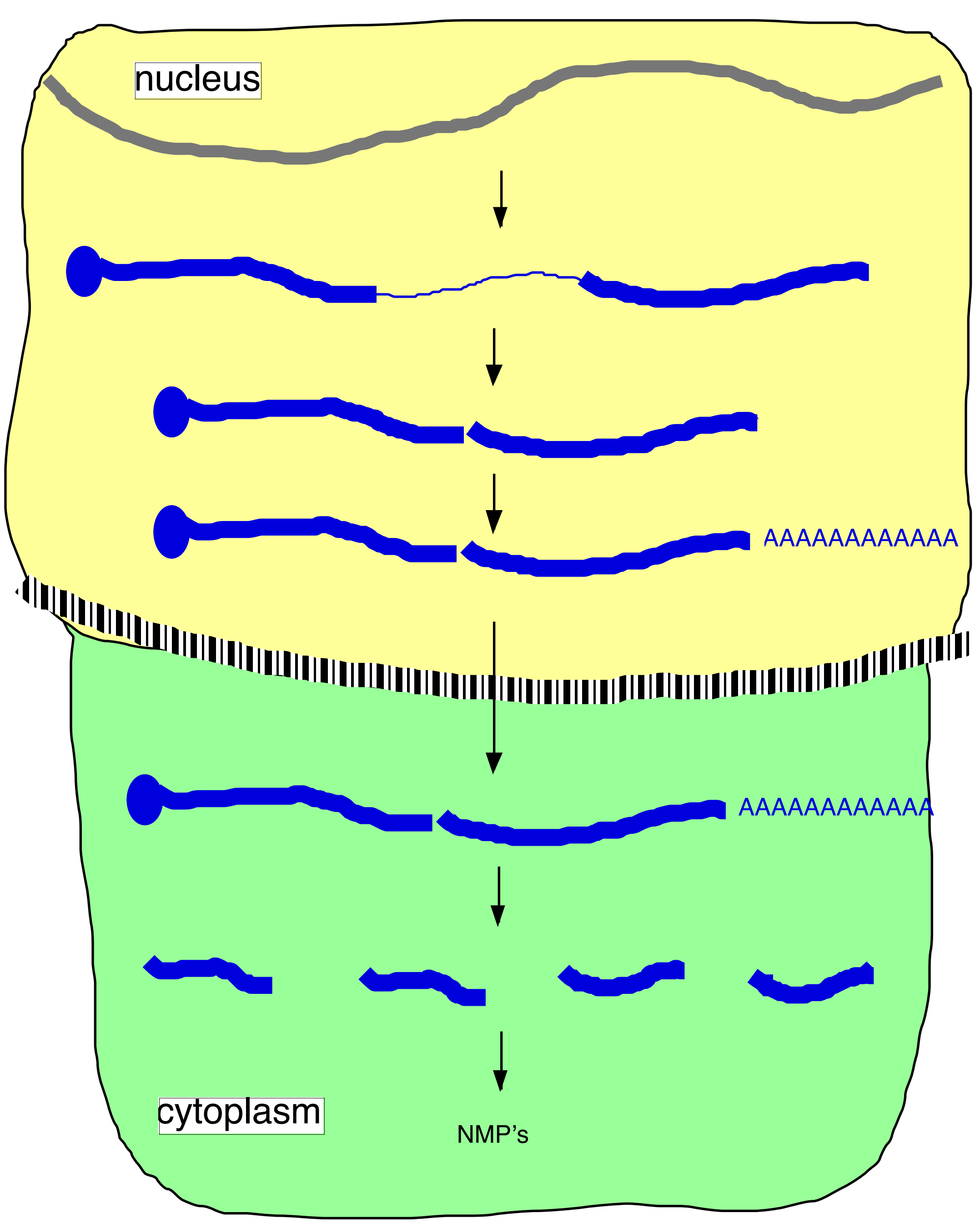
The fundamental question: What are the rate-limiting steps in the production of mRNA in a plant cell?

Why ask this question?

- **[mRNA] is usually the parameter that determines the level of expression of a given gene**
- **understanding how [mRNA] is determined lends insight into the bases for action of important genes**
- **understanding how [mRNA] is determined is important for applying biotechnological tools to problems in plant science**

How do we ask this question?

- **theoretical considerations**
- **direct experimentation**



nucleus
AAAAAAAAAAAA
AAAAAAAAAAAA
cytoplasm
NMP's

the predominant form of a given mRNA species in a cell is the cytoplasmic form

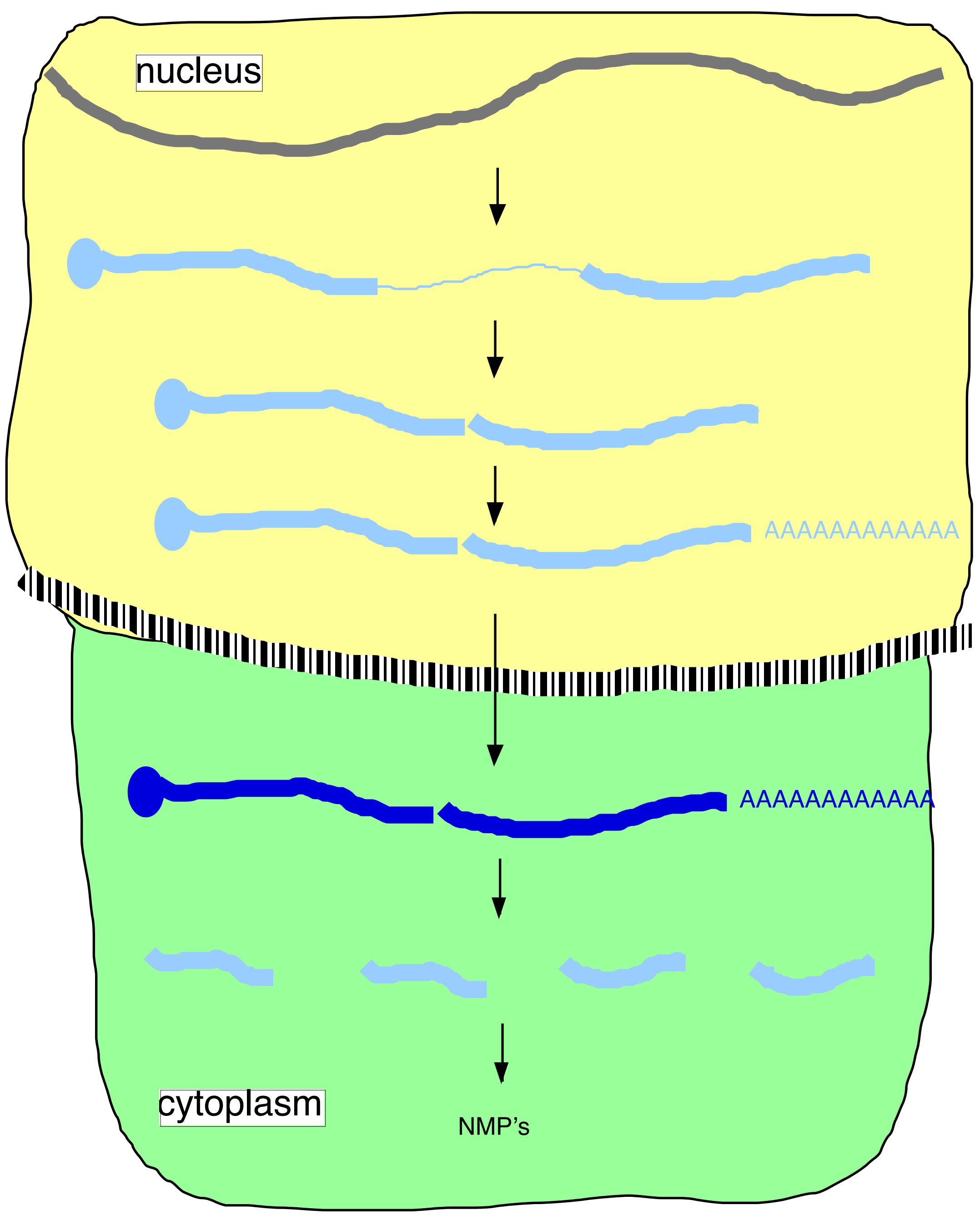
→ cytoplasmic mRNA is produced more rapidly than it is turned over

→ RNA turnover in the cytoplasm is a rate-limiting step in the course of mRNA biogenesis

partially processed forms of an mRNA do not accumulate

→ mRNA processing is more "rapid" that the production of the primary transcript

→ production of the primary transcript is rate-limiting in the course of mRNA biogenesis


nucleus
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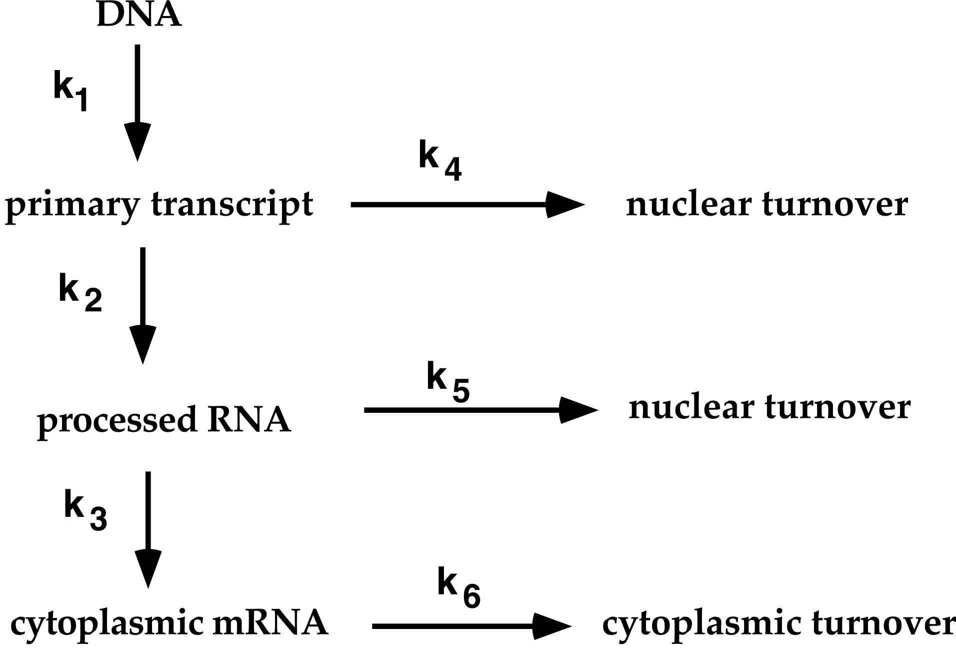

DNA
k1
primary transcript
k4
nuclear turnover
k2
processed RNA
k5
nuclear turnover
k3
cytoplasmic mRNA
k6
cytoplasmic turnover

$$\frac{d[\text{primary RNA}]}{dt} = k_1\,[\text{DNA}] - (k_2 + k_4)\,[\text{primary RNA}]$$

$$\frac{d[\text{primary RNA}]}{dt} = 0 \quad ==>$$

$$[\text{primary RNA}] = \frac{k_1\,[\text{DNA}]}{k_2 + k_4}$$

$$\frac{d[\text{processed RNA}]}{dt} = k_2\,[\text{primary RNA}] - (k_3 + k_5)\,[\text{processed RNA}]$$

$$\frac{d[\text{processed RNA}]}{dt} = 0 \quad ==>$$

$$[\text{processed RNA}] = \frac{k_2\,[\text{primary RNA}]}{k_3 + k_5}$$

$$\frac{d[\text{mRNA}]}{dt} = k_3\,[\text{processed RNA}] - k_6\,[\text{primary RNA}]$$

$$\frac{d[\text{mRNA}]}{dt} = 0 \quad ==>$$

$$[\text{mRNA}] = \frac{k_3\,[\text{processed RNA}]}{k_6}$$

$$\boxed{[\text{mRNA}] = \frac{k_1\,k_2\,k_3}{(k_2 + k_4)\,(k_3 + k_5)\,k_6}\,[\text{DNA}]}$$

Some special cases:

when nuclear RNA turnover is slow (e.g $k_2 >> k_4$ and $k_3 >> k_5$), then

$$[mRNA] = \frac{k_1}{k_6}\,[DNA]$$

when nuclear RNA turnover is fast (e.g. $k_2 << k_4$ and $k_3 << k_5$), then:

$$[mRNA] = \frac{k_1\,k_2\,k_3}{k_4\,k_5\,k_6}\,[DNA]$$

Identifying the important steps that determine changes in [mRNA]

For a change from state A to state B, where $[mRNA]_A \neq [mRNA]_B$:

$$\frac{[mRNA]_B}{[mRNA]_A} = \frac{(k_{1B} / k_{6B})}{(k_{1A} / k_{6A})}$$

we can ask: do any of the ratios (k_{1B} / k_{1A}, k_{6B} / k_{6A}, etc.) equal ($[mRNA]_B / [mRNA]_A$?

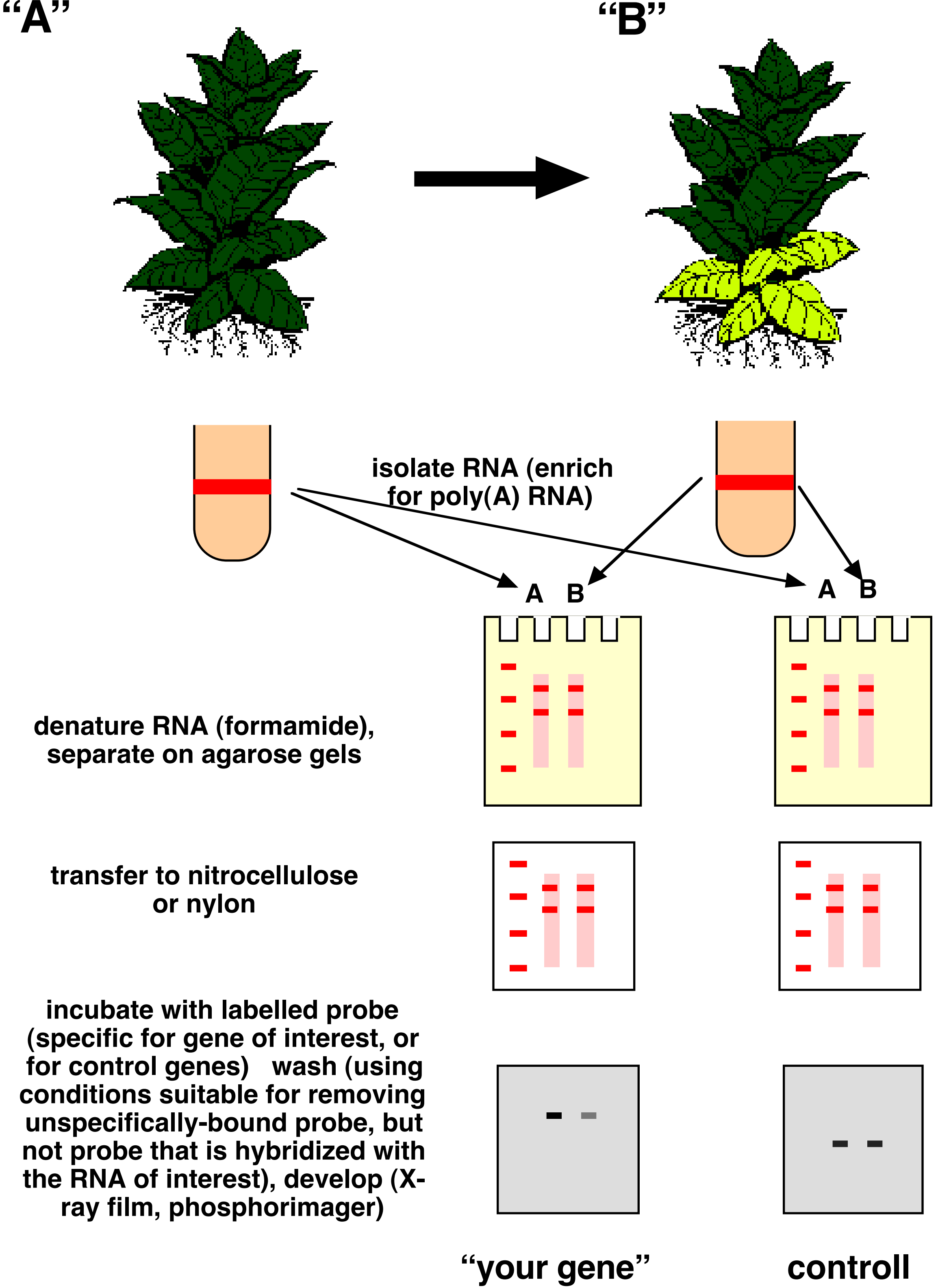
If any of these do, then the step responsible for Δ[mRNA] has been identified.

Experimental determination of Δ[mRNA], Δk_1, and Δk_6:

Δ[mRNA]: northern blot, nuclease protection, RT/PCR

Δk_1: nuclear run-off

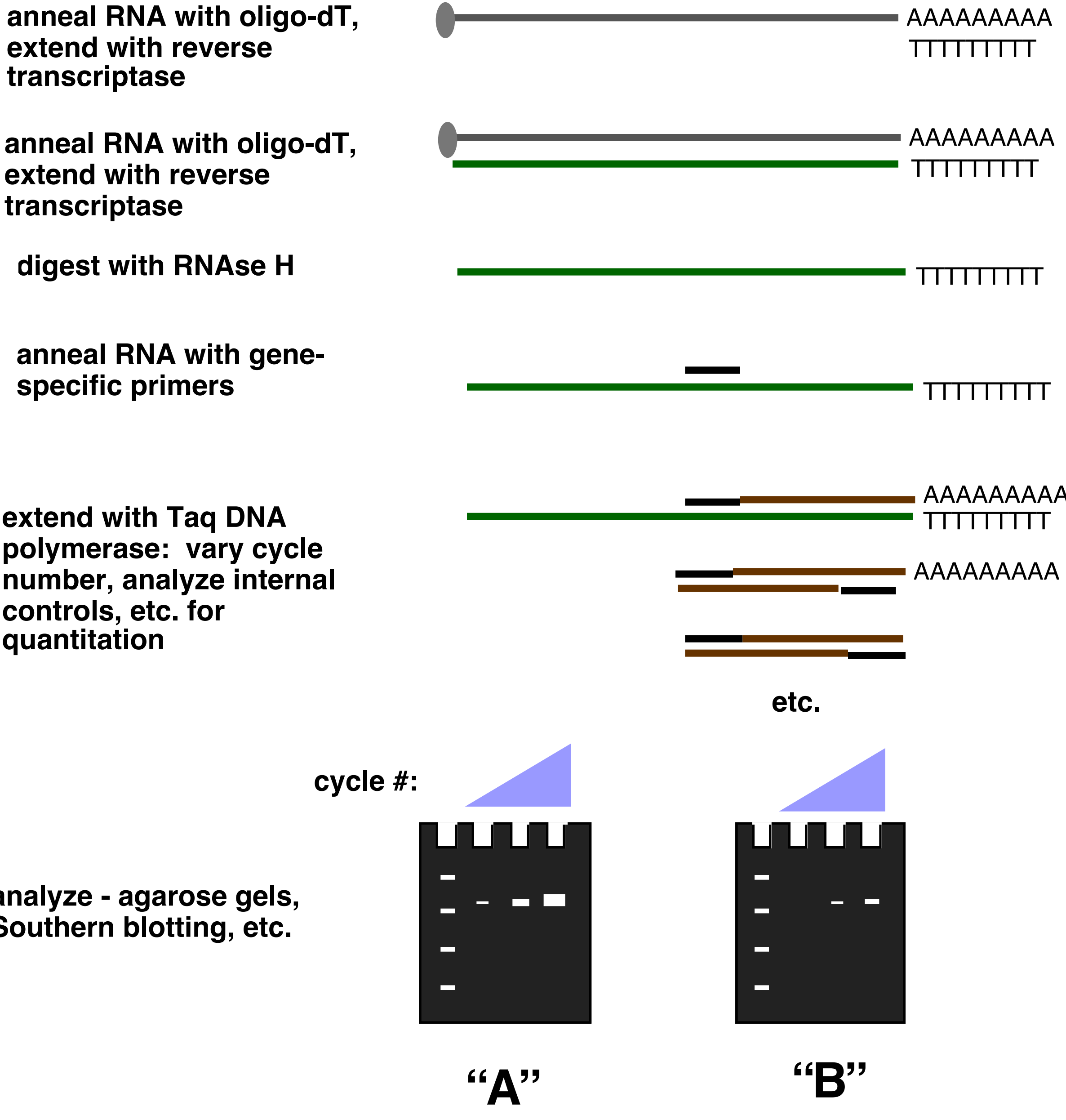
Δk_6: direct measurement of transcript stability



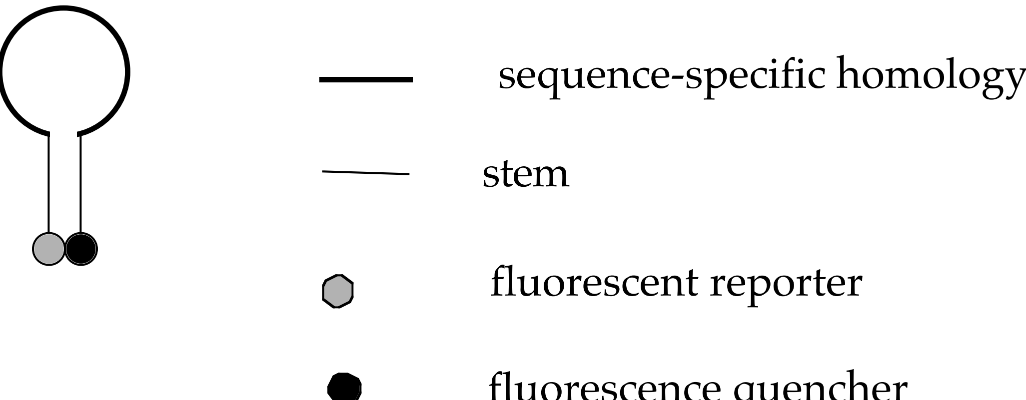
“A”
“B”
isolate RNA (enrich
for poly(A) RNA)
A B
A B
denature RNA (formamide),
separate on agarose gels
transfer to nitrocellulose
or nylon
incubate with labelled probe
(specific for gene of interest, or
for control genes) wash (using
conditions suitable for removing
unspecifically-bound probe, but
not probe that is hybridized with
the RNA of interest), develop (X-
ray film, phosphorimager)
“your gene”
controll

Nucleatse protection assay

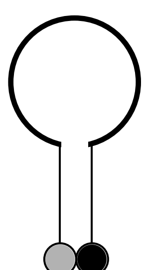
Reverse transcription + polymerase chain reaction (RT/PCR) for determination of [mRNA]



Molecular beacons and quantitation of PCR reactions



fluorescence



no fluorescence

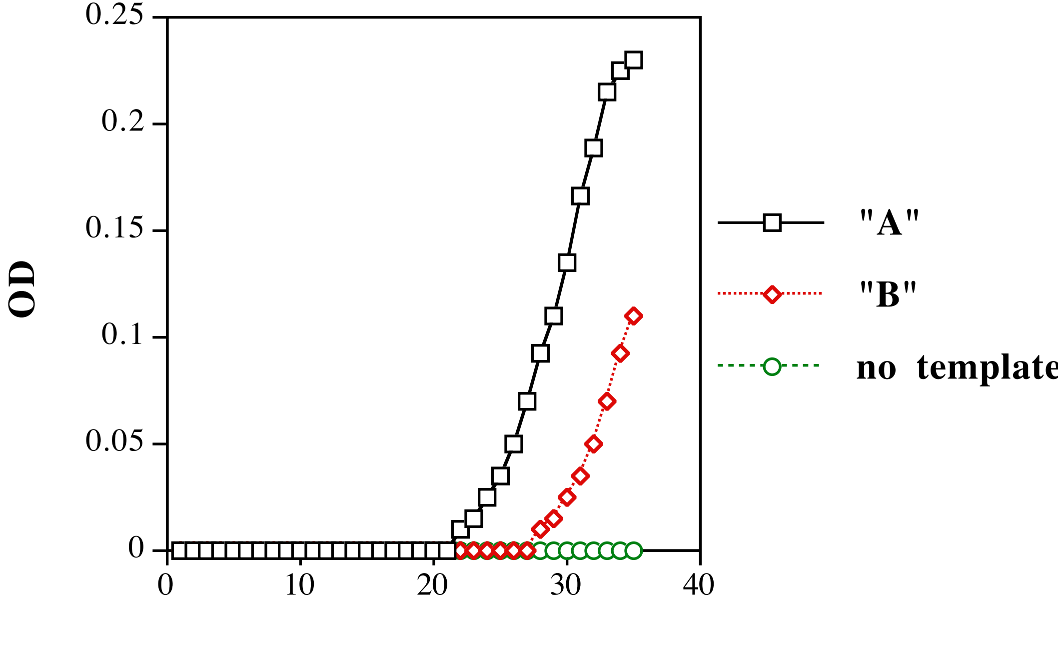
Molecular beacons and quantitation of PCR reactions

fluorescent reporter

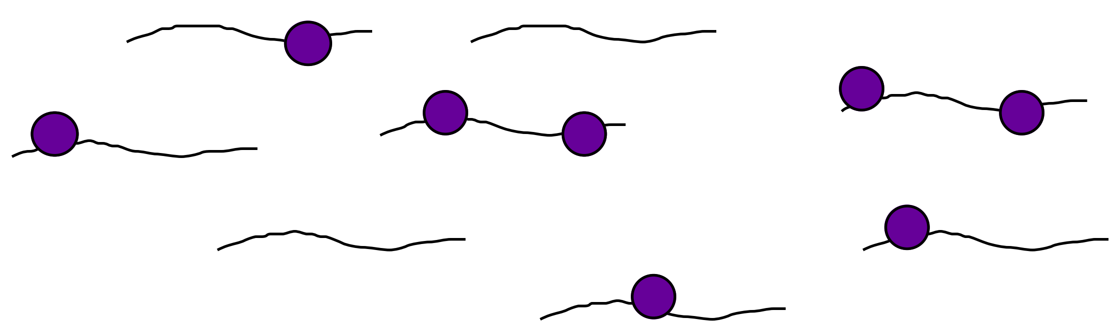
fluorescence quencher

Reporter and quencher are constrained on primer -> no fluorescence

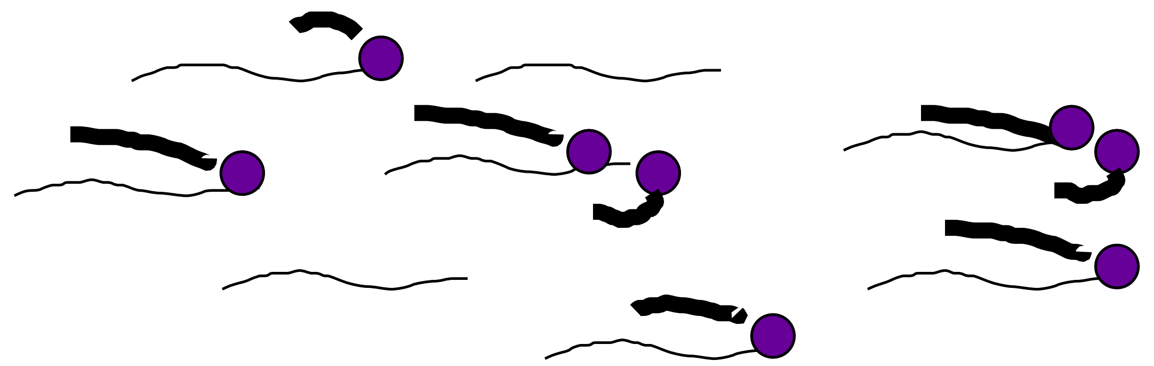
5'->3' exonuclease activity of Taq DNA polymerase releases reporter -> **fluorescence!**


OD
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
cycle
"A"
"B"
no template

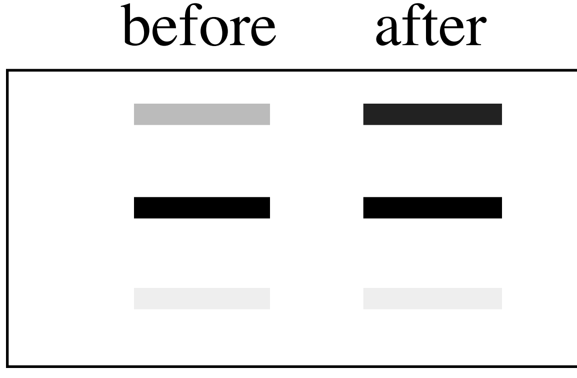
“stop”, isolate nuclei (rapidly)



incubate nuclei with ^{32}P-UTP



hybridize labeled RNA with immobilized DNA



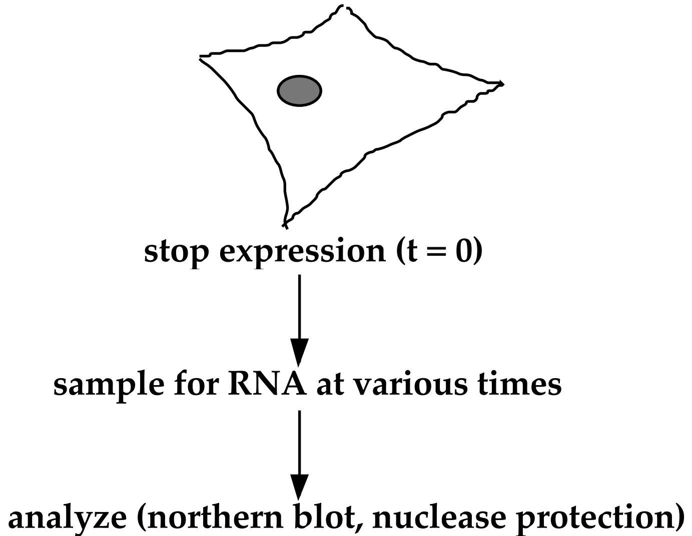
“sample”

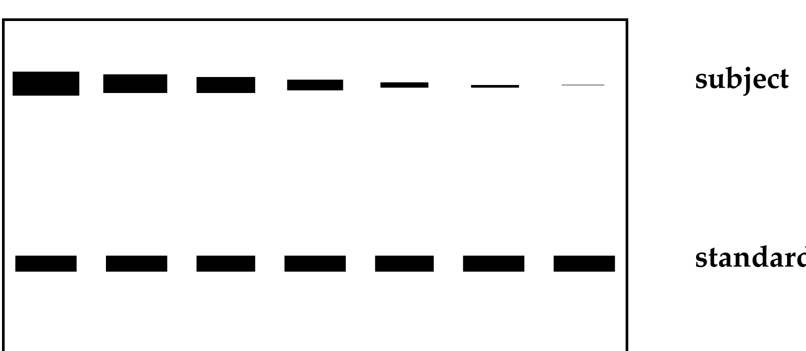
positive control

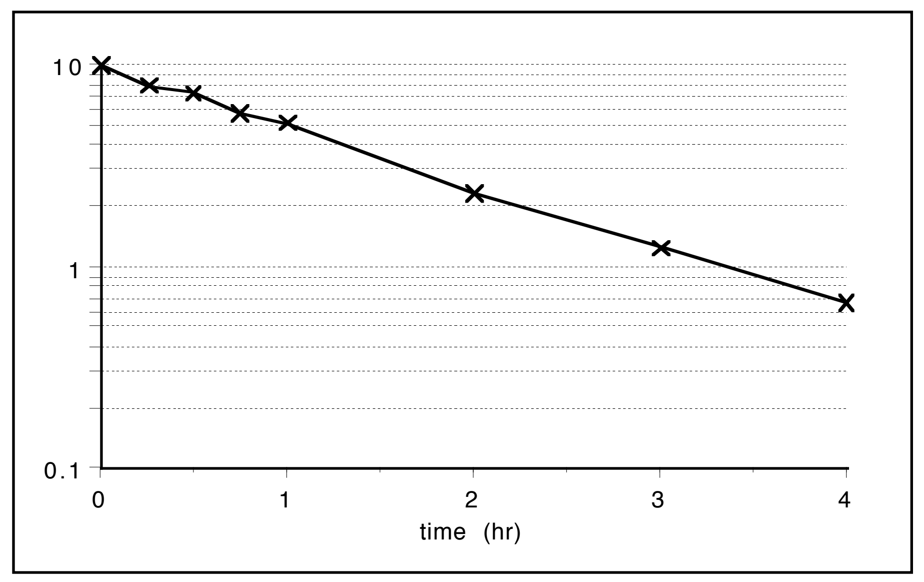
negative control

Assumptions that underly the nuclear run-off (or run-on) method

- **Nuclei isolation is rapid and provides a "snapshot" of the population of polymerases at the desired time**
- **Internal pools of NTPs are small (or equilibration with exogenous label is rapid), specific activities do not change**
- **A representative set of polymerases will complete transcription upon addition of transcription reagents**
- **Transcription re-initiation will not occur after addition of transcription reagents**
- **Transcriptional pausing and termination will be random events**


stop expression (t = 0)
sample for RNA at various times
analyze (northern blot, nuclease protection)


subject
standard


10
1
0.1
0
1
2
3
4
time (hr)

stopping gene expression

-> transcription inhibitors

pros: generally applicable (can use to study any endogenous gene)

can look at many different mRNAs in a single experiment

cons: pleiotropic effects of inhibitors on stability

no good internal standards

-> regulated promoters

pros: can normalize data to internal standards that are not affected

pleiotropic effects of inhibitors are avoided

cons: restricted to specific genes <u>or</u>

must use chimeric genes and transgenics (must rule out potential promoter effects on stability)

What about the period before steady state is reached?

How are different steady states attained?

Consider the simplified case where steady-state levels are determined solely by k_1 and k_6 (note that we can "group" k_1 and [DNA] into a revised constant k_1 - that is what is done in the following):

$$\frac{d[mRNA]_t}{dt} = k_1 - k_6[mRNA]_t$$

$$[mRNA]_t = \frac{k_1}{k_6} - \left(\frac{k_1}{k_6} - [mRNA]_0\right)e^{-k_6 t}$$

where $[mRNA]_0$ - the initial [mRNA]

k_1/k_6 - the final steady-state [mRNA]

($[mRNA]_t$ when $t \Rightarrow \infty$)

How do the absolute values of k_1 and k_6 affect transitions?

consider cases where $[mRNA]_0$ = 2 and k_1 and k_6 are of different initial and final values

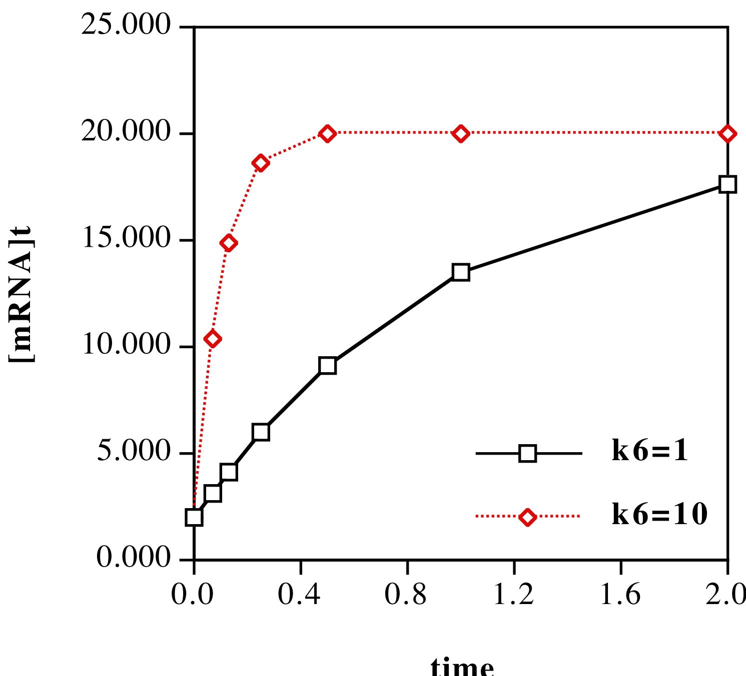
k_1=2, k_6=1

increase k_1 to 20 -> [mRNA] will increase by a factor of 10 (to 20)

if k_1=20 and k_6=10, $[mRNA]_0$ is still 2

increase k_1 to 200, [mRNA] will still increase to 20

however, the speed with which the changes take place will vary dramatically:



Likewise, if k_1=2, k_6=1

decrease k_1 to 0.2 -> [mRNA] will decrease by a factor of 10 (to 0.2)

if k_1=20 and k_6=10, $[mRNA]_0$ is still 2

decrease k_1 to 2, [mRNA] will still decrease to o.2

however, the speed with which the changes take place will vary dramatically: