin as efficiently as the (L) *Hind*III ends.

FUTURE EXPERIMENTS

The experiment should be repeated again to test the validity of the results with several improvements. The agarose gel clearly shows that the *Vsp*I-cut λ ladder is a possible way to investigate whether or not biotin-labeling is size-dependent; however, possibly a higher amount of deoxynucleotides and Klenow DNA polymerase are required as well as a longer incubation time. After labeling the double digest with biotin, the next step is to cleave the two fragments with *Sal*I and *Vsp*I. This step should be kept, however, it would be beneficial to use *Sal*I from Fermentas as it could be used simultaneously with *Vsp*I because both work optimally in the same buffer. If the experiment can be optimized with sufficient amount of deoxynucleotides and Klenow DNA polymerase, and incubated for a longer period, the right half of the digested pBR322 fragments may show up and further analysis can be done to see if (R) end of *Nde*I-digested fragment is available for biotin-labeling.

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