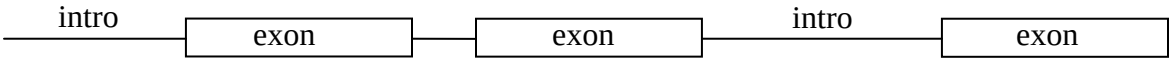
Lecture notes:

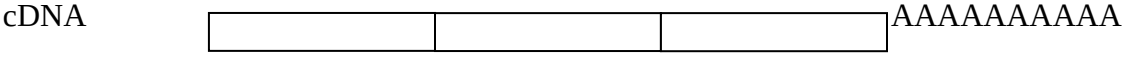
RT-PCR: Cloning cDNAs

Genes in higher organisms are not continuous sequences; they exist on the chromosomes as separate pieces of coding region that must be stitched together.

The various bits of DNA that actually code for protein are called **exons** and the intervening sequences ("junk" DNA) that separate them are called **introns**. When mRNA is first transcribed from a gene, it carries a literal transcription of the whole gene region, exons and introns both.



During subsequent processing of the mRNA, the introns are **spliced** out and the exons stitched together to make a single continuous coding region. If this spliced mRNA is **reverse transcribed** into DNA, that DNA is called a **cDNA** (c for complementary). Making a cDNA copy of a mRNA can be done using **reverse transcriptase**, and polymerase made by retroviruses.



All processed mRNAs also are polyadenylated (have a poly-A tail).

If you isolate TOTAL RNA from a cell, the majority of RNA you will get is rRNA and tRNA. mRNA makes up only a small fraction of all the RNA in a cell.

Reverse transcription, like all DNA synthesis, requires a primer.

- **Sequence-specific primer**: will reverse transcribe only a specific mRNA
- **Oligo dT primer:** will prime reverse transcription of any mRNA (will bind to any poly-A tail)
- **Random hexamers:** a mixture of 6 nt long primers containing all possible sequences of 6 nt; these will hybridize all over the place and prime reverse transcription of any RNA (in various sizes)

In our experiment, both RT and PCR are performed in the same tube. One primer acts as a sequence-specific primer for first strand cDNA synthesis AND as one of the primers for PCR. The second primer in the mix acts as the other PCR primer.

Using RT-PCR, we expect to amplify a 700 bp band from an antibody mRNA isolated from hybridoma cells which express this gene at a high level.

Notes: Topo – TA cloning

Strategies for cloning PCR products:

- When the sequence of your product is known, AND appropriate restriction sites are available, you can directly digest the PCR product and use **sticky end ligation** to clone it into a plasmid. {We did this with the λ DNA PCR.}
- If appropriate restriction sites are NOT available, you can **add restriction sites to 5' ends of primers.** These sequences are not expected to hybridize to the target DNA but will be incorporated into each PCR product.
    - PROBLEMS: Some restriction enzymes will not cut near ends; adding sequence to the primers can lead to nonspecific amplification of unwanted PCR products
- **TA ligation**. *Taq* (though not all polymerases used for PCR) adds a single A overhang to the 3' end of each DNA it synthesizes. This tiny sticky end can be used for cloning into a vector with single T overhangs.
- **Blunt end ligation**. The single A overhangs can be removed from *Taq* PCR products using enzymes with single strand nuclease activity.

Topoisomerases are a class of enzymes responsible for winding and unwinding DNA. Type I topoisomerases break one strand of a DNA molecule, conserve the energy of bond breakage by covalently attaching themselves to the DNA. Using the energy thus stored, topo I can then seal the nick or re-ligate the DNA backbone, forming a new phosphodiester bond and releasing itself in the process.

Vaccinia virus produces an unusual topoisomerase that is **sequence specific** (other topoisomerases generally are not). It will bind to its recognition sequence and make a single strand nick in the DNA at a specific site.

The Topo TA cloning kit we are using comes with a plasmid already cut by topo I. Therefore each cut end of the plasmid has a topo I enzyme covalently attached to it (2 enzymes per plasmid, one for each strand as topo I only works on one strand of the DNA.). *Vaccinia* topoisomerase was used; the recognition sites on each strand in the plasmid were cleverly aligned so that cleavage of each strand effectively provides a single T overhang for binding to the A overhang on a PCR product.

Ligations in this system are very fast because the covalently bound topoisomerase also performs the ligation. Unlike reactions performed by DNA ligase, no exogenous ATP is required.