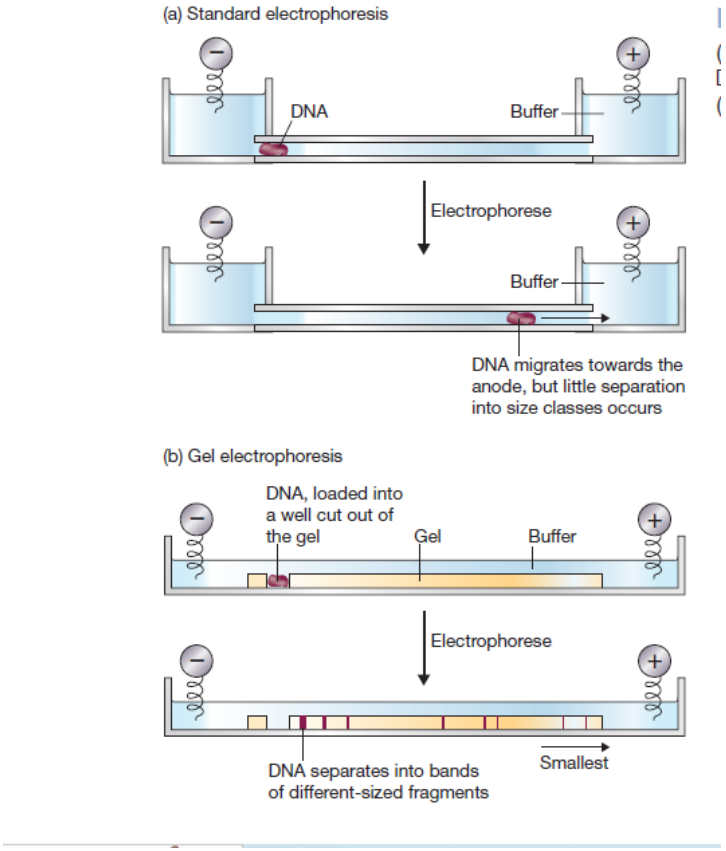
Analyzing the result of restriction endonuclease cleavage

- A restriction digest results in a number of DNA fragments, the sizes of which depend on the exact positions of the recognition sequences for the endonuclease in the original molecule. A way of determining the number and sizes of the fragments is needed if restriction endonucleases are to be of use in gene cloning. 1. Whether or not a DNA molecule is cut at all can be determined fairly easily by testing the viscosity of the solution. Larger DNA molecules result in a more viscous solution than smaller ones, so cleavage is associated with a decrease in viscosity. However, working out the number and sizes of the individual cleavage products is more difficult. In fact, for several years this was one of the most tedious aspects of experiments involving DNA.
- 2.Eventually the problems were solved in the early 1970s when the technique of gel electrophoresis was developed.



- **Electrophoresis:** is a technique that uses differences in electrical charge to separate the molecules in a mixture. DNA molecules have negative charges, and so when placed in an electric field they migrate toward the positive pole (Figure 4.12a). The rate of migration of a molecule depends on two factors, its shape and its charge-to-mass ratio. Unfortunately, most DNA molecules are the same shape and all have very similar charge-to-mass ratios. Fragments of different sizes cannot therefore be separated by standard electrophoresis.
- The size of the DNA molecule does, however, become a factor if the electrophoresis is performed in a gel. A gel, which is usually made of agarose, polyacrylamide, or a mixture of the two, comprises a complex network of pores, through which the DNA molecules must travel to reach the positive electrode. The smaller the DNA molecule, the faster it can migrate through the gel. **Gel electrophoresis** therefore separates DNA molecules according to their size.

- In practice the composition of the gel determines the sizes of the DNA molecules that can be separated. A 0.5 cm thick slab of 0.5% agarose, which has relatively large pores, would be used for molecules in the size range 1–30 kb, allowing, for example, molecules of 10 and 12 kb to be clearly distinguished. At the other end of the scale, a very thin (0.3 mm) 40% polyacrylamide gel, with extremely small pores, would be used to separate much smaller DNA molecules, in the range 1–300 bp, and could distinguish molecules differing in length by just a single nucleotide.
- Visualizing DNA molecules in an agarose gel
- The easiest way to see the results of a gel electrophoresis experiment is to stain the gel with a compound that makes the DNA visible. Ethidium bromide (EtBr), is also routinely used to stain DNA in agarose and polyacrylamide gels (Figure 2). Bands showing the positions of the different size classes of DNA fragment are clearly visible under ultraviolet irradiation after EtBr staining, so long as sufficient DNA is present. Unfortunately, the procedure is very hazardous because ethidium bromide is a powerful mutagen. EtBr staining also has limited sensitivity, and if a band contains less than about 10 ng of DNA then it might not be visible after staining.

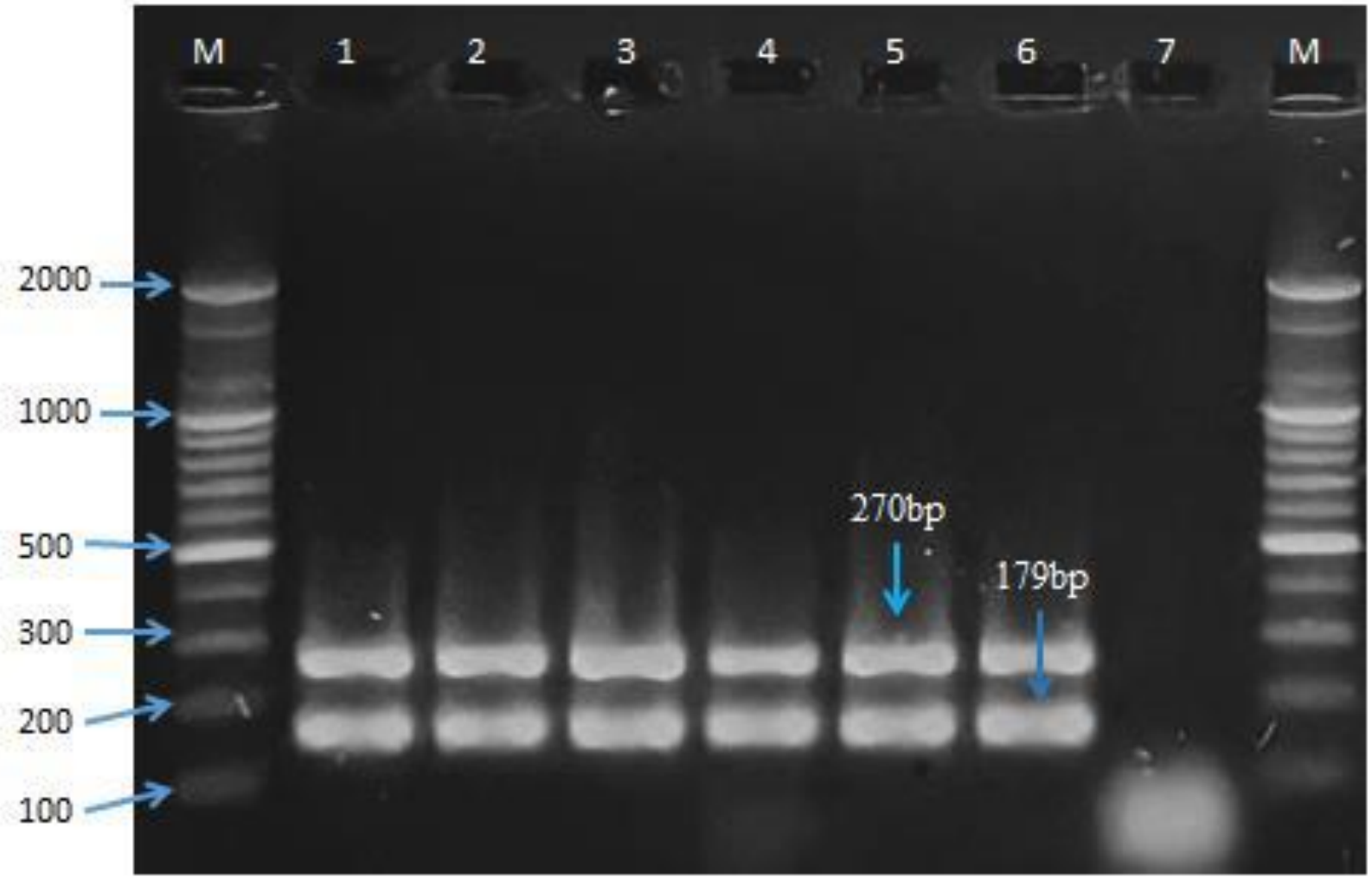
- Why Ethidium bromide dye regards a mutagenic agent? Explain

Its mutagenic properties arise from its ability to intercalate between the base pairs of DNA molecules.

When ethidium bromide is added to a DNA sample and exposed to UV light, it becomes fluorescent, allowing researchers to visualize the DNA bands under UV light. However, ethidium bromide molecules can insert themselves between the base pairs of DNA, causing structural distortions and potentially interfering with DNA replication and transcription processes. This intercalation can lead to mutations in the DNA sequence. Additionally, ethidium bromide can also induce DNA damage directly through the generation of free radicals when exposed to UV light. These free radicals can cause DNA strand breaks and other types of damage, further contributing to its mutagenic effects.

- For this reason, non-mutagenic dyes that stain DNA green, red, or blue are now
- used in many laboratories. Most of these dyes can be used either as a post-stain after electrophoresis for EtBr, or alternatively, because they are non-hazardous, they can be included in the buffer solution in which the agarose or polyacrylamide is dissolved when the gel is prepared. Some of these dyes require ultraviolet irradiation in order to make the bands visible, but others are visualized by illumination at other wavelengths, for example under blue light, removing a second hazard as ultraviolet radiation can cause severe burns. The most sensitive dyes are able to detect bands that contain less than 1 ng DNA.

- Non-mutagenic dyes are preferred alternatives to mutagenic dyes like ethidium bromide for staining nucleic acids in gel electrophoresis. These dyes typically work by binding specifically to DNA or RNA molecules without causing mutations or significant structural alterations. Here are some common mechanisms used by non-mutagenic dyes: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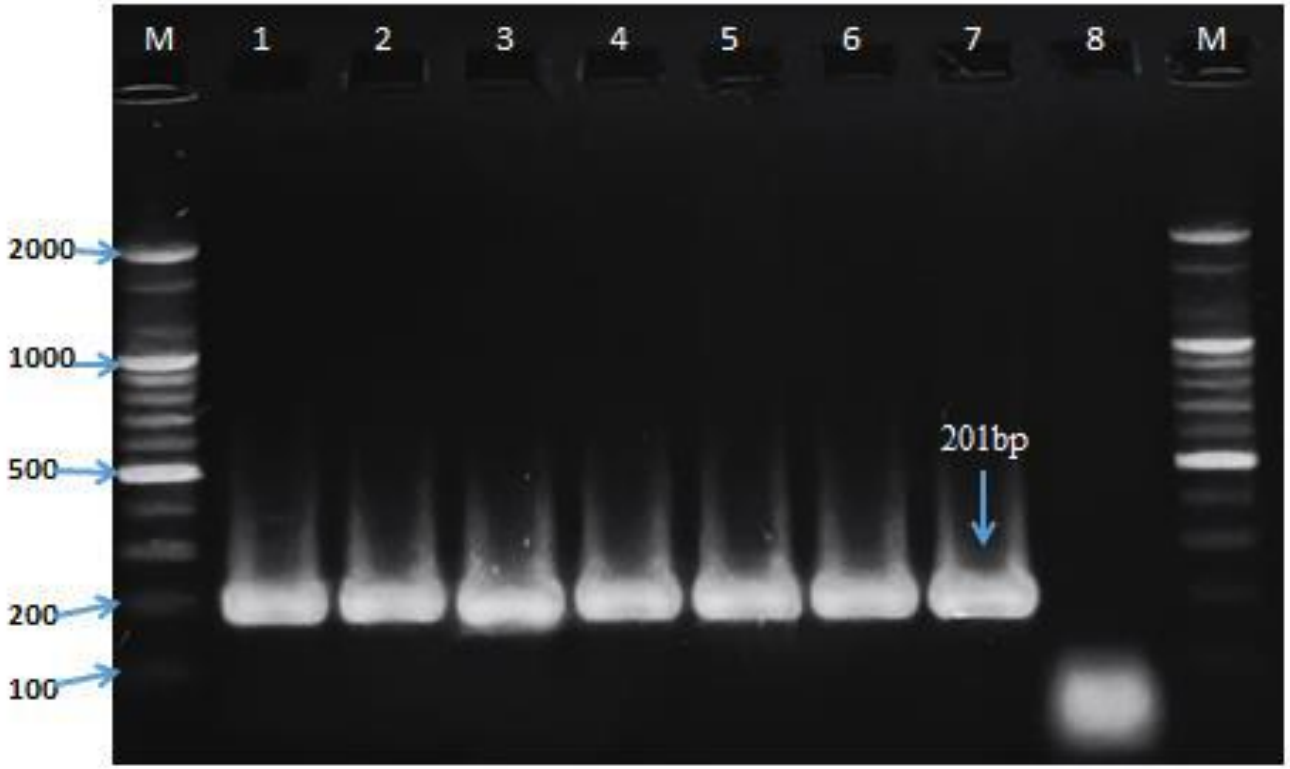
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- Intercalating dyes: Similar to ethidium bromide, some non-mutagenic dyes intercalate between the base pairs of DNA or RNA. However, they are designed to have minimal mutagenic effects. These dyes insert themselves between the stacked base pairs without significantly distorting the DNA structure or interfering with replication and transcription processes.
- Exclusion dyes: Instead of intercalating between base pairs, exclusion dyes bind to the sugar-phosphate backbone of DNA or RNA molecules. They do not penetrate into the interior of the double helix but rather attach to the exterior. These dyes typically have charged groups that interact with the negatively charged phosphate groups of nucleic acids, allowing for specific binding.
- Non-specific binding dyes: Some non-mutagenic dyes bind to nucleic acids through non-specific interactions such as electrostatic attractions or hydrogen bonding. These dyes may not have a specific binding site on the nucleic acid molecule but still provide sufficient staining for visualization in gel electrophoresis.

- Non-covalent binding: Non-mutagenic dyes usually bind to DNA or RNA molecules through reversible non-covalent interactions. This means they can easily dissociate from the nucleic acids after staining, reducing the risk of interfering with downstream applications like PCR or cloning.
- Minimal phototoxicity: Non-mutagenic dyes are designed to have minimal phototoxicity. They are less prone to induce DNA damage or generate free radicals when exposed to UV light compared to mutagenic dyes like ethidium bromide.
- Common examples of non-mutagenic dyes used in gel electrophoresis include SYBR Safe, GelRed, GelGreen, and SYBR Gold. These dyes provide sensitive and reliable detection of nucleic acids while minimizing the risks associated with mutagenic staining agents.

- **Estimation of the sizes of DNA molecules**
- Gel electrophoresis separates different sized DNA molecules, with the smallest molecules traveling the greatest distance toward the positive electrode. If several DNA fragments of varying sizes are present (the result of a successful restriction digest, for example), then a series of bands appears in the gel. How can the sizes of these fragments be determined?
- The most accurate method is to make use of the mathematical relationship that links migration rate to molecular mass. The relevant formula is:
- $D = a - b(\log M)$

where *D* is the distance moved,

M is the molecular mass,

and *a* and *b* are constants that depend on the electrophoresis conditions.

- Because extreme accuracy in estimating DNA fragment sizes is not always necessary, a much simpler though less precise method is more generally used. A standard restriction digest, comprising fragments of known size, is usually included in each electrophoresis gel that is run. Restriction digests of e DNA are often used in this way as size markers. For example, *Hin*dIII cleaves e DNA (E-DNA," often written as eDNA, stands for environmental DNA. It refers to genetic material obtained directly from environmental samples such as soil, water, or air, rather than from individual organisms) into eight fragments, ranging in size from 125 bp for the smallest to over 23 kb for the largest. As the sizes of the fragments in this digest are known, the fragment sizes in the experimental digest can be estimated by comparing the positions of the bands in the two tracks (Figure 3).

- **Ligation**
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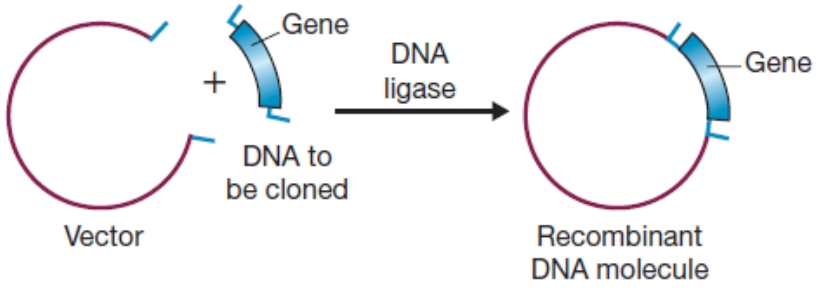
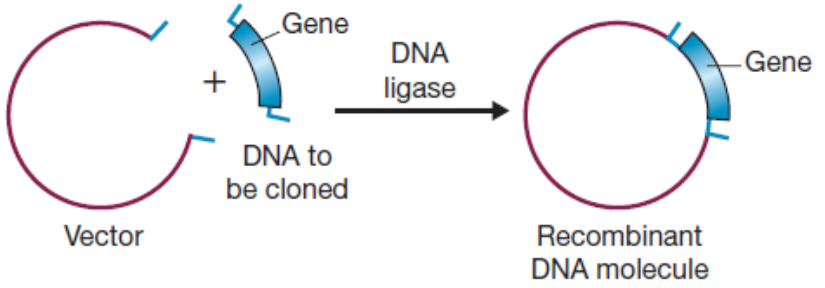
- **Ligation – joining DNA molecules together**
- The final step in construction of a recombinant DNA molecule is the joining together of the vector molecule and the DNA to be cloned (Figure 4.19). This process is referred to as ligation, and the enzyme that catalyzes the reaction is called DNA ligase.

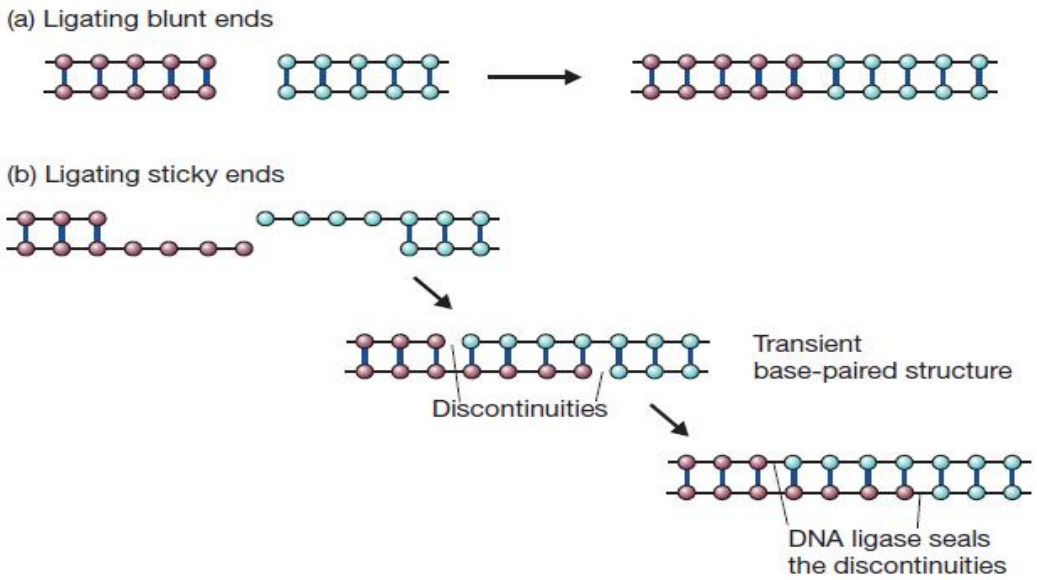


4.3.1 The mode of action of DNA ligase

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 double-stranded molecule (see Figure 4.4a). A discontinuity is quite simply a position where a phosphodiester bond between adjacent nucleotides is missing (contrast this with a nick, where one or more nucleotides are absent). Although discontinuities may arise by chance breakage of the cell's DNA molecules, they are also a natural result of processes such as DNA replication and recombination.

Ligases therefore play several vital roles in the cell. In the test tube, purified DNA ligases, as well as repairing single-strand discontinuities, can also join together individual DNA molecules or the two ends of the same molecule. The chemical reaction involved in ligating two molecules is exactly the same as discontinuity repair, except that two phosphodiester bonds must be made, one for each strand (Figure 4.20a).

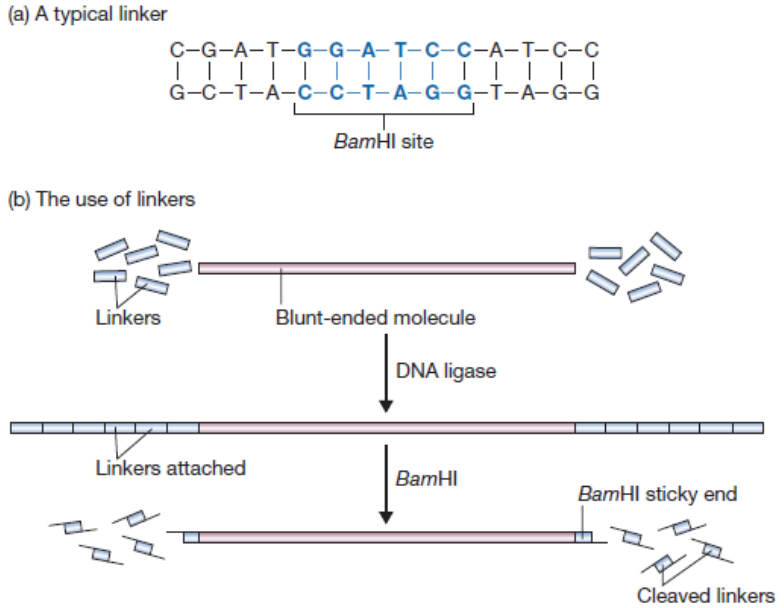




- **Sticky ends increase the efficiency of ligation**
- The ligation reaction shows two blunt-ended fragments being joined together. Although this reaction can be carried out in the test tube, it is not very efficient. This is because the ligase is unable to “catch hold” of the molecule to be ligated, and has to wait for chance associations to bring the ends together. If possible, blunt end ligation should be performed at high DNA concentrations, to increase the chances of the ends of the molecules coming together in the correct way.
- In contrast, ligation of complementary sticky ends is much more efficient. This is because compatible sticky ends can base pair with one another by hydrogen bonding (Figure 4.20b), 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. These transient, base-paired structures do, however, increase the efficiency of ligation by increasing the length of time the ends are in contact with one another.

- **Putting sticky ends onto a blunt-ended molecule**
- For the reasons detailed in the preceding section, compatible sticky ends are desirable on the DNA molecules to be ligated together in a gene cloning experiment. Often these sticky ends can be provided by digesting both the vector and the DNA to be cloned with the same restriction endonuclease, or with different enzymes that produce the same sticky end, but it is not always possible to do this. A common situation is where the vector molecule has sticky ends, but the DNA fragments to be cloned are blunt-ended. Under these circumstances one of three methods can be used to put the correct sticky ends onto the DNA fragments.

- **Linkers**
- The first of these methods involves the use of **linkers**. These are short pieces of doublestranded DNA, of known nucleotide sequence, that are synthesized in the test tube.
- A typical linker is shown in Figure. 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. Although a blunt end ligation, this particular reaction can be performed very efficiently because synthetic oligonucleotides, such as linkers, can be made in very large amounts and added into the ligation mixture at a high concentration



- ***Adaptors***
- There is one potential drawback with the use of linkers. Consider what would happen if the blunt-ended molecule shown in Figure 4.21b contained one or more *Bam*HI recognition sequences. If this was the case, the restriction step needed to cleave the linkers and produce the sticky ends would also cleave the blunt-ended molecule (Figure 4.22). The resulting fragments will have the correct sticky ends, but that is no consolation if the gene contained in the blunt-ended fragment has now been broken into pieces.
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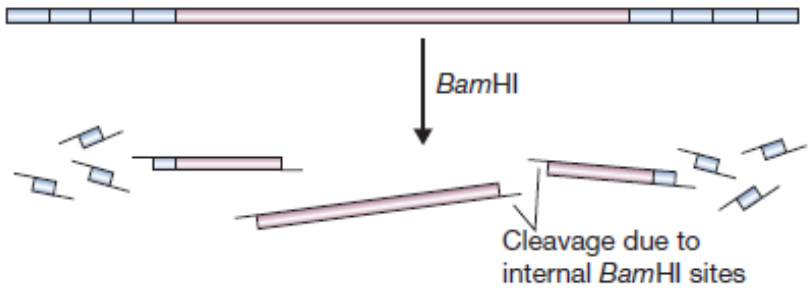

- The second method of attaching sticky ends to a blunt-ended molecule is designed to avoid this problem. **Adaptors**, like linkers, are short synthetic oligonucleotides. But unlike linkers, an adaptor is synthesized so that it already has one sticky end (Figure 4.23a). The idea is of course to ligate the blunt end of the adaptor to the blunt ends of the DNA fragment, to produce a new molecule with sticky ends. This may appear to be a simple method but in practice a new problem arises. The sticky ends of individual adaptor molecules could base pair with each other to form dimers (Figure 4.23b), so that the new DNA molecule is still blunt-ended (Figure 4.23c). The sticky ends could be recreated by digestion with a restriction endonuclease, but that would defeat the purpose of using adaptors in the first place.