INVERTIS UNIVERSITY BAREILLY

502 LECTURE 2

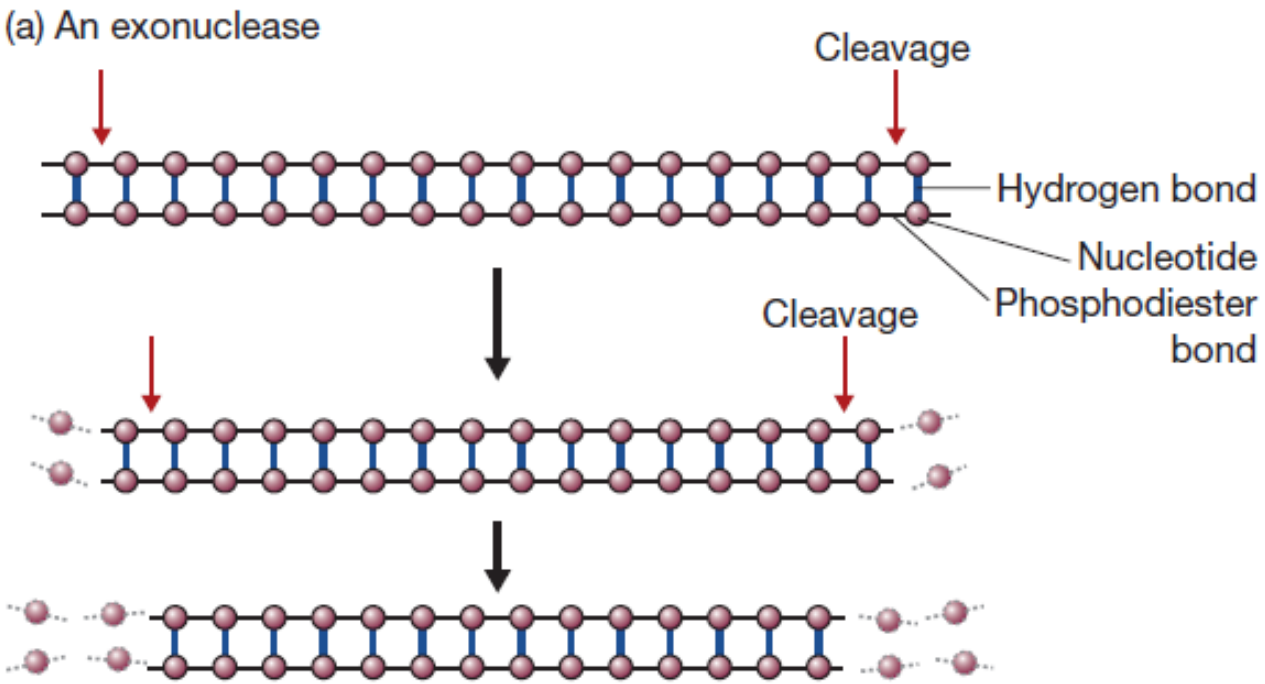
DNA MANIPULATIVE ENZYMES

- After purification of DNA molecules from different samples, the next step in a gene cloning experiment is construction of the recombinant DNA molecule.
- And to produce recombinant DNA, the vector and the DNA (gene) to be cloned, must be cut at specific points and then joined together in a controlled manner.
- Cutting and joining are two examples of DNA manipulative techniques, and by these technique DNA molecules can be:
  - shortened,
  - lengthened,
  - copied into RNA or into new DNA molecules,
  - and modified by the addition or removal of specific chemical groups.
- These manipulations (*in vitro*), provide the foundation not only for gene cloning, but also for study DNA biochemistry, gene structure, and the control of gene expression.

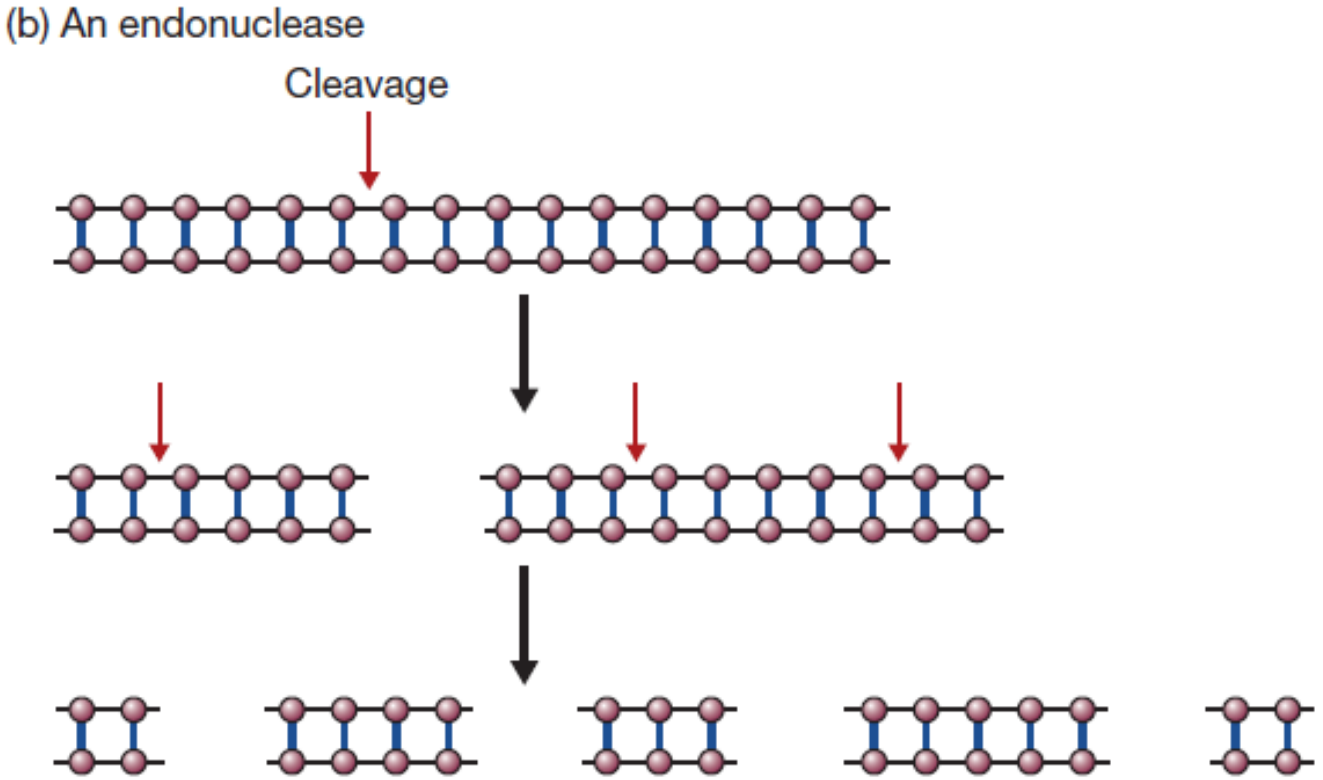
- Almost all DNA manipulative techniques make use of different purified enzymes.
- Within the cell these enzymes participate in processes like DNA replication & transcription, breakdown of unwanted or foreign DNA (e.g., invading virus DNA), repair of mutated DNA, and **recombination** between different DNA molecules.
- After purification from cell extracts, many of these enzymes can be used to carry out their natural reactions, or something closely related reactions, under artificial conditions.
- The cutting & joining manipulations, basics of gene cloning, are carried out by enzymes called **restriction endonucleases** (for cutting) & **ligases** (for joining).
- DNA manipulative enzymes can be grouped into four classes
  1. **Nucleases** are enzymes that cut, shorten, or degrade nucleic acid molecules.
  2. **Ligases** join nucleic acid molecules together.
  3. **Polymerases** make copies of molecules.
  4. **Modifying enzymes** remove or add chemical groups.

NUCLEASES:

- Nucleases degrade DNA molecules by breaking the phosphodiester bonds that link one nucleotide to the next nucleotide.
- There are two different kinds of nuclease: **Exonucleases and Endonucleases**
- **Exonucleases:** removes nucleotides one by one from the end of DNA.

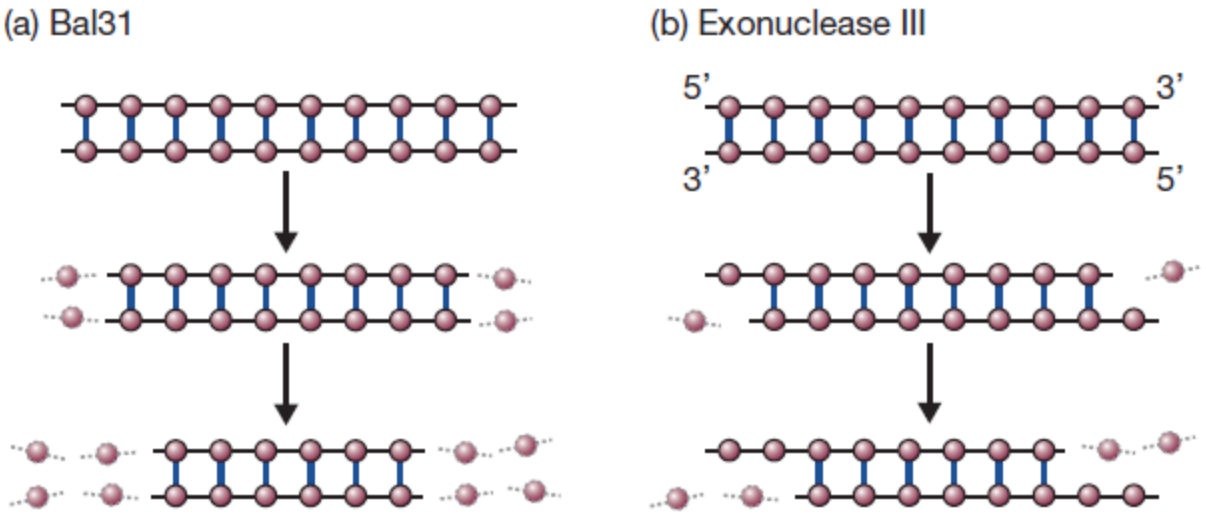


- **Endonucleases:** able to break internal phosphodiester bonds within DNA.



- Types of exonucleases: different types of exonucleases are present on the basis of the activity when a dsDNA molecule is attacked by them.

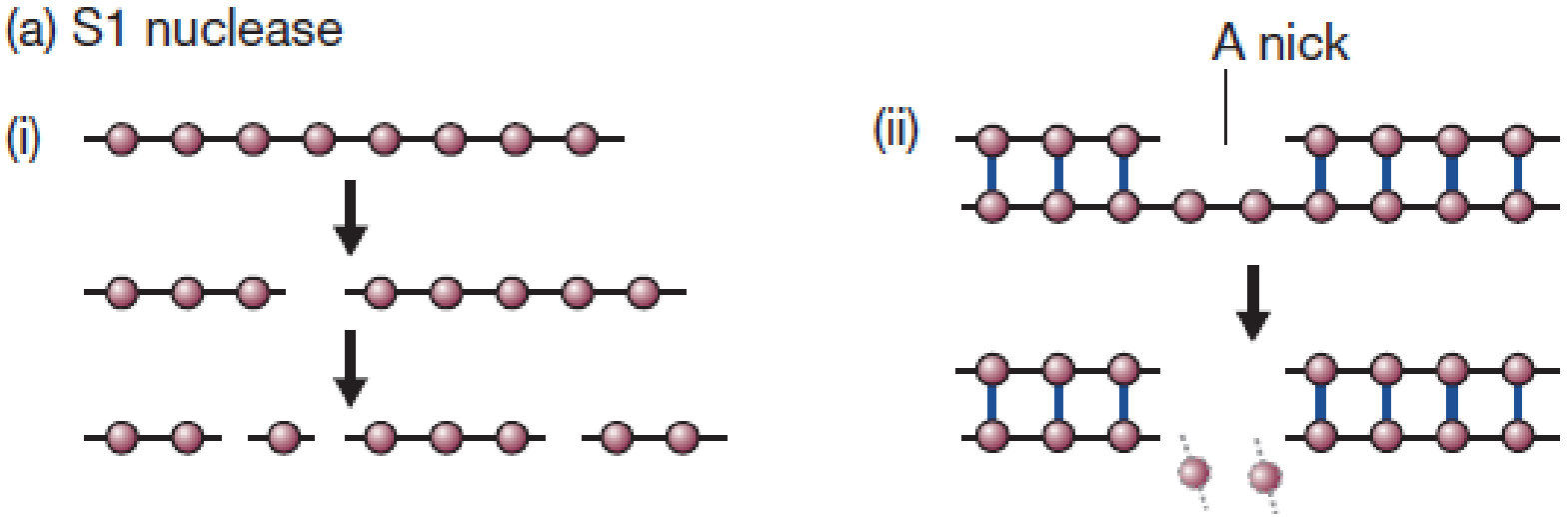
- The enzyme called Bal31 (from bacterium *Alteromonas espejiana*) is an example of an exonuclease that removes nucleotides from both strands of dsDNA.
- The greater the length of exposure time of Bal31 on DNA molecules, the shorter the resulting DNA fragments will be.
- In contrast, enzymes such as *E. coli* exonuclease III degrade just one strand of dsDNA molecule, leaving single-stranded DNA as the product.



(a) Bal31 - removes nucleotides from both strands of a dsDNA molecule.

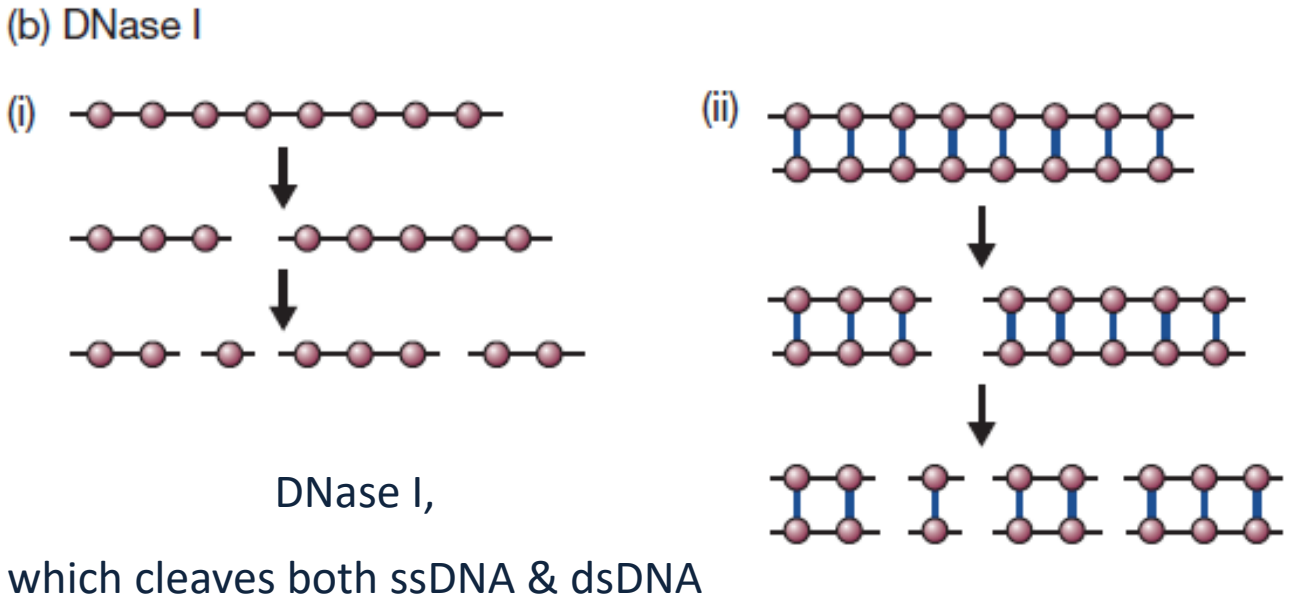
(b) Exonuclease III - removes nucleotides only from the 3′ terminus.

- Similarly, endonucleases had also been characterized:
- S1 endonuclease (from *Aspergillus oryzae*) only cleaves single strands.



S1 nuclease, which cleaves only single-stranded DNA, including single-stranded nicks in mainly double-stranded molecules.

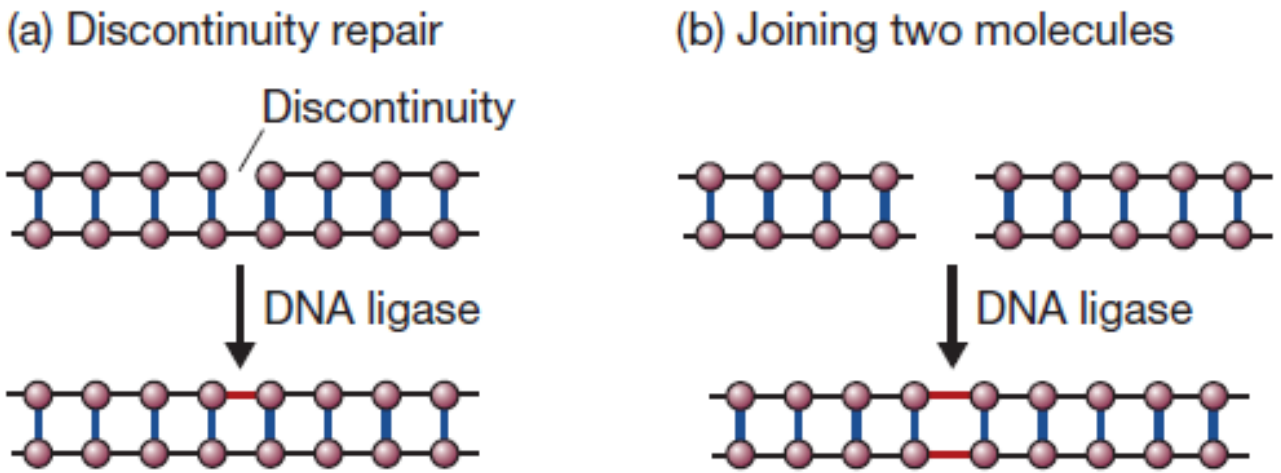
- deoxyribonuclease I (DNase I) (prepared from cow pancreas), cuts both ssDNA and dsDNA.



- DNase I has non-specific activity: thus it attacks on DNA at any internal phosphodiester bond and prolonged DNase I exposure results is a mixture of mononucleotides and very short oligonucleotides.
- On the other hand, the special group of enzymes k/a ***restriction endonucleases*** cleave dsDNA only at a limited number of specific recognition sites**.**

LIGASES

- In the cell the function of DNA ligase is to repair single-stranded breaks ("discontinuities") that arise in dsDNA molecules during; for ex, DNA replication.
- DNA ligases from most organisms can also join together two individual fragments of dsDNA.



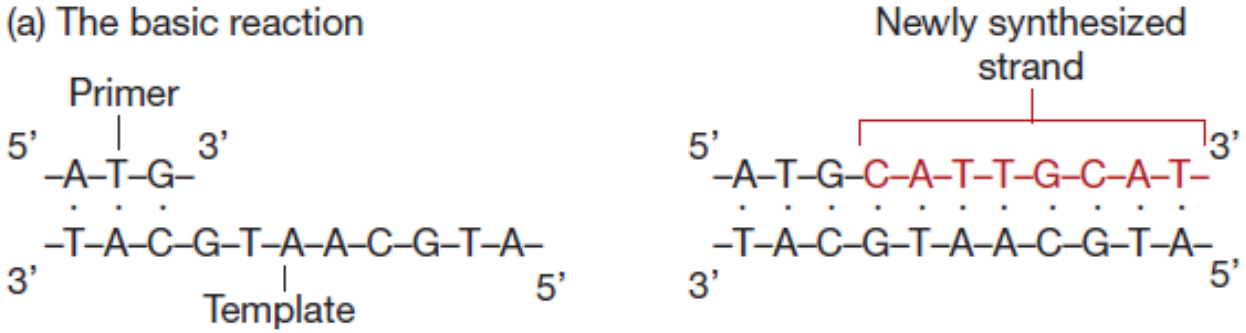
DNA ligase:

(a) Repair of a discontinuity - a missing phosphodiester bond in one strand.

(b) Joining two molecules dsDNA together.

POLYMERASES

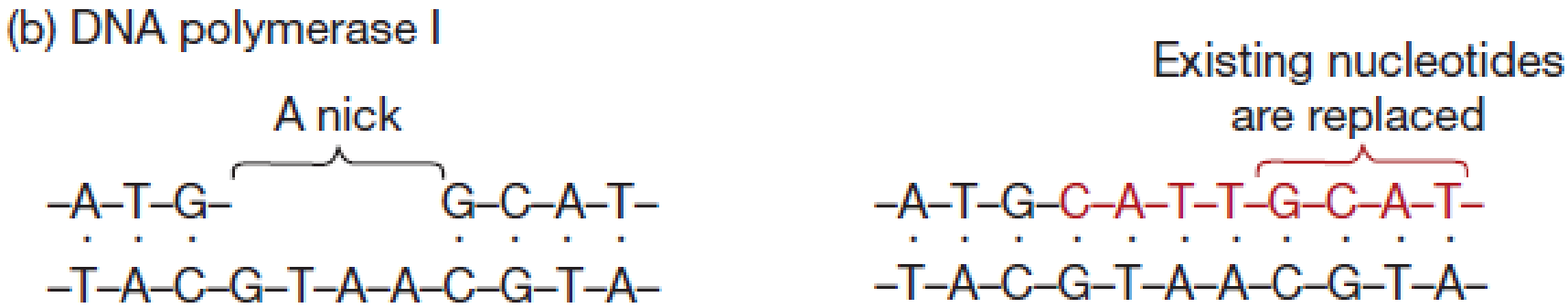
- DNA polymerases are enzymes that synthesize a new DNA strand complementary to an existing DNA or RNA template.



- Most polymerases can function only if the **template** possesses a double-stranded region that acts as a **primer** for initiation of polymerization.
- Four types of DNA polymerase are used routinely in genetic engineering.
  1. DNA polymerase I (usually prepared from *E. coli*)
  2. Klenow fragment
  3. *Taq* DNA polymerase
  4. reverse transcriptase

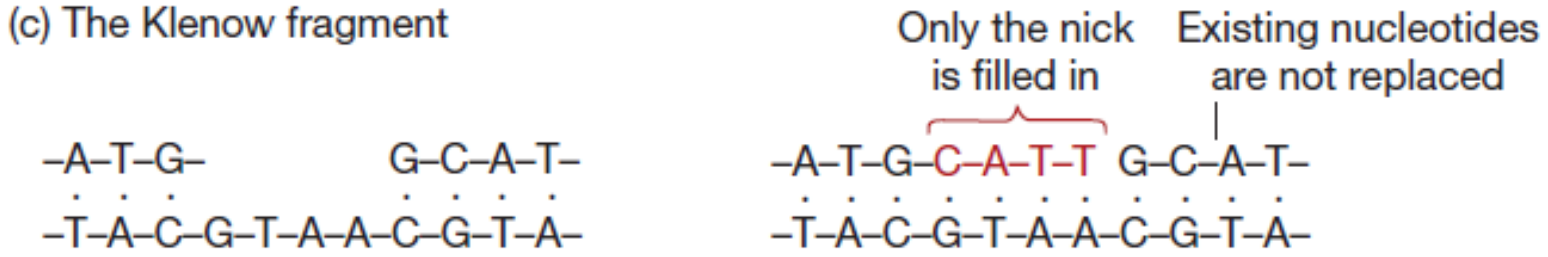
1. DNA polymerase I (usually prepared from *E. coli*): This enzyme attaches to a short ssDNA region (or **nick**) in a mainly dsDNA molecule, and then synthesizes a completely new strand, degrading the existing strand as it proceeds.

- Thus it is an example of dual activity enzyme i.e. polymerization & degradation.



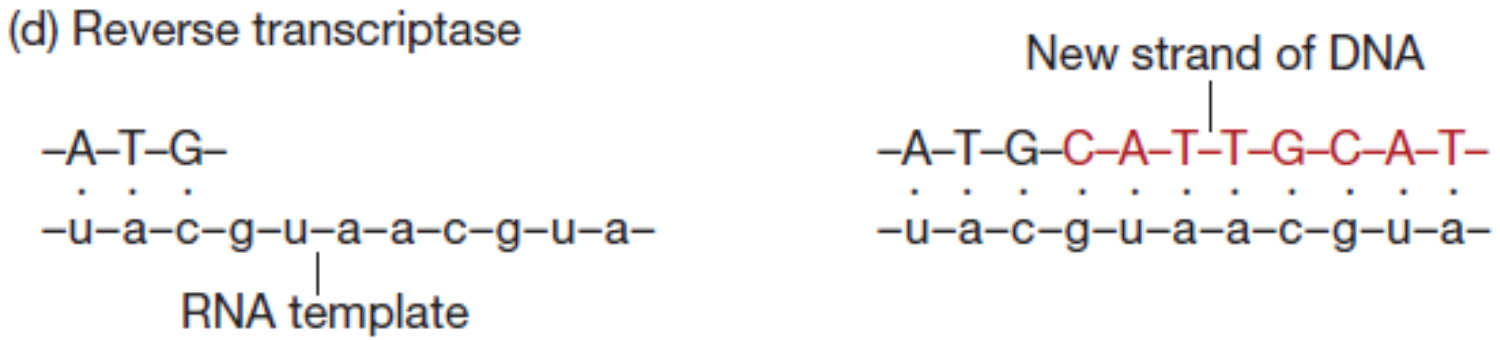
- The polymerase and nuclease activities of DNA polymerase I are controlled by different parts of the enzyme molecule.
- The nuclease activity is contained in the first 323 amino acids of the polypeptide.
- So removal of this segment leaves a modified enzyme that functions as polymerase and is unable to degrade DNA.

- This modified enzyme is k/a **Klenow fragment**, can still synthesize a complementary DNA strand on a single-stranded template.



- Several other enzymes i.e. natural polymerases and their modified versions have similar properties to the Klenow fragment.
- And these are majorly used in DNA sequencing.
- The ***Taq* DNA polymerase:** used in the PCR is the DNA polymerase I enzyme of the bacterium *Thermus aquaticus*.
- This organism lives in hot springs, & many of its enzymes, including the *Taq* DNA polymerase, are thermo-stable (resistant to denaturation by heat treatment).
- This special feature of *Taq* DNA polymerase makes it suitable for PCR because it is stable when temperature of the reaction is raised to 94°C to denature the DNA.

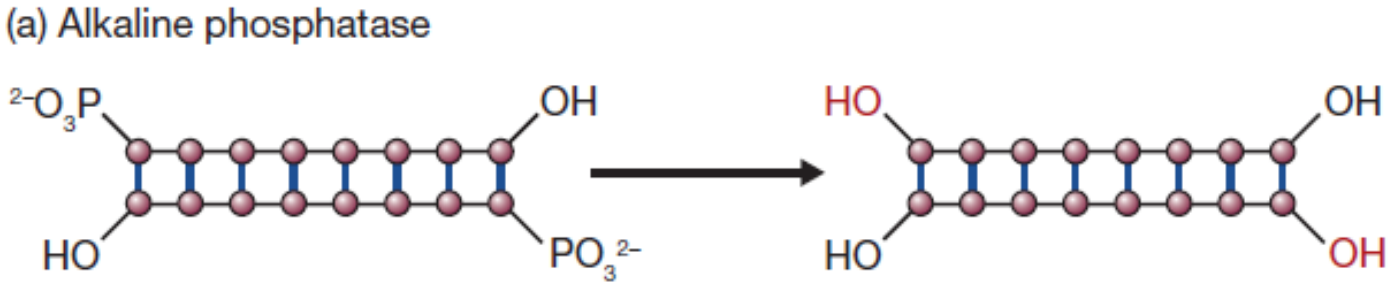
- The another DNA polymerase important in genetic engineering is **reverse transcriptase.**
- This enzyme is involved in the replication of several kinds of virus.
- Reverse transcriptase is unique in that it uses as a template not DNA but RNA.



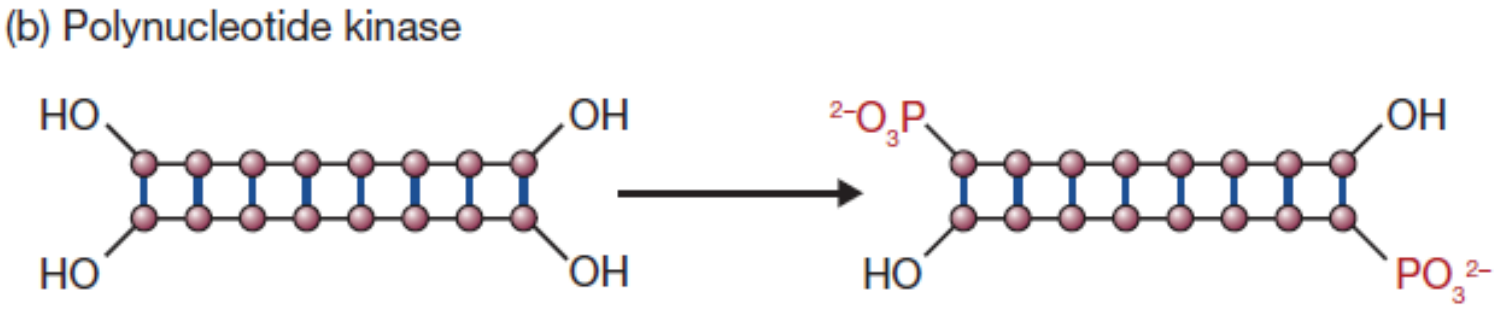
- The ability of this enzyme to synthesize a DNA strand complementary to an RNA template is central to the technique called complementary DNA (cDNA) cloning.

DNA MODIFYING ENZYMES:

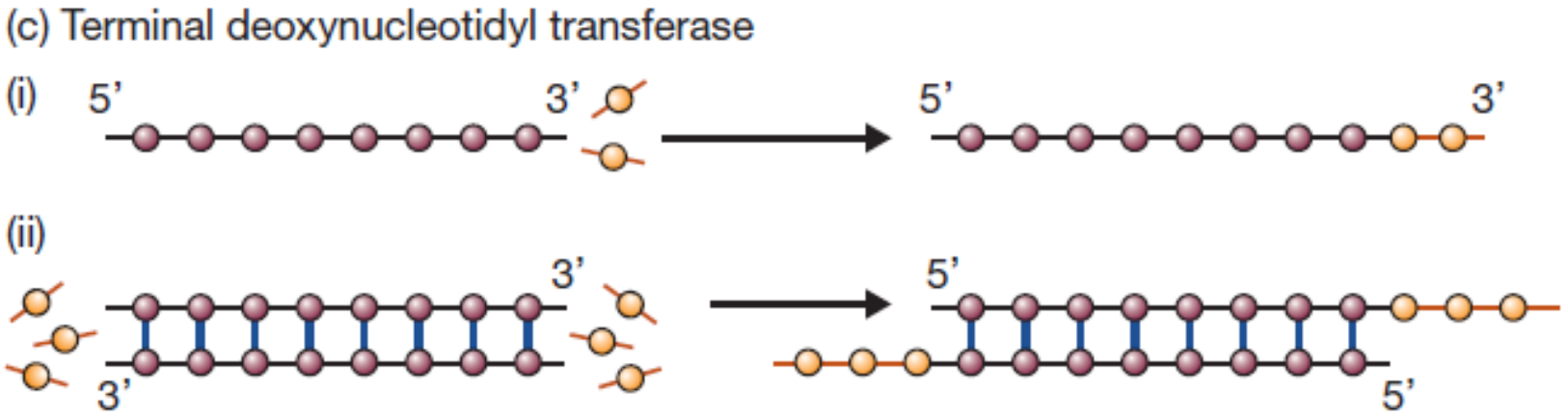
- There are numerous enzymes that modify DNA molecules by addition or removal of specific chemical groups.
- The most important are as follows:
- **Alkaline phosphatase** (from *E. coli or* calf intestinal tissue): removes phosphate group present at the **5′ terminus** of a DNA molecule.



- **Polynucleotide kinase** (from *E. coli* infected with T4 phage): has the reverse effect to alkaline phosphatase, adding phosphate groups onto free 5′ terminal.

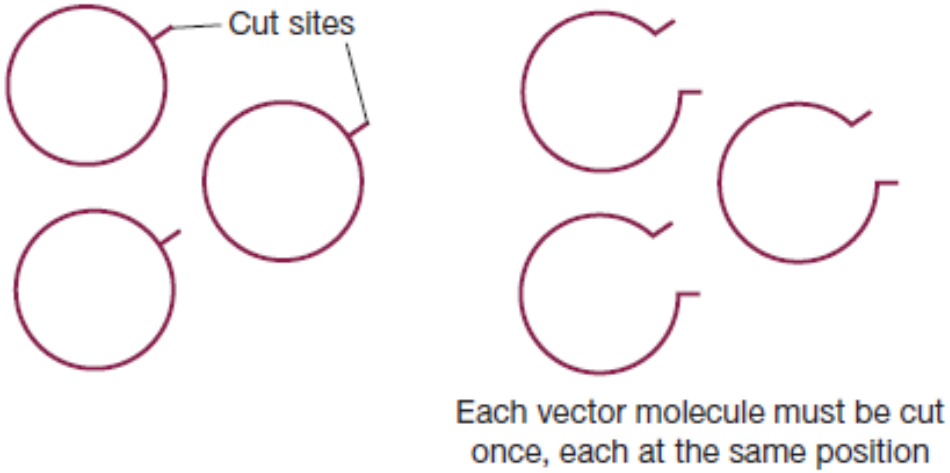


- **Terminal deoxynucleotidyl transferase** (from calf thymus tissue): adds one or more deoxyribonucleotides onto the **3′ terminus** of a DNA molecule.



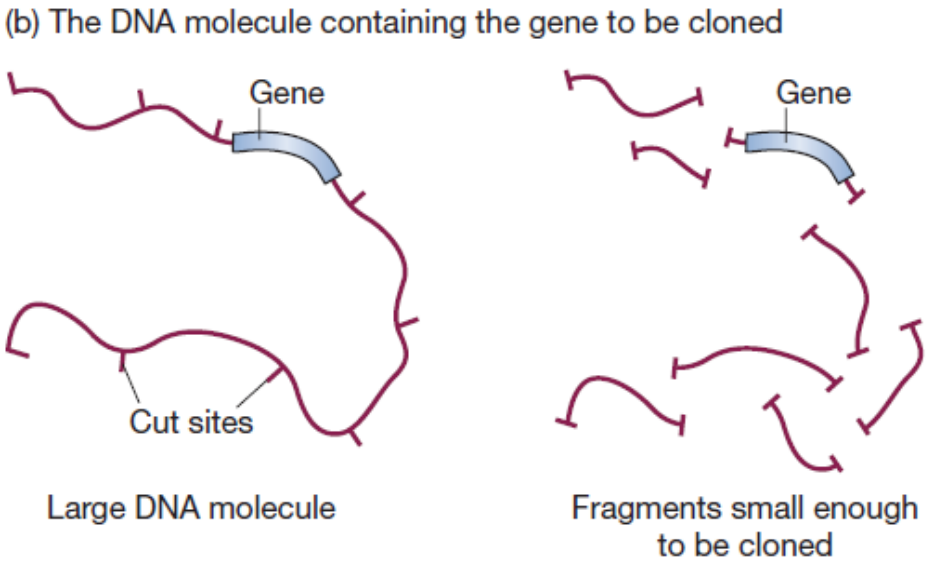
RESTRICTION ENDONUCLEASES

- Gene cloning requires that DNA molecule to be cut in reproducible fashion.



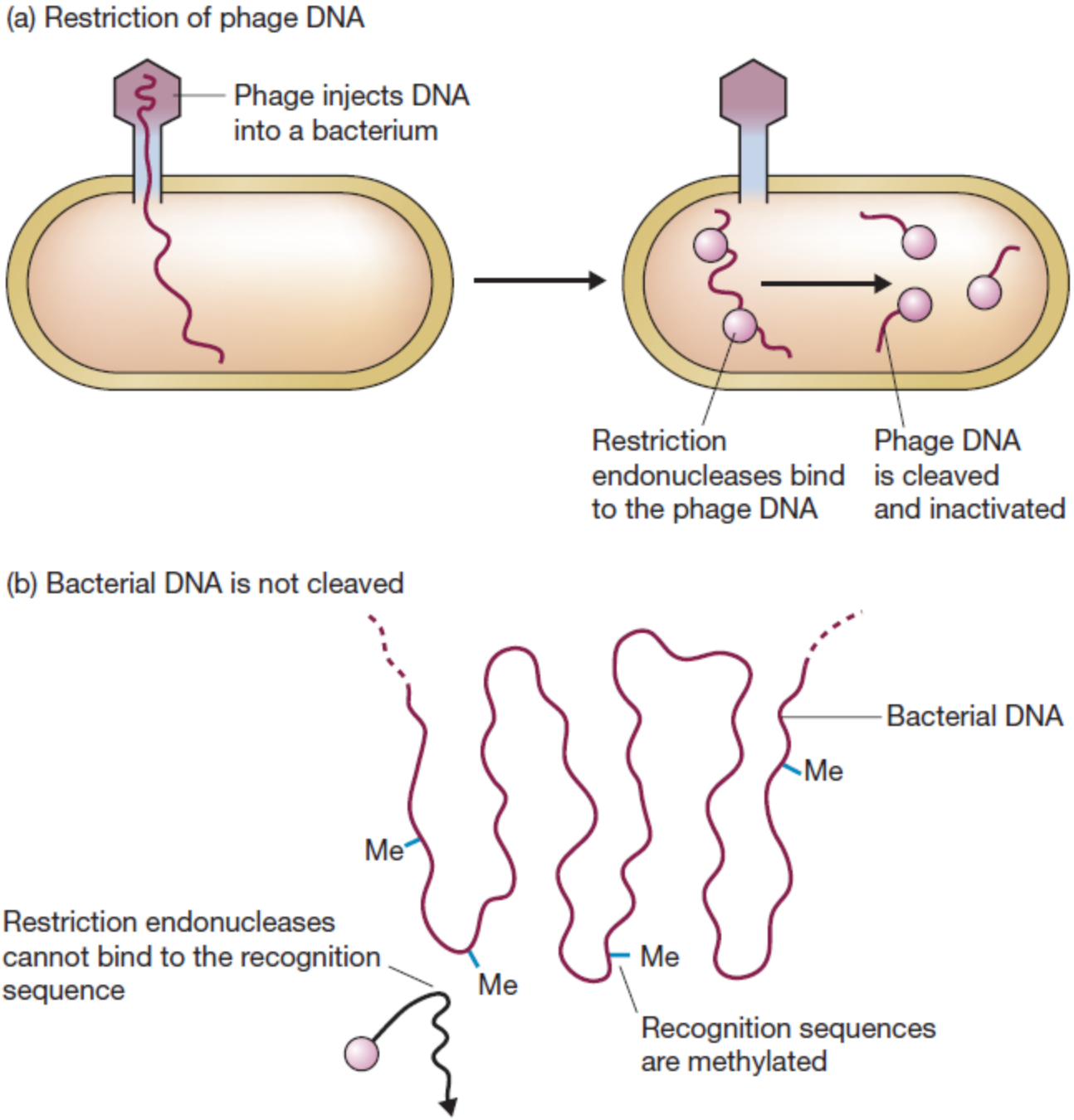
- Each vector molecule must be cleaved at a single position and at exactly the same position on circle, to open up the circle so that new DNA can be inserted.
- A molecule that is cut more than once will be broken into two or more separate fragments and will be of no use as a cloning vector.
- It should be clear that a very special type of nuclease is needed to carry out this manipulation.

- It is also necessary to cleave the DNA that is to be cloned.
- If our aim is to clone ***single gene***, (consist of only 2-3 kb of DNA), then that gene will have to be cut out of the large (often greater than 80 kb) DNA molecules.
- Large DNA molecules should be broken into small fragments which vectors can carry.
- Most vectors show a preference for DNA fragments that fall into a size range: most plasmid-based vectors, for example, are very inefficient at cloning DNA molecules more than 8 kb in length.
- Purified restriction endonucleases allow the molecular biologist to cut DNA molecules in the precise, reproducible manner required for gene cloning.
- The discovery of these enzymes, (led to Nobel Prizes for W. Arber, H. Smith, & D. Nathans in 1978), was the key breakthroughs in development of RDT.



THE DISCOVERY AND FUNCTION OF RESTRICTION ENDONUCLEASES:

- The final discovery of restriction endonucleases was made in early 1950s.
- At that time, it was shown that some bacterial strains are immune to bacteriophage infection & this immunity referred as **host-controlled restriction**.
- Restriction occurs because bacterium produces an enzyme that degrades phage DNA before it has time to replicate and direct synthesis of new phage particles.
- The bacterium's own DNA is protected from attack because it carries additional methyl groups that block the degradative enzyme action.
- These degradative enzymes are k/a restriction endonucleases and are synthesized by many species of bacteria: over 2500 different ones have been isolated and more than 300 are available for use in the laboratory.
- Three different classes of restriction endonuclease are recognized.
- Types I and III are complex and have only a limited role in RDT.
- Type II restriction endonucleases are cutting enzymes and thus important in RDT.


(a) Restriction of phage DNA
Phage injects DNA into a bacterium
Restriction endonucleases bind to the phage DNA
Phage DNA is cleaved and inactivated
(b) Bacterial DNA is not cleaved
Bacterial DNA
Me
Me
Me
Me
Restriction endonucleases cannot bind to the recognition sequence
Recognition sequences are methylated

TYPE II RESTRICTION ENDONUCLEASES

- The central feature of type II restriction endonucleases is that: each enzyme has a specific recognition sequence at which it cuts a DNA molecule.
- A particular enzyme cleaves DNA at the recognition sequence and nowhere else.
- For example, the restriction endonuclease called *Pvu*I (from *Proteus vulgaris*) cuts DNA only at the hexanucleotide sequence i.e. CGATCG.
- A second enzyme from the same bacterium, called *Pvu*II, cuts at a different hexanucleotide i.e. CAGCTG.
- Many restriction endonucleases recognize hexanucleotide target sites, but others cut at four, five, eight, or even longer nucleotide sequences.
- *Sau*3A (from *Staphylococcus aureus* strain 3A) recognizes GATC, and *Alu*I (*Arthrobacter luteus*) cuts at AGCT.

- Many other restriction endonucleases can also degenerate recognition sequences, **meaning that:** they cut DNA at any one of a family of related sites.
- For Example: *Hin*fI (*Haemophilus influenzae* strain Rf), recognizes GA**N**TC, so cuts at GA**A**TC, GA**T**TC, GA**G**TC, and GA**C**TC.
- Recognition sequences for frequent restriction endonucleases are as follows:

ENZYME	ORGANISM	RECOGNITION SEQUENCE*	BLUNT OR STICKY END
*Eco*RI	*Escherichia coli*	GAATTC	Sticky
*Bam*HI	*Bacillus amyloliquefaciens*	GGATCC	Sticky
*Bgl*II	*Bacillus globigii*	AGATCT	Sticky
*Pvu*I	*Proteus vulgaris*	CGATCG	Sticky
*Pvu*II	*Proteus vulgaris*	CAGCTG	Blunt
*Hin*dIII	*Haemophilus influenzae* R_d	AAGCTT	Sticky
*Hin*fI	*Haemophilus influenzae* R_f	GANTC	Sticky
*Sau*3A	*Staphylococcus aureus*	GATC	Sticky
*Alu*I	*Arthrobacter luteus*	AGCT	Blunt
*Taq*I	*Thermus aquaticus*	TCGA	Sticky
*Hae*III	*Haemophilus aegyptius*	GGCC	Blunt
*Not*I	*Nocardia otitidis-caviarum*	GCGGCCGC	Sticky
*Sfi*I	*Streptomyces fimbriatus*	GGCCNNNNNGGCC	Sticky

- Sequence shown in the table is that for one strand, given in the 5′ to 3′ direction.
- "N" indicates any nucleotide.
- Note that almost all recognition sequences are palindromes i.e. both strands are considered they read the same in each direction, for example:

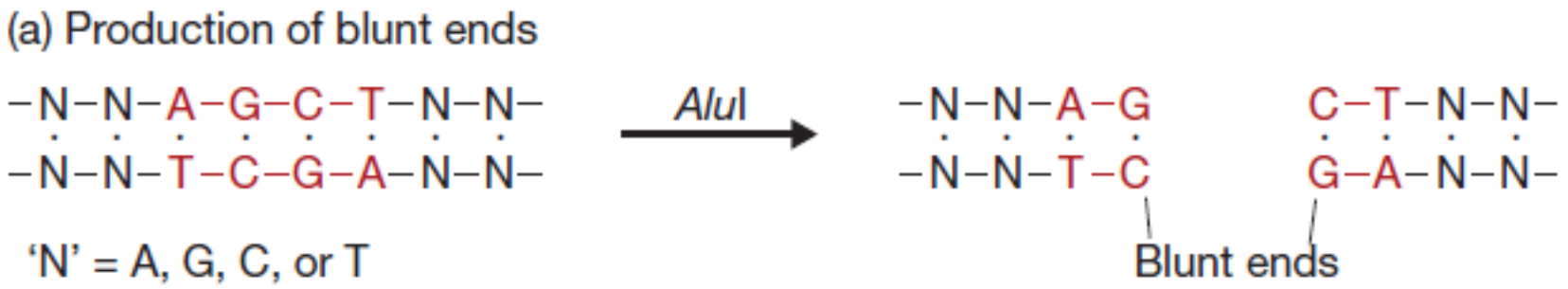
*Eco*RI	*Escherichia coli*	GAATTC	Sticky

*Eco*RI

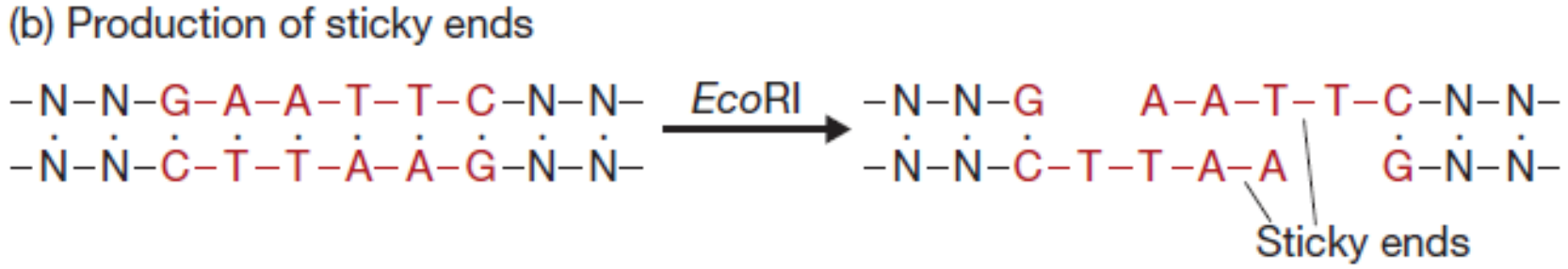
5′–GAATTC–3′
||||||
3′–CTTAAG–5′

BLUNT ENDS AND STICKY ENDS

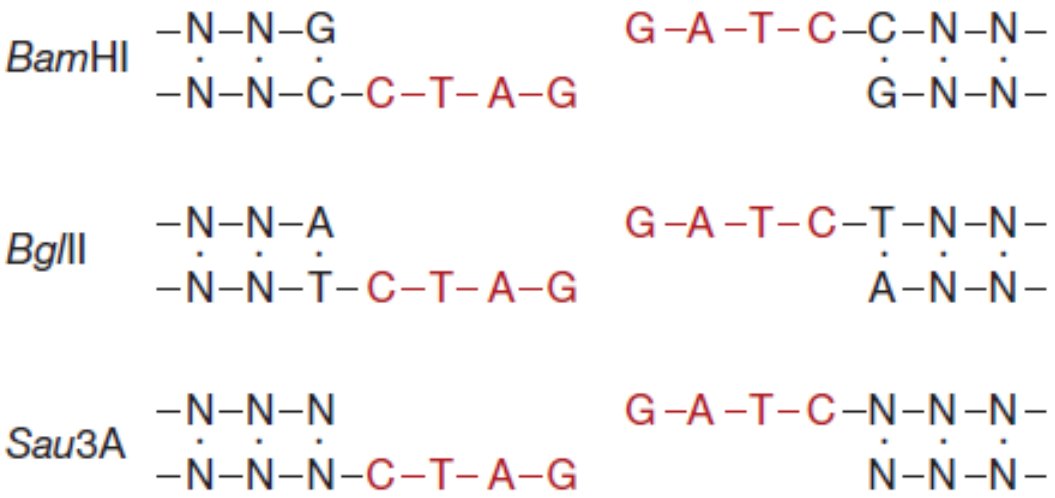
- Many restriction endonucleases make a simple double-stranded cut in the middle of the recognition sequence, resulting in a **blunt end** or **flush end**.
- For example: *Alu*I (A G C T) cuts DNA and leaved blunt ends.



- Other restriction endonucleases are not cut DNA at exactly the same position.
- The cleavage is staggered (by two or four nucleotides) so that resulting DNA fragments have short ssDNA overhangs at each end k/a sticky or cohesive ends.



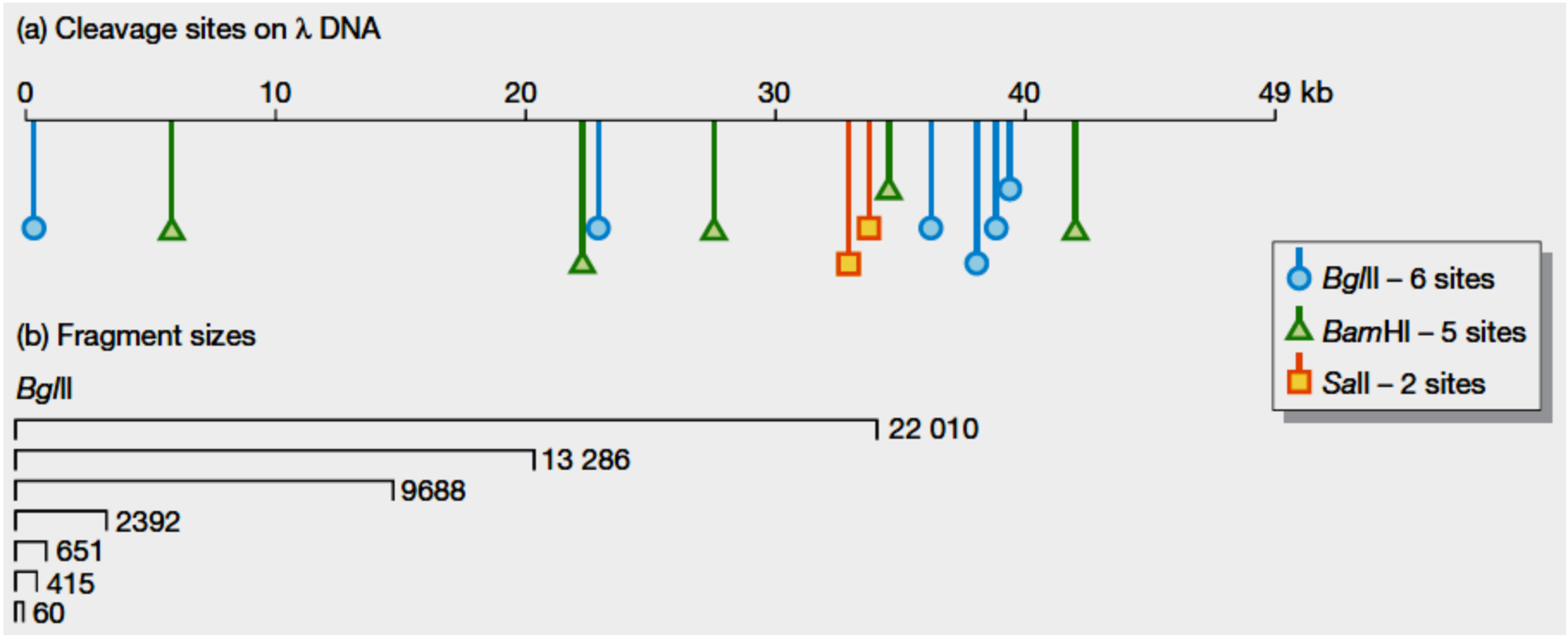
- These are called sticky or cohesive ends, as base pairing between them can stick the DNA molecule back together again.
- **Important feature of sticky end enzymes:** Restriction endonucleases with different recognition sequences may produce the same sticky ends.
- *Bam*HI (recognition sequence GGATCC) and *Bgl*II (AGATCT) produce same sticky ends i.e. GATC sticky ends.
- Fragments of DNA produced by cleavage with either of these enzymes can be joined to each other.



THE FREQUENCY OF RECOGNITION SEQUENCES IN A DNA MOLECULE:

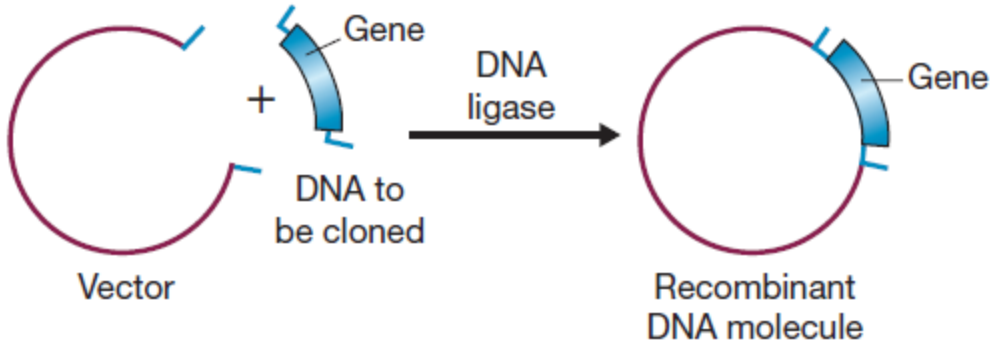
- The number of recognition sequences for a particular restriction endonuclease in a DNA molecule of known length can be calculated mathematically.
- A tetranucleotide (e.g., GATC) should occur once every $4^4 = 256$ nucleotides,
- and a hexanucleotide (e.g., GGATCC) once every $4^6 = 4096$ nucleotides.
- These calculations assume that nucleotides are ordered in a random fashion and all four nucleotides are present in equal proportions (i.e., the GC content = 50%).
- In practice, neither of these assumptions is entirely valid.
- For example, the λ DNA molecule, at 49 kb, should contain about 12 sites for a restriction endonuclease with a hexanucleotide recognition sequence.
- In fact, many of these recognition sites occur less frequent (e.g., six for *Bgl*II, five for *Bam*HI, and only two for *Sal*I), a reflection of the fact that the GC content for λ DNA is rather less than 50%.

- Furthermore, generally restriction sites are not evenly spaced out along a DNA molecule.
- If they were, then digestion with a particular restriction endonuclease would give fragments of roughly equal sizes.
- Although mathematics may give an idea of how many restriction sites to expect in a given DNA molecule but only experimental analysis can provide true picture.



DNA LIGASE - JOINING DNA MOLECULES TOGETHER:

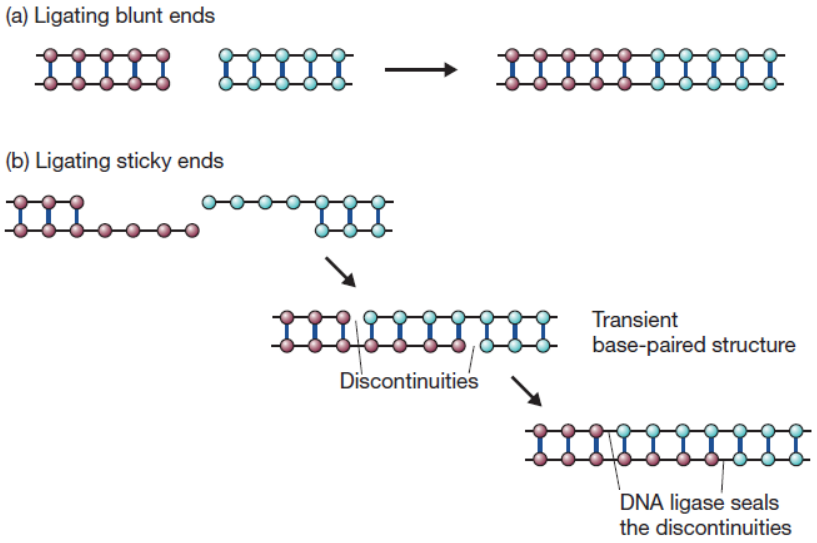
- The final step in construction of a **recombinant DNA molecule** is the joining vector molecule and the DNA to be cloned, process k/a ligation and the enzyme that catalyzes the reaction is called DNA ligase.



Mode of action of DNA ligase:

- All living cells produce DNA ligases, but the enzyme used in genetic engineering is usually purified from *E. coli* bacteria that have been infected with T4 phage.
- Within the cell the enzyme carries out the very important function of repairing any discontinuities that may arise in one of the strands of a dsDNA molecule.

- Discontinuity means position where phosphodiester bond between two adjacent nucleotides is missing (Nick means one or more nucleotides are absent).
- Although discontinuities may arise by chance, this is also a natural result of processes such as DNA replication and recombination.
- Ligases therefore play several vital roles in the cell.
- In test tube, purified DNA ligases (along with repairing ssDNA discontinuities) it can also join together individual DNA molecules or two ends of same molecule.
- The chemical reaction involved in ligating two molecules is exactly the same as discontinuity repair.



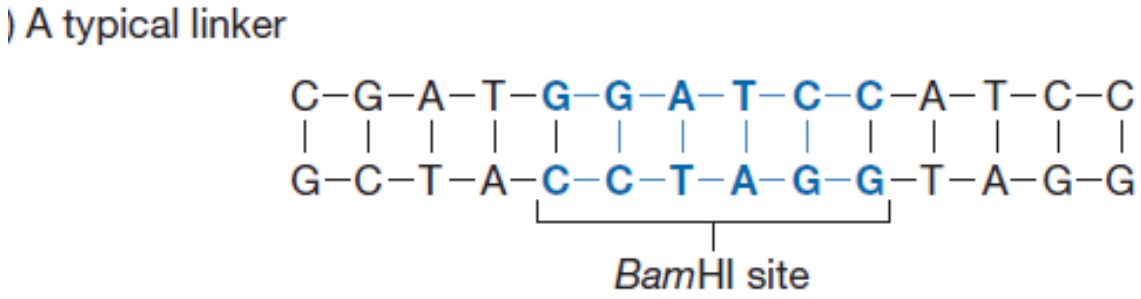
Sticky ends increase the efficiency of ligation:

- Joining of two blunt-ended fragments in a test tube it is not very efficient because the ligase is unable to “catch hold” of the molecule to be ligated.
- i.e. ligase has to wait for chance associations to bring the ends together, thus blunt end ligation is performed at high DNA concentrations to increase chances.
- In contrast, ligation of complementary sticky ends is much more efficient.
- Because compatible sticky ends can base pair with one another by hydrogen bonding, forming a relatively stable structure for the enzyme to work on.
- If the phosphodiester bonds are not synthesized fairly quickly then the sticky ends fall apart again.

- **Joining of sticky ends onto a blunt-ended DNA molecule**
- In general, complementary sticky ends are desirable on the DNA molecules to be ligated together in a gene cloning experiment.
- For cloning, these sticky ends can be provided by digesting both vector & DNA to be cloned with:
  - Either the same restriction endonuclease,
  - Or with different enzymes that produce the same sticky end
- But it is not always possible to do this.
- Therefore a common situation arises is which the vector molecule has sticky ends, but the DNA fragments to be cloned are blunt-ended.
- Under these circumstances one of the following method can be used:
  - **Linkers**
  - **Adaptors**
  - **Producing sticky ends by homopolymer tailing**

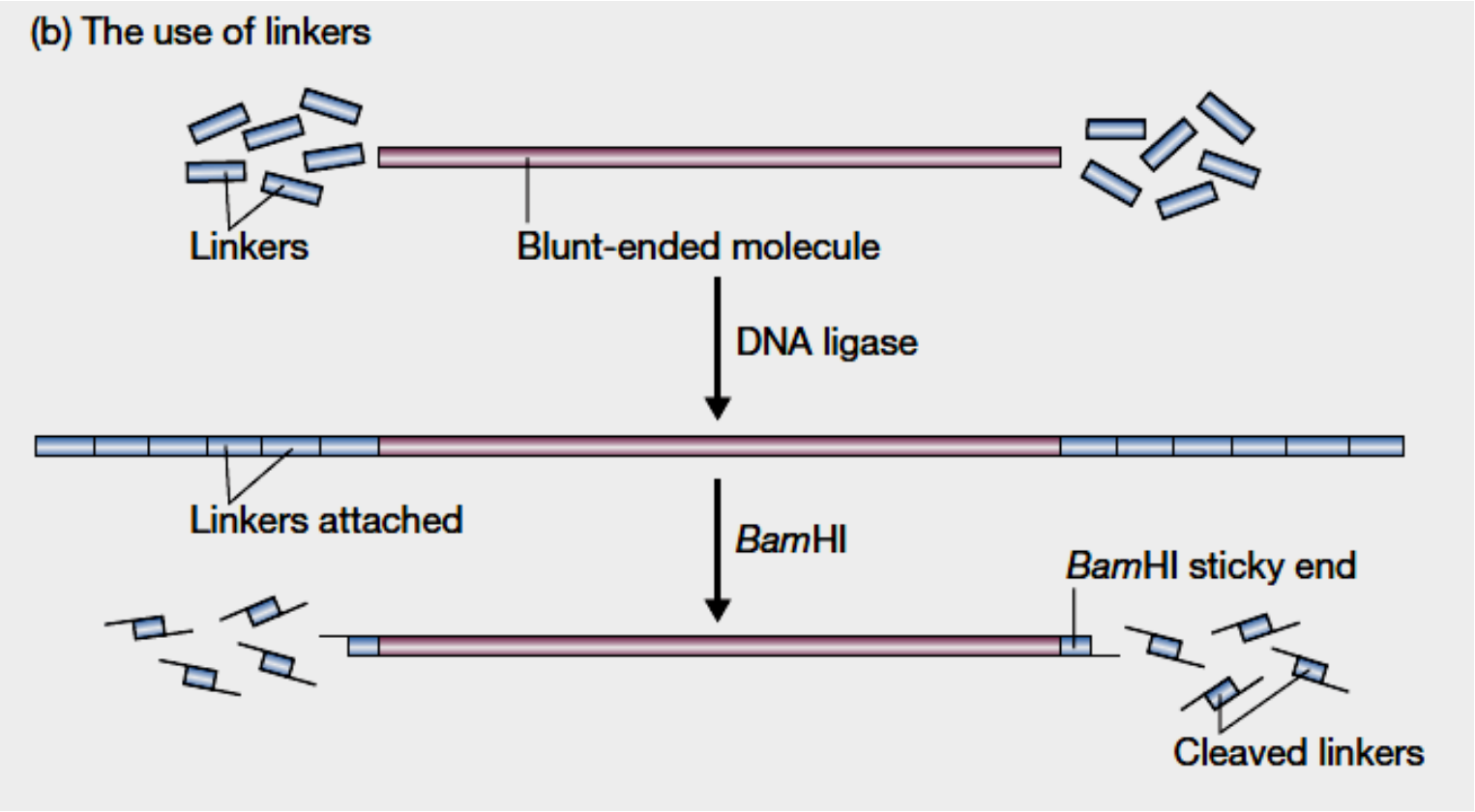
USE OF LINKERS:

- **Linkers** are short dsDNA (known sequence) synthesized in the test tube.



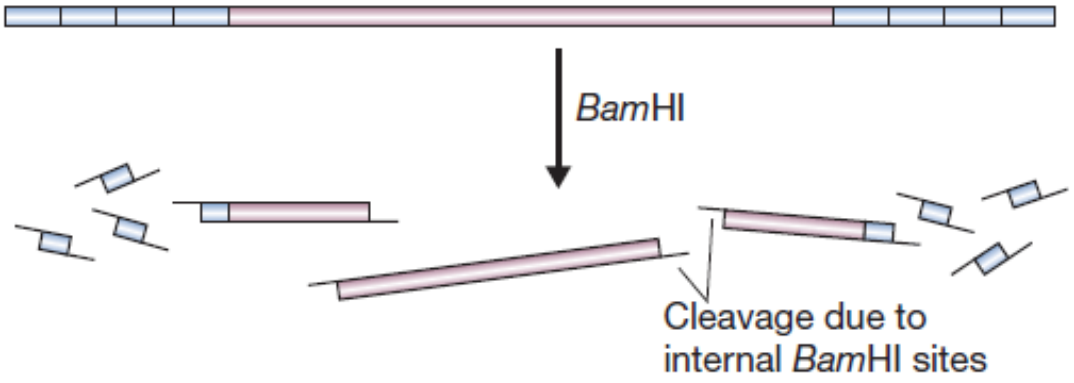
- It is blunt-ended, but contains a restriction site, *Bam*HI in the example shown.
- DNA ligase can attach linkers to the ends of larger bluntended DNA molecules.
- This reaction can be performed very efficiently because linkers are present in very high concentration for this ligation to blunt ended dsDNA to be cloned.
- Many linker will attach to each end of dsDNA molecule

- Digestion of this with *Bam*HI cleaves linkers chains at their sequences, producing a large number of cleaved linkers and the DNA fragment with *Bam*HI sticky ends.
- Thus modified dsDNA fragment is now ready for ligation into a cloning vector restricted with *Bam*HI.



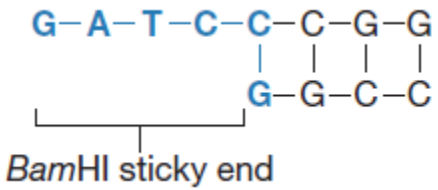
USE OF ADAPTORS:

- There is one drawback with the use of linkers i.e. if the blunt-ended DNA to be cloned have one or more *Bam*HI recognition sequences.
- And in this case, the restriction step needed to cleave the linkers to produce sticky ends would also cleave the blunt-ended DNA molecule.



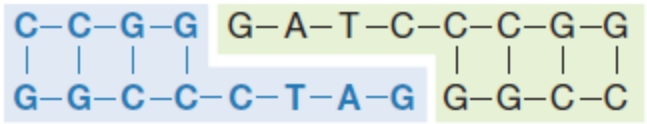
- Thus the use of adaptors for attaching sticky ends to a blunt-ended molecule is designed to avoid the above problem.
- **Adaptors** are also short synthetic oligonucleotides, and synthesized in a manner that it contains one sticky end and another blunt.

- The idea is of course to ligate the blunt end of the adaptor to the blunt ends of the DNA fragment, to produce a new molecule with sticky ends.

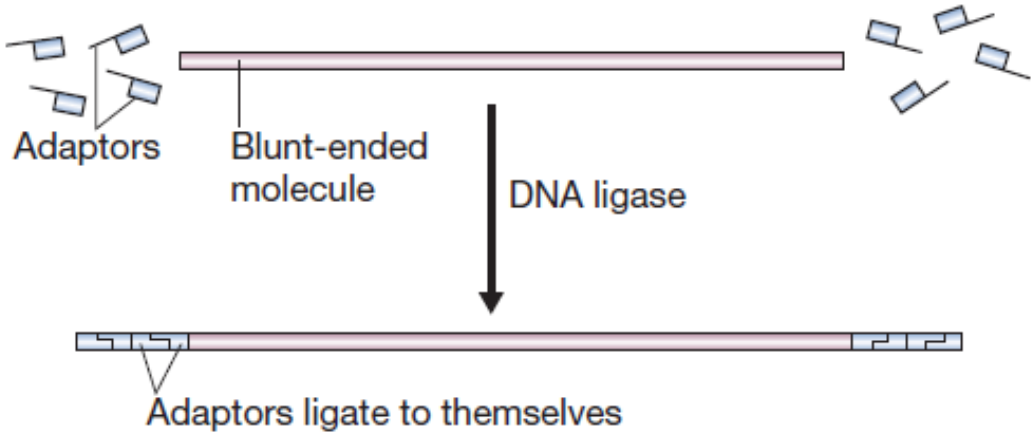


- But in practice the sticky ends of individual adaptor molecules could base pair with each other to form dimers and thus new DNA molecule is still blunt-ended.

Adaptors could ligate to one another

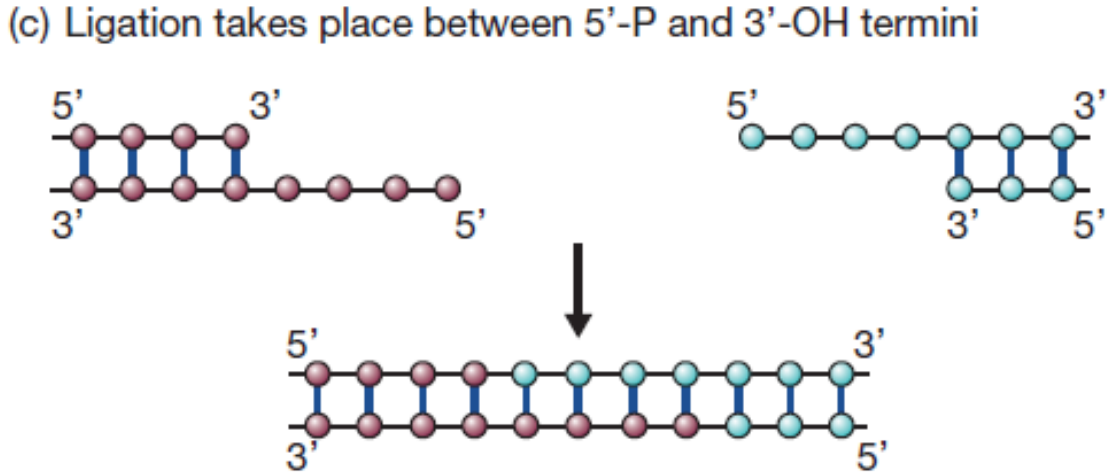


(c) The new DNA molecule is still blunt-ended

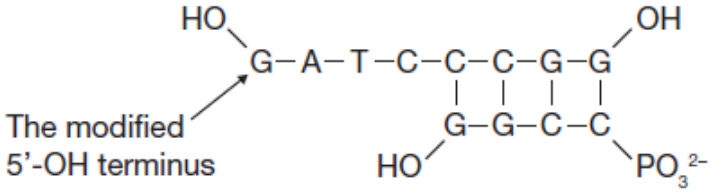


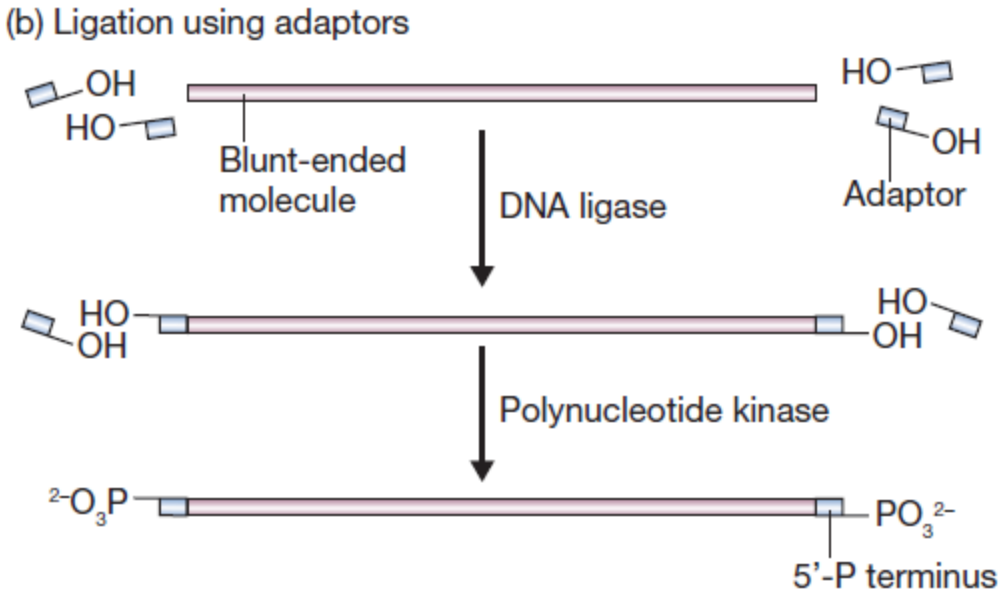
- The sticky ends could be recreated by digestion with a restriction endonuclease, but that would crush the purpose of using adaptors in place of linkers.

- The solution to this problem present in the chemical structure of the ends of the adaptor molecule.
- Normally the two ends of a polynucleotide strand are chemically distinct i.e. one end, referred to as the 5′ terminus carries a phosphate group (5′-P) and other the 3′ terminus has a hydroxyl group (3′-OH).
- In the double helix the two strands are antiparallel, so each end of a dsDNA molecule consists of one 5′-P terminus and one 3′-OH terminus.
- And ligation takes place between the **5′-P and 3′-OH** ends.



- To overcome the problem i.e. self ligation among adaptors.
- Adaptor molecules are synthesized so that the blunt end is the same as "natural" DNA, but the sticky end is different.
- The 3′-OH terminus of the sticky end is the same as usual, but the 5′-P terminus is modified: it lacks the phosphate group, and is in fact a 5′-OH terminus
- DNA ligase is unable to form a phosphodiester bridge b/w 5′-OH and 3′-OH ends.
- Although base pairing is always occurring b/w sticky ends of adaptor molecules but the association is never stabilized by ligation.



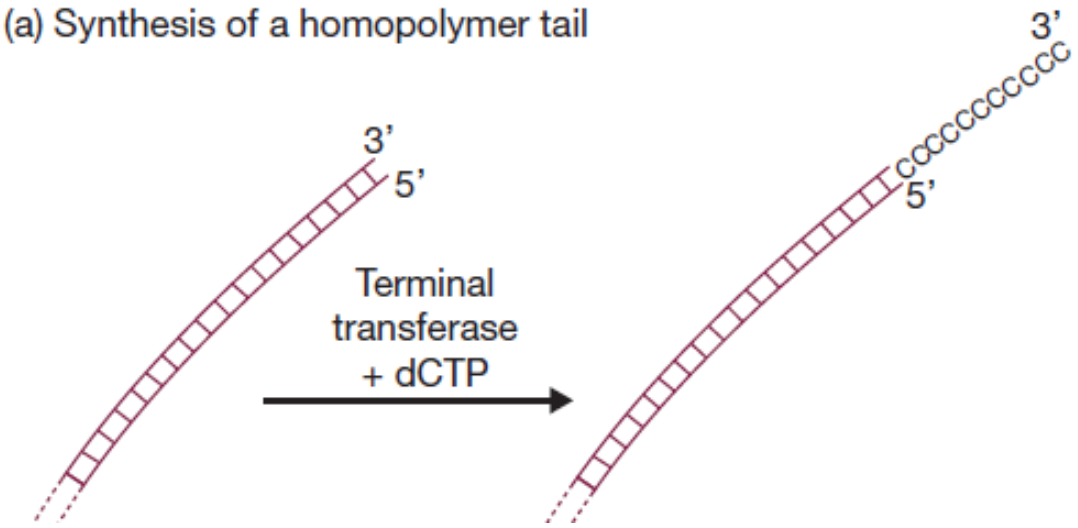


- Thus adaptors can be ligated to blunt-ended DNA but not to themselves.
- After the attachment of adaptors:
- The abnormal 5’-OH terminus is converted to the natural 5’-P.
- By the treatment of enzyme polynucleotide kinase, producing a sticky-ended fragment that can be inserted into an appropriate vector.

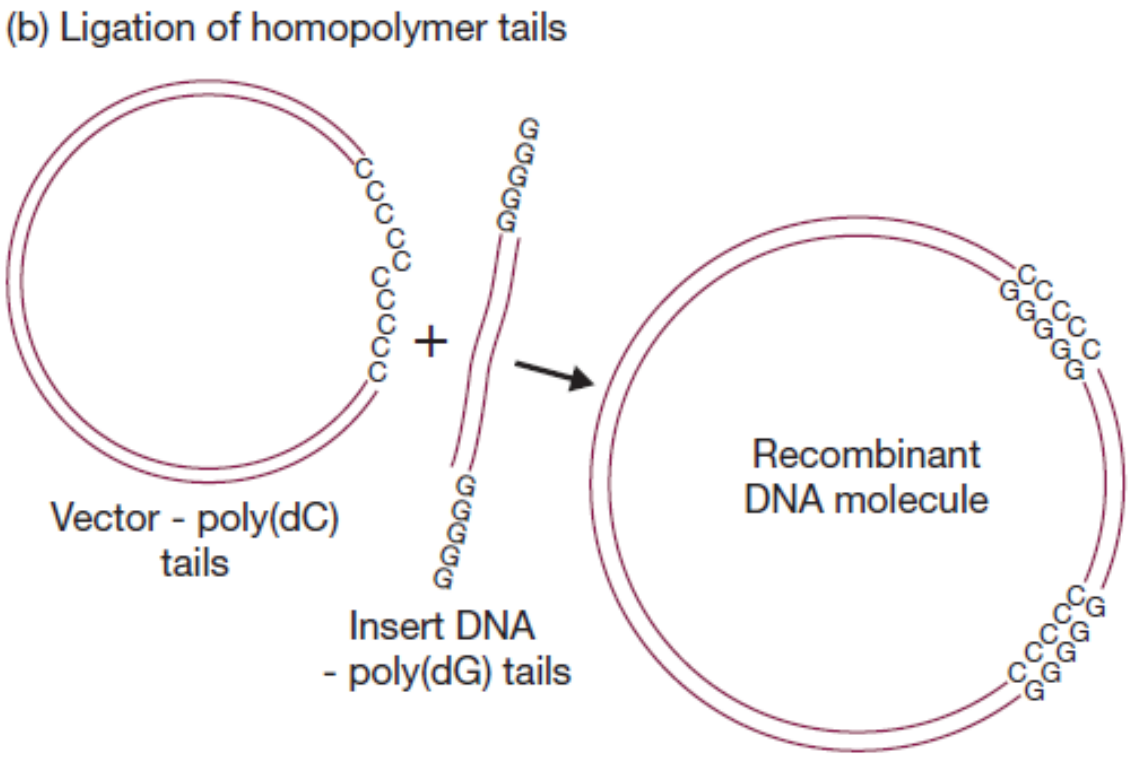
PRODUCING STICKY ENDS BY HOMOPOLYMER TAILING

- Homopolymer tailing can also produce sticky ends on blunt-ended DNA molecule.
- A homopolymer is simply a polymer in which all the subunits are the same.
- A DNA strand made up of **example** poly deoxyguanosine is a homopolymer.

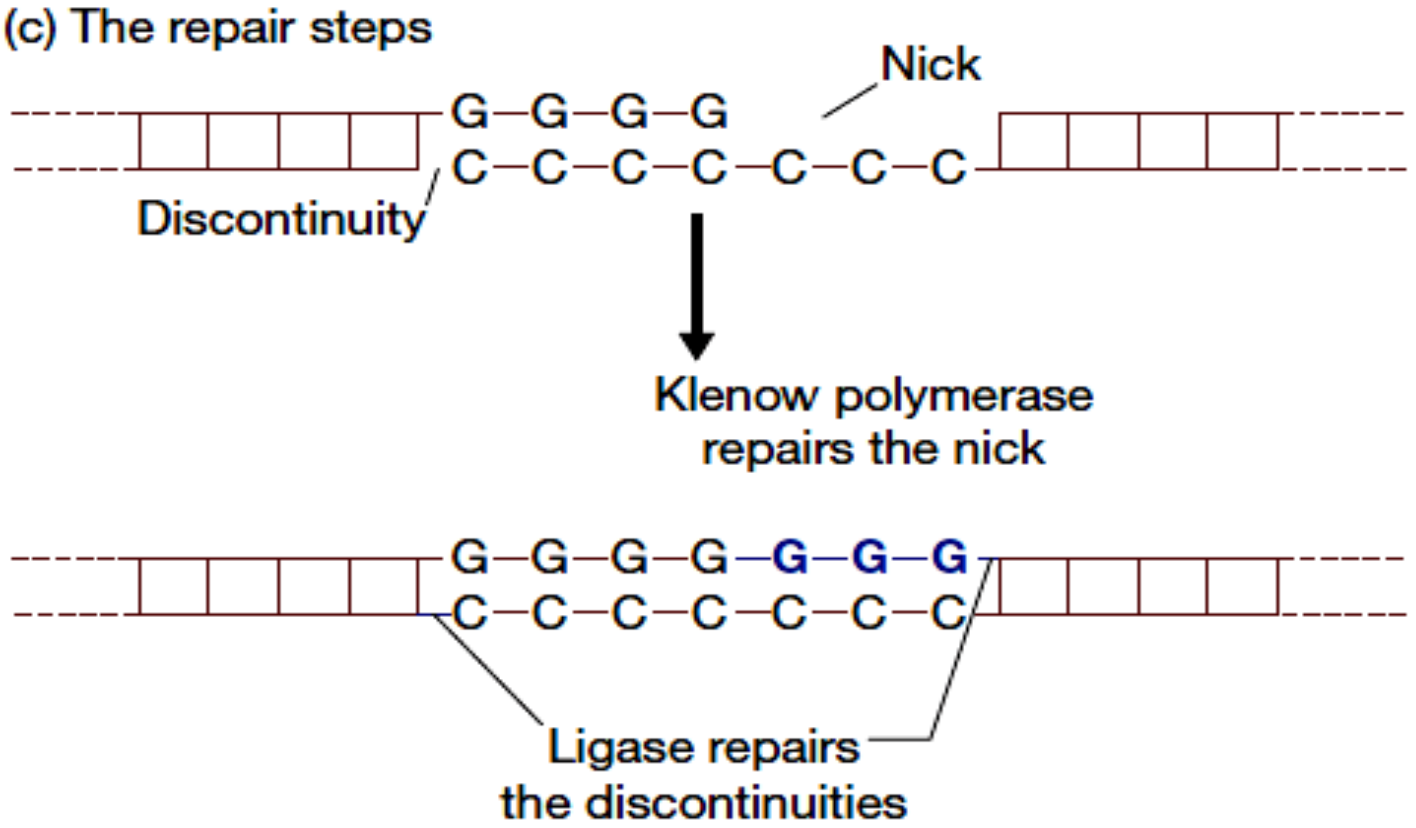
- Tailing involves using the enzyme terminal deoxynucleotidyl transferase, to add nucleotides on 3′-OH termini of dsDNA molecule.
- If this reaction is carried out in the presence of just one deoxyribonucleotide, a homopolymer tail is produced.



- For ligation, produced homopolymers (poly-C) must be complementary to vector.
- Poly-C tails are attached to the vectors having poly-G tail.
- Base pairing b/w two occurs when the DNA molecules are mixed.



- In practice, the poly-G and poly-C tails are not of exactly the same length.
- Thus the produced recombinant molecules have nicks and discontinuities.



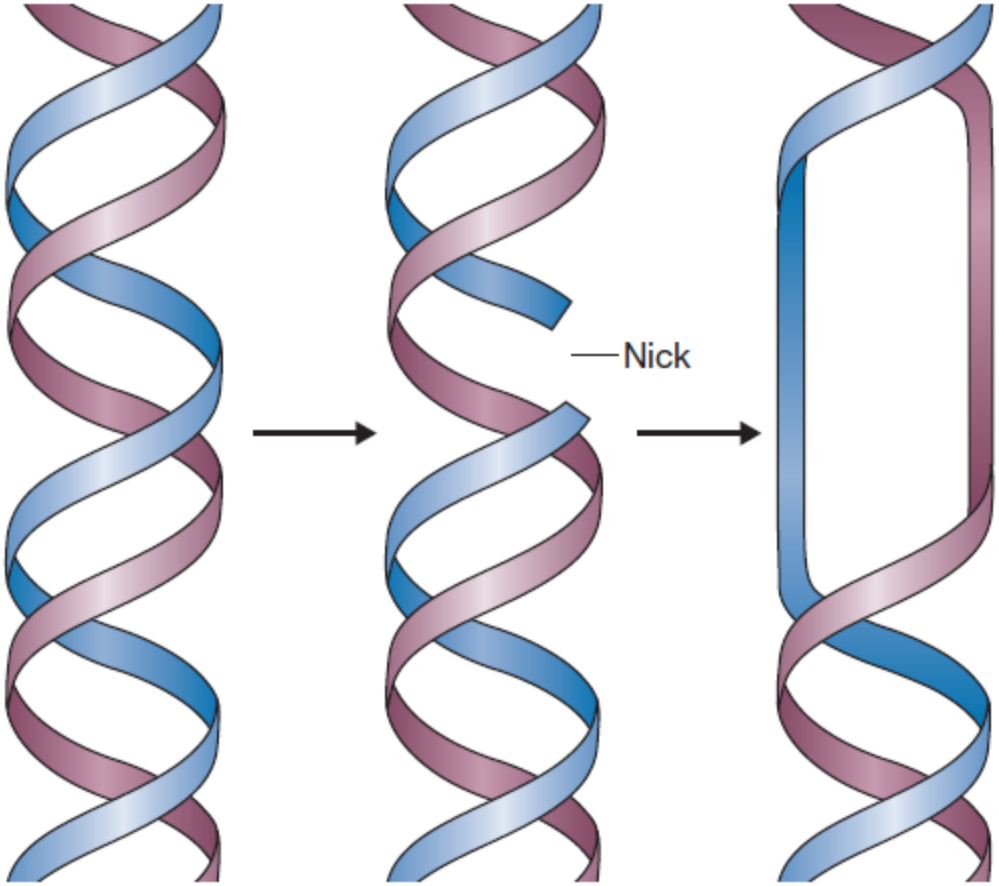
- Repair is therefore a two-step process:

1. using Klenow polymerase to fill in the nicks
2. followed by DNA ligase to synthesize the final phosphodiester bonds.

- This repair reaction does not always have to be performed in the test tube.
- Because, if the complementary homopolymer tails are longer than about 20 nucleotides, then quite stable base-paired associations are formed.
- A recombinant DNA molecule is stable enough to be introduced into the host cell.
- Once inside the host, the cell's own DNA polymerase and DNA ligase repair the recombinant DNA molecule and thus completing the construction begun in the test tube.

BLUNT END LIGATION WITH A DNA TOPOISOMERASE

- This is more sophisticated and easier technique in which a special type of enzyme called a **DNA topoisomerase** is used.
- In the cell, DNA topoisomerases are involved in processes that require **turns of the dsDNA helix to be removed** or **added to dsDNA molecule**.
- Turns are removed during DNA replication in order to unwind the helix and enable each polynucleotide to be replicated.
- DNA topoisomerases are able to separate the two strands of a DNA molecule without actually rotating the double helix.
- DNA topoisomerases achieve this by causing single- or double-stranded breakages in the DNA backbone.
- DNA topoisomerases therefore have both nuclease and ligase activities.

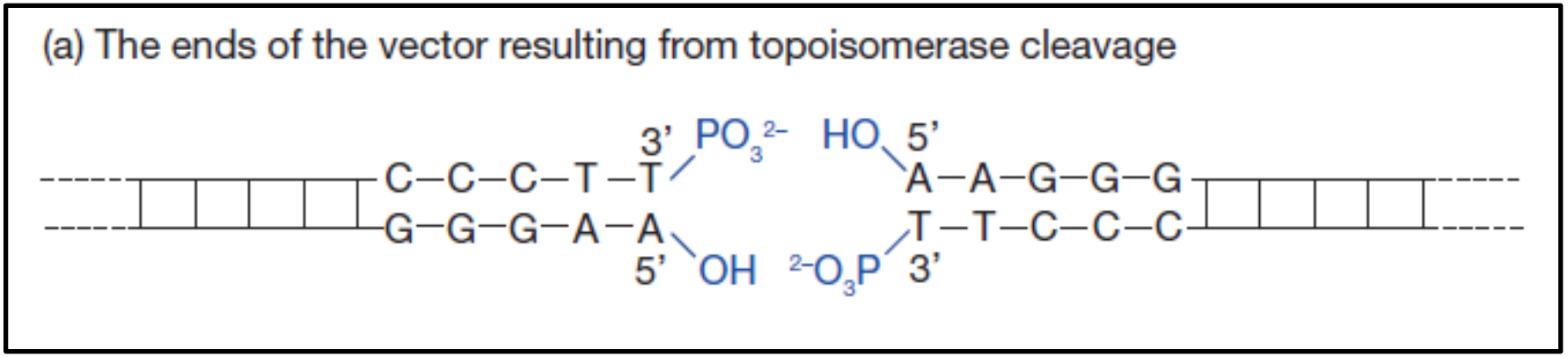


Type 1 DNA topoisomerase, which removes or adds turns to a double helix by making a transient break in one of the strands.

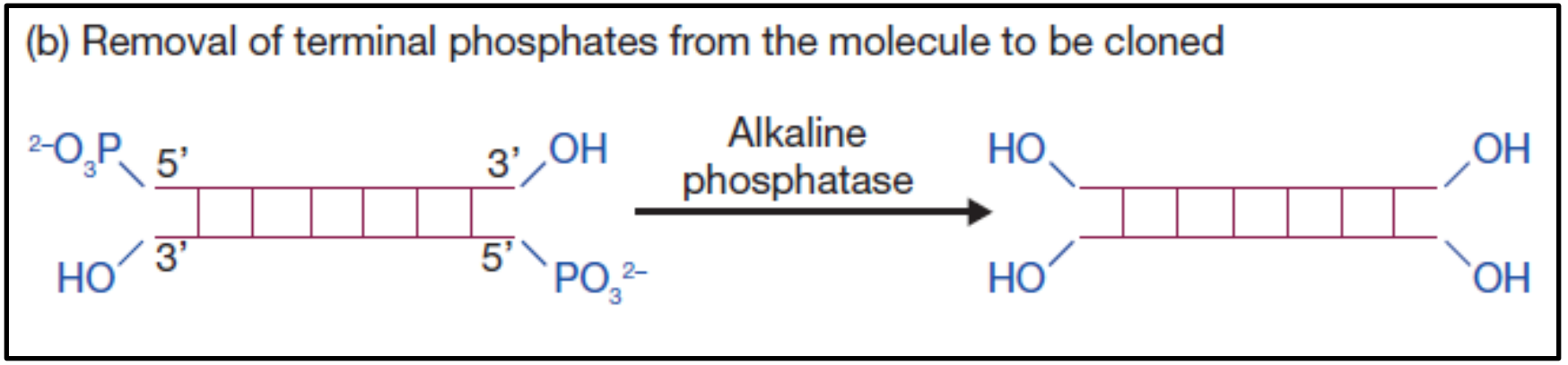
- For blunt end ligation with topoisomerase, special cloning vector is needed.
- This is a plasmid that has been linearized by nuclease activity of DNA topoisomerase enzyme from vaccinia virus.
- Vaccinia topoisomerase cuts DNA at sequence CCCTT (present once in plasmid).
- After cutting the plasmid, topoisomerase enzymes remain covalently bound to the resulting blunt ends.
- The reaction can be stopped at this point and vector is stored until it is needed.
- Cleavage by topoisomerase results in 5'-OH and 3'-P termini.

(In normal cases 5' contains -P and 3' contains -OH terminal.

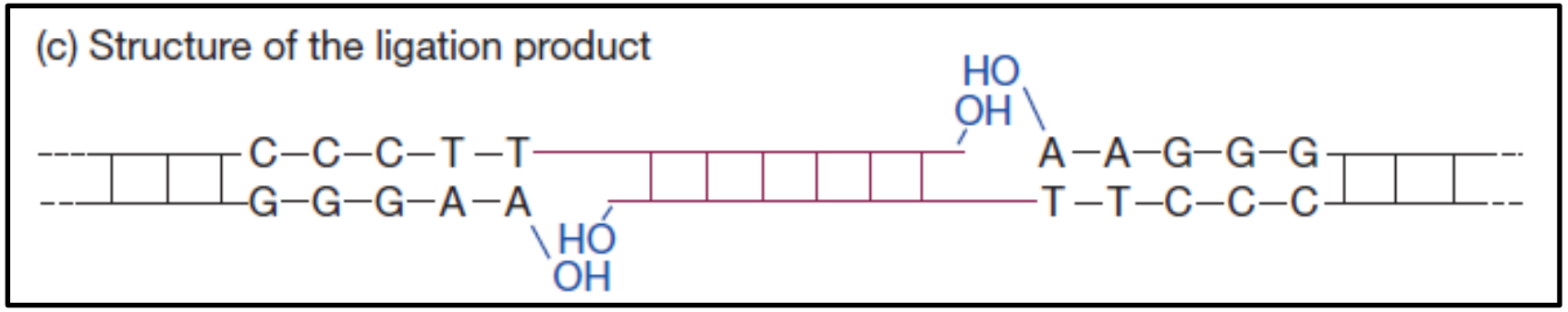
(VECTOR WITH CUTS)



- The blunt-ended dsDNA molecule, which is to be cloned, have been produced from a larger DNA by cutting with restriction enzyme.
- The blunt-ended dsDNA molecule have 5′-P and 3′-OH ends.
- Before mixing these molecules with the vector, their 5′-P must be removed to give 5′-OH ends, so that they can ligate to the 3′-P termini of the vector.
- The molecules are therefore treated with alkaline phosphatase.



- Adding the phosphatased molecules to the vector reactivates the bound topoisomerases, which proceed to the ligation phase of their reaction.
- Ligation occurs between the 3′-P ends of the vectors and the 5′-OH ends of the dsDNA molecule to be cloned.
- The blunt-ended dsDNA molecule therefore become inserted into the vectors.
- But only one strand is ligated at each junction point.
- But this is not a problem b/z this discontinuities will be repaired by cellular enzymes of the host cells when they have been introduced in host bacteria.



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THANKYOU