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Investigation of the Ligation Efficiency of *Nde*I Digested Fragments

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It has been observed that fragments cut by *Nde*I type II restriction enzyme have low ligation efficiency. To investigate this observation, λ DNA was digested to produce *Nde*I fragments. In ligation trials using T4 DNA ligase, *Nde*I fragments were shown to have lower ligation efficiency than *Hind*III fragments. The effect of different ligation incubation times on the efficiency was also examined. Although the cohesive ends of *Nde*I digested fragments would normally ligate in one hour, higher ligation efficiency was observed when *Nde*I fragments were allowed to ligate for 22 hours or more. In addition, a specific fragment was found to be present in all various lengths of ligation incubation time, which suggests that it did not ligate as effectively as other fragments. It is not clear why this fragment did not ligate as efficiently as others.

Restriction enzymes are commonly found in prokaryotic cells (2). Since the first restriction enzyme was discovered in 1960s, many other restriction enzymes have been found and characterized. These enzymes catalyze the cleavage of DNA at specific base sequences that usually exhibit two-fold symmetry around a given point. However, their role in cells is still a topic under discussion. Many believe the main function of restriction enzymes is to protect cellular DNA from viral DNA attacks (2), while others suggest that restriction enzymes are involved in cellular DNA recombination and transposition (2); some even propose that restriction enzymes can act as selfish elements along with other modification enzymes (2).

Restriction enzymes have become an indispensable tool in genetic studies such as gene analysis and cloning. It is therefore essential to understand the properties of restriction enzymes and their products. It has been observed in cloning experiments that PCR fragments digested by *Nde*I restriction endonuclease have poor ligation efficiency compared to other commonly used restriction enzymes such as *Hind*III (W. Ramey, personal communication). *Nde*I, a type II restriction endonuclease purified from *Neisseria denitrificans*, is a four base cutter with recognition site at 5'-CA↓TATG-3' and creates overhangs in cut DNA fragments (4). Currently, there are no published reports regarding the low ligation efficiency of *Nde*I digested DNA fragment.

To investigate this problem, the previously observed low ligation efficiency of *Nde*I digested DNA was first confirmed. Since the ligation incubation time is an important factor that influences the activity of restriction enzymes (3), the effect of the various incubation times on the ligation efficiency of *Nde*I digested DNA was investigated to determine whether the efficiency would be improved by prolonged incubation.

MATERIALS AND METHODS

Digestion of λ DNA by restriction enzymes: Three hundred to five hundred nanograms of λ DNA (#25250-028, Invitrogen) were used in each reaction. *Nde*I (#111S) and *Hind*III (#15207-012) were purchased from New England Biolabs and Invitrogen respectively. The React buffers are supplied with the enzymes. Five units of restriction enzyme and 2 µl of 10x React buffers were used in each reaction. The reactions were made up to a volume of 20 µl in distilled water and incubated at 37°C for 1 hour. Enzymes were heated for 20 min at 70°C after digestion.

Purification of DNA: Prior to the ligation reaction, digested samples were purified from unwanted proteins and salts by a standard phenol extraction and ethanol precipitation according as previously described (1).

Ligations: Ligations were performed on *Nde*I and *Hind*III digested fragments using T4 DNA ligase (#15224-017, Invitrogen) supplied with ligase buffer. One unit of enzyme and one mircolitre of ligase buffer were used in each reaction. *Hind*III digested λ DNA fragments (#15612-013, Invitrogen) was used as a control. Ligations were performed at 22°C for varying periods of time from 2 hours to 43 hours.

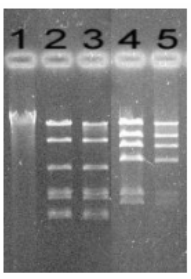
Agarose gel electrophoresis: Six times loading buffer, made from 1.2 mL bromophenol blue and 150 µl glycerol, was added to each sample to a final volume of 24 µl for *Nde*I and *Hind*III digestions and ligations reactions. Nine microliters of loading buffer was added to undigested λ DNA and supplied *Hind*III digested fragments. All samples were heated to 65°C for 5 minutes prior loading. Agarose gel electrophoresis was performed with 0.8% gel in 1x TBE (10x TBE buffer was prepared using 108 g Tris base, 55 g Boric acid, and 9.3 g Tetrasodium EDTA up to 1L with distilled water). Electrophoresis was performed at 100 volts for 75 or 105 minutes. Gel was stained in 0.2 µg/mL ethidium bromide bath for 30 minutes prior to visualization using a UV transilluminator.

RESULTS

Confirmation of ligation *Nde*I digested fragments: λ DNA was digested with *Nde*I and *Hind*III enzymes for 1 hour at 37°C. The enzymes were heated at 70°C for 20 minutes. *Nde*I and *Hind*III digestions were purified using phenol extraction and ethanol precipitation to remove unwanted proteins and salts. From figure 1, a minor loss of DNA is seen in the purified digests but the bands can still be clearly observed. The digests were ligated using T4 DNA ligase at 22°C for 22 hours.

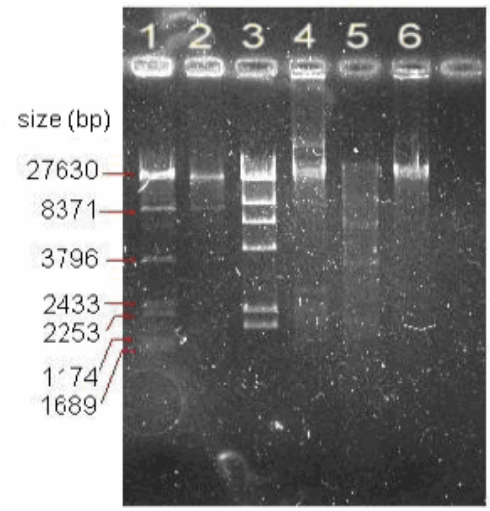
*Nde*I digested λ DNA fragments ligate less effectively compared to the *Hind*III (Fig. 2). While many of the small bands of the *Nde*I digest disappear after ligation, the band at 8371 bp is still evident. In the *Hind*III ligation, three bands of large molecular weight are observed (Fig. 2, lane 4); these bands have molecular weights that are equal to or larger than the original λ DNA.

***Nde*I ligation for various time periods:** In order to determine if the length of incubation time affects the ligation efficiency of *Nde*I fragments, digested fragments were ligated for various lengths of time, including 2, 8, 16, 22 and 43 hours (Fig. 3, Lanes 3 to 7 respectively). The *Nde*I ligations for 22 and 43 hours show more ligation because the bands around 2000 and 1000 bp are fainter than those observed in the ligations incubated for less than 22 hours. A new band of approximately 6600bp is observed in the *Nde*I ligation samples but is not found in the digestion. This band is more apparent in the ligations for 22 and 43 hours than in shorter incubation times.



Lane 1: λ DNA
Lane 2: *Nde*I digested λ DNA
Lane 3: Purified *Nde*I digested λ DNA
Lane 4: *Hind*III digested λ DNA
Lane 5: Purified *Hind*III digested λ DNA

FIG. 1 Purification of *Nde*I and *Hind*III digested λ DNA. 500ng of λ DNA was digested with *Nde*I and *Hind*III restriction enzymes and subsequently purified by phenol extraction and ethanol precipitation. Samples were run on 0.8% agarose gel.



Lane 1. *Nde*I digested λ DNA
Lane 2. Ligation of *Nde*I fragments
Lane 3. *Hind*III digested λ DNA
Lane 4. Ligation of *Hind*III fragments
Lane 5. Predigested *Hind*III λ DNA (control)
Lane 6. Ligation of predigested *Hind*III λ DNA (control)

FIG. 2 *Nde*I digested λ DNA and subsequent ligations with T4 DNA ligase. Ligation reactions were incubated at 22˚C overnight for 20 hours. Samples were run on 0.8% agarose gel.

DISCUSSION

Results from figure 2 suggest that some *Nde*I digested fragments ligated together because the bands of lower molecular weights in Lane 2 cannot be seen. Phenol extraction and ethanol precipitation were performed on ligation samples in Lane 2 and 4; digestion samples in Lane 1 and 3 were taken prior to these purification procedures. Therefore, there is a possibility that the lower bands could not be observed in *Nde*I ligation (Lane 2) because of DNA loss during purification. However, as ligation is evident in Fig. 3, the banding pattern in *Nde*I ligation sample (Lane 2) in figure 2 is more likely to have resulted from ligation than DNA loss during the purification process.

*Hind*III restriction enzyme serves as a control throughout the experiment. Ligation is observed in all *Hind*III digested fragments samples as small fragments ligated to form large fragments thus generating a thicker band of similar size to λ DNA (Fig 2 lane 4, and Fig. 3, Lanes 9 and 11); yet the presence of faint low molecular bands in the ligation of *Hind*III digests indicates that some of these fragments remain unligated (Fig. 2 lane 4 and Fig. 3 lane 11). This corresponds to the property of *Hind*III fragments that they do not ligate at 100%, as written in the Invitrogen catalogue. Nonetheless, the overall ligation efficiency of *Hind*III digest is still very high. In contrast with *Nde*I, the low molecular weight bands of *Hind*III ligation are significantly lighter than its digest (Fig. 3). This illustrates that the ligation of *Hind*III fragment is more efficient than *Nde*I fragments. In addition, the presence of three bands of size bigger than undigested λ DNA is also seen in these ligations. Since the overhangs of the *Hind*III digested fragments are identical, ligase can randomly join these fragments as long as their overhangs are complementary to each other. Thus, it is possible for a given end of large fragment to encounter a complementary end on another large fragment to form a hybrid that is longer than the original λ DNA.

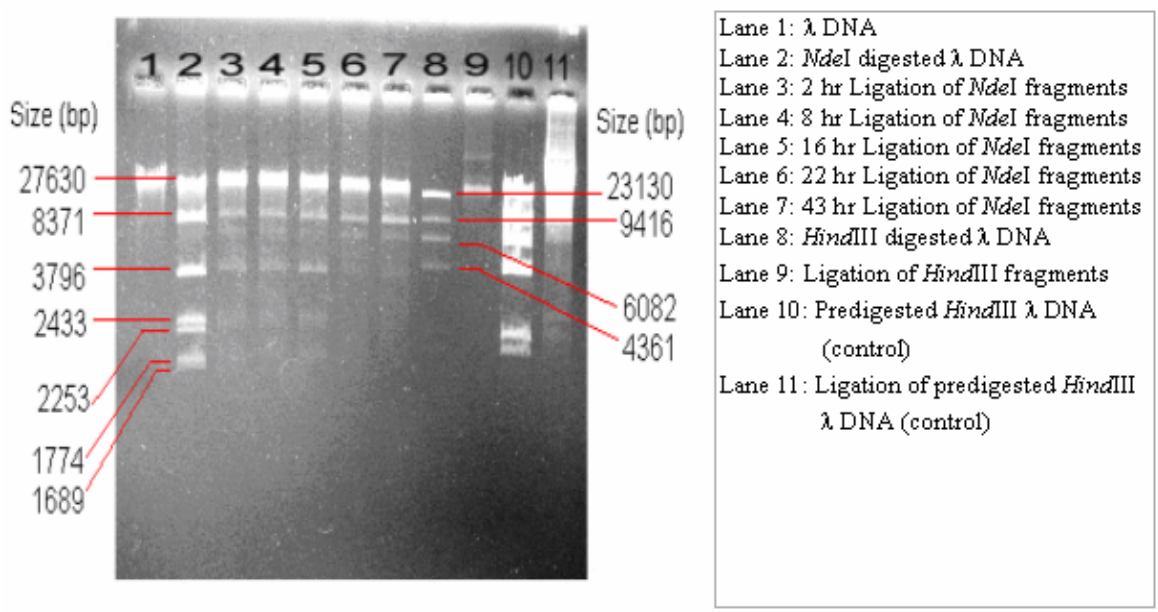


FIG. 3 Variations of ligation incubation time of *Nde*I digested λ DNA. Ligation samples were incubated at 22°C for 2, 8, 16, 22, and 43 hours. Samples were run on 0.8% agarose gel.

Ligation occurred in all five *Nde*I digested samples as seen in figure 3 lanes 3 to 7. Ligation efficiency is highest in samples incubated for 22 and 43 hours. In the 2, 8, and 16 hour ligation samples, the ligation appears to be less efficient since faint low molecular bands are present. The faint bands represent DNA fragments which may be 3796 bp, 2433 bp, 2253 bp, 1774 bp, and 1689 bp in length; these bands are also present in *Nde*I digestions.

A DNA fragment of 8371 bp in length is observed in all *Nde*I ligation samples (Fig 2 lane 2, and Fig. 3 lanes 3-7). The intensity of this band remains relatively constant throughout the *Nde*I ligation samples (Lane 3-7, Fig. 3) even in the samples with highest ligation efficiencies. This observation is unexpected. It appears that the 8371 bp fragment is not preferred by the T4 DNA ligase in the ligation reaction for unknown reasons. Since the 8371 bp fragment lies on the end of the original λ DNA, it requires only a single cut by *Nde*I as opposed to two cuts on either side. The fact that the end fragment only contains one *Nde*I compatible end may cause its ligation to be slower than those fragments with two *Nde*I compatible ends. However, slow ligation was not observed in the end fragments of *Hind*III samples; this suggests that additional factors may have contributed to the observation in *NdeI* ligations. It is possible that there may be unusual sequences around this last restriction site which causes *Nde*I to cut differently thus result in a lower ligation efficiency compared to other fragments.

In summary, the ligation efficiency of *Nde*I digested fragments increases as the ligation incubation time increase. Highest efficiency was observed at 22 and 43 hours. In contrast to *Hind*III, *Nde*I digested fragments have a lower ligation efficiency. The presence of a band of approximately 8371 bp in all *Nde*I ligation samples suggests that it is not preferred by the T4 DNA ligase in the ligation reaction.

Restriction enzymes are primarily used in vitro for the construction of recombinant DNA for cloning.

Gel analysis of the ligation reaction is the preliminary step to ensure the restriction enzyme digested insert has successfully ligated into the vector. Thus it is important to analyze the ligation efficiency of *Nde*I digested inserts with plasmid vector in order to increase the yield of bacterial transformants. This could be done by ligating an *Nde*I digested insert into a cloning vector flanked by *Nde*I restriction sites, following by electrophoresis analysis.

FUTURE EXPERIMENTS

We suspect that the key to understanding the low ligation efficiency of *Nde*I digest lies in the 8371 bp fragment which is consistently found in all ligation reactions. Due to time constraints, we were unable to analyze this fragment. Since the structure of the termini correlates significantly to ligation efficiency, further studies should examine the DNA sequence at the ends of the fragment which may encode sequences that may possibly interfere with the ligation process.

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