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Vaccines and Related Biological Products Advisory Committee
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Issues Associated With Residual Cell-Substrate DNA

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Topics Covered

- History of cell-substrate DNA in biological products
- Methods used to quantify DNA
- Perceived safety issues associated with DNA
- Review of assays and published data on the biological activity of DNA
- Development of quantitative assays to assess risk
- Extrapolations from data to assist in the regulatory process
- How such data can be used used to assess safety
- Summary, Conclusions, Unresolved Issues

Some Landmarks in Cell Substrates and DNA Levels

1954: Proscription on use of cell lines for vaccine manufacture; “normal” cells to be used (US Armed Forces Epidemiological Board)

1986: WHO established DNA limit for vaccines manufactured in cell lines at ≤100 pg per dose

1996: WHO/IABs and WHO Expert Committee, for vaccines produced in cell lines, DNA limit raised to ≤10 ng per dose

Vaccines and DNA

- Viral vaccines and biological products contain contaminating residual DNA from cell substrate
- The amount of residual cell-substrate DNA in a vaccine will depend on the vaccine and the manufacturing process
  - protein/subunit (*e.g.*, HBV)
  - inactivated virus (*e.g.*, IPV, influenza virus)
  - live, attenuated virus (*e.g.*, OPV, MMR, varicella)

Methods and Sensitivities of DNA Detection

Spectrophotometry	> 0.1 µg/mL
Hybridization	
randomly labelled DNA	50 pg (10^{-12} g)
biotinylated probes	2 µg
repetitive DNA (SINE, Alu)	5 pg
Immunological methods	5 – 10 pg
PCR methods	
unique sequence DNA	fg (10^{-15} g)
repetitive DNA (SINE, Alu)	ag (10^{-18} g)

Is DNA a Risk?

Assessments Vary From:

DNA is an inert contaminant whose amount needs to be measured but is not a safety concern
[Pettriciani and Horaud, Biologicals 23: 233-238, 1995]

To:

DNA is a biologically active molecule whose activities pose a significant risk to vaccinees; thus, the amount of DNA needs to be limited and its activities reduced

The Route for DNA into Cells

Pathway to Consequence

- Binding of DNA to cells
- Uptake of DNA into cell
- Transfer of DNA to nucleus
- Expression of DNA
- Integration of DNA

Activities Associated with Residual Cell-Substrate DNA

- Oncogenic Activity
  - Consequences of integration into host genome
    Disruption of tumor-suppressor gene (*e.g.*, *p53*)
    Activation of dominant proto-oncogene
  - Introduction of a dominant oncogene (*e.g., ras*)
- Infectivity Activity
  - Capacity to generate infectious agent (*e.g.*, DNA virus, retroviral DNA)

NOTES BY FDA: The risks associated with residual cell-substrate DNA have been debated for 40 years without resolution. The potential risks are considered to be twofold.
First, DNA could have an oncogenic potential.
Second, the DNA could be infectious.
The oncogenic activity has historically been the one that has drawn the most attention from regulators.
There are several ways by which DNA could be oncogenic
- The cell-substrate DNA could possess one or more dominant activated oncogenes, such as *ras*.
- The other way is by integration of the cell-substrate DNA into the host chromosome. The consequences of this integration could be:
   1. To disrupt a tumor-suppressor gene, such as p53.
   2. To integrate near a cellular oncogene and alter the normal expression of this gene.

The infectivity risk arises if the cell-substrate DNA contains a genome of an infectious virus. Thus, if the genome of this virus is inoculated into the vaccinee, it could establish an infection in the human, and this could have pathogenic consequences. The infectious genome could be a DNA virus, either integrated or extra-chromosomal, or could be the DNA provirus of a retrovirus.
{Such a mechanism was originally seen in leukemias in chickens, where an increased expression of the myc gene was frequently observed, and has recently be seen in the gene therapy studies for X-linked SCID, where the Lmo-2 gene was affected and several children have leukemia.}
[However, this mechanism has not been considered likely by several Advisory Committees, and CBER cannot consider it likely, ***as we have allowed milligram amounts of DNA*** *to be injected as DNA vaccines. Thus, the major oncogenic risk is through the introduction of oncogenes.]*

DNA Integration Has Been Considered a Low Risk

- Estimates of the probability of integration of a DNA molecule inducing an oncogenic event are low (10^{-19} – 10^{-23})
- There are no limits for some types of cellular DNA, *e.g.*, primary cells, diploid cell strains
- Levels of plasmid DNA vaccines up to 8 mg per dose have been permitted by CBER

Difficult to imagine mechanisms by which some types of cellular or plasmid DNA pose a higher integration risk than others

Major Issues Associated with Residual Cell-Substrate DNA

- Oncogenic Activity
  - Consequences of integration into host genome
    Disruption of tumor-suppressor gene (*e.g.*, *p53*)
    Activation of dominant proto-oncogene
  - Introduction of a dominant oncogene (*e.g., ras*)
- Infectivity Activity
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Assays to Assess the Biological Activities of DNA

- Oncogenic Activity
  - *in vitro*: Transformation (immortalization, loss of contact inhibition, acquisition of anchorage independence)
  - *in vivo*: Tumor induction
- Infectivity Activity
  - *in vitro*: Establishment of virus infection
  - *in vivo*: Establishment of virus infection

Difficulty of Testing Cellular DNA

- The Dilution Factor

  A single-copy gene or virus is 10^5- to 10^6-fold less abundant for equivalent amounts of cellular DNA as compared with a plasmid DNA clone containing the same gene/virus

  Therefore, the amount of mammalian genomic DNA equivalent to 1 µg of a cloned gene or virus is 1×10^5 to 1×10^6 µg (0.1 g to 1 g)

- No Validated Assays Exist

Review of Published Studies on Biological Activity of DNA

Published Studies on DNA Oncogenicity

- Viral oncogenes
  - v-*src* in chickens
  - polyoma DNA
- Cellular oncogenes
  - H-*ras*

Oncogenicity of *src* DNA in Chickens

Fung *et al*. (1983)

- Cloned RSV DNA (2 µg) induced tumors in 6/6 chickens inoculated s.c. in their wing-web
- Cloned v-*src* DNA (2 µg) induced tumors in 7/10 chickens inoculated s.c. in their wing-web

Halpern *et al*. (1990)

- Cloned v-*src* DNA (20 µg) induced tumors in chickens
  52/60 (87%) inoculated s.c. in their wing-web
  8/36 (22%) inoculated i.v.

Conclusion

2 µg (2.5×10^{11} molecules) of cloned v-*src* is oncogenic in chickens

Oncogenicity of Polyoma Virus DNA *in vivo*

- **Polyoma virus DNA in newborn hamsters**

i.p.	0.5 µg	supercoiled	5/52 (10%)
s.c.	0.5 µg	supercoiled	14/73 (19%)
s.c.	0.5 µg	linear	29/64 (45%)

- **Cloned polyoma virus DNA in newborn hamsters**

s.c.	0.5 µg	supercoiled	11/20 (55%)
s.c.	2 µg	linear	33/55 (60%)
s.c.	0.2 µg	linear	2/9 (22%)

Conclusion

0.2 µg (1.9 x 10^{10} molecules) of polyoma virus DNA is oncogenic in newborn hamsters

Oncogenicity of a Cellular Oncogene in Mice

Burns *et al*. (1991)

- Activated H-*ras* (T24) gene (10 µg) inoculated by scarification of mouse skin
- Lymphangiosarcomas developed in 33/34 animals within 12 months; usually within 12 weeks
- Normal c-*ras* failed to induce tumors (0/10 animals)

Conclusion

10 µg (1.1×10^{12} molecules) of activated *ras* is oncogenic in adult mice

Summary of *in vivo* Infectivity with Viral Genomes

Viral DNA	DNA/Route	Genomes for Infection
Retroviruses	15 - 500 µg i.m.	1.1×10^{12} - 2.3×10^{13}
Polyoma Virus	5×10^{-5} µg s.c.	1.3×10^{7}

Conclusions

- Infectivity of different retroviral DNAs is similar
  - Depending on the route of inoculation, 15 µg can be infectious
- Infectivity of polyoma virus DNA is higher (~ 50 pg)

Comparison of Oncogenicity & Infectivity

DNA	Oncogenicity	Infectivity
Polyoma Virus	0.2 µg (3.6 x 10^{10} genomes)	ID_{50} 1.3 x 10^{-4} µg (2.3 x 10^{7} genomes)
SV40	1 µg (1.7 x 10^{11} genomes)	ND
Retroviruses	NR	15 - 30 µg (1 - 2 x 10^{12} genomes)
v-*src*	2 µg (2.5 x 10^{11} molecules)	NR
Activated *ras*	10 µg (9.1 x 10^{11} molecules)	NR

ND not done; NR not relevant

Conclusion: DNA infectivity > DNA oncogenicity ~10^{3} fold

Cell Substrates and WHO Recommended DNA Limits

- Primary Cells: No limits
- Diploid Cell Strains: No limits
- Cell Lines: 10 ng per dose

Operational Principles for Regulatory Decisions for Cell-Substrate DNA

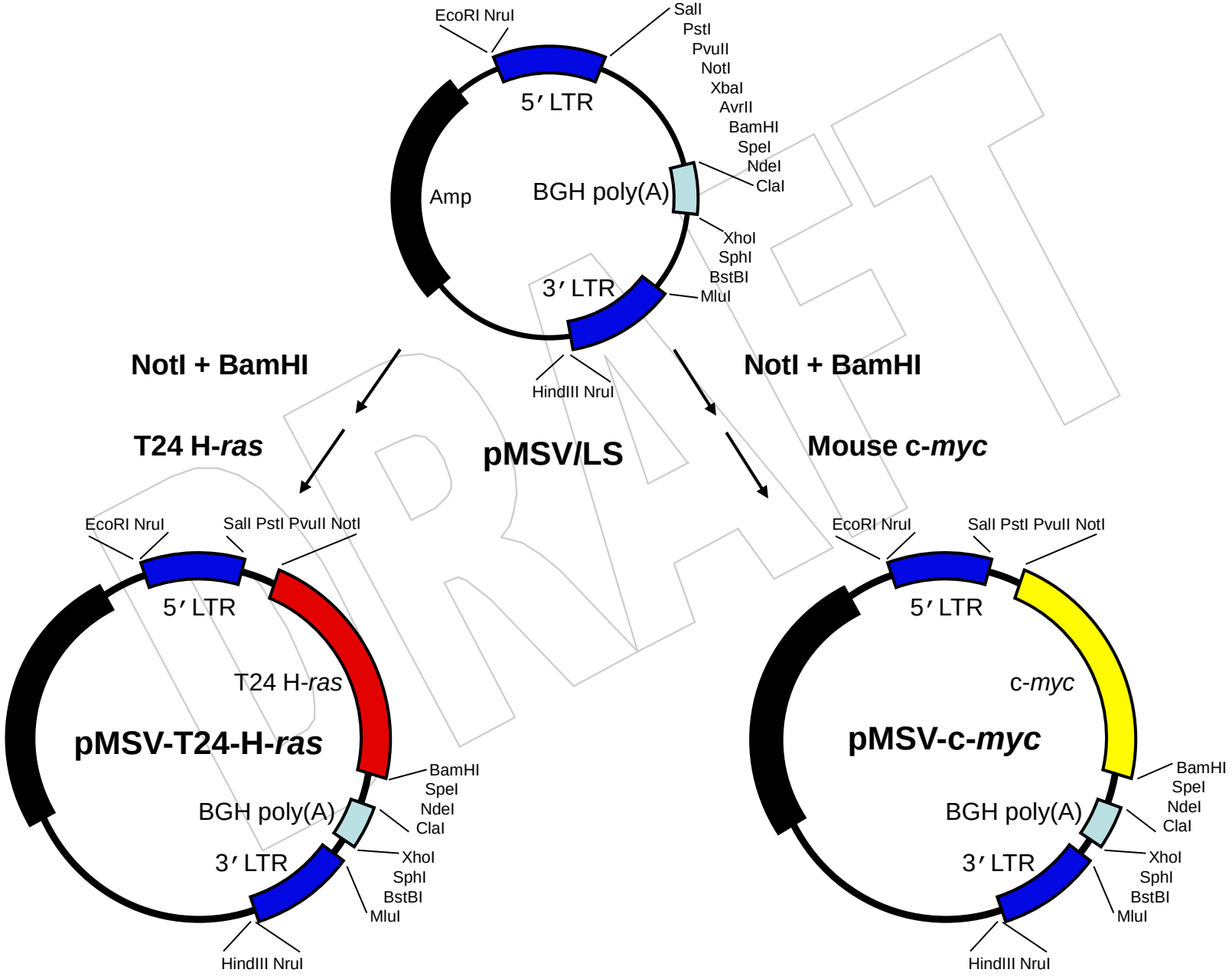
- Evaluations of risk need to be based on quantitative experimental data on the biological activity of DNA
- As long-term human safety data are usually unattainable, it is prudent to make estimates based on the most sensitive model systems
- As more data are obtained, risk estimates may change and recommendations may be revised

Development of Sensitive and Quantitative Animal Models to Assess DNA Oncogenicity

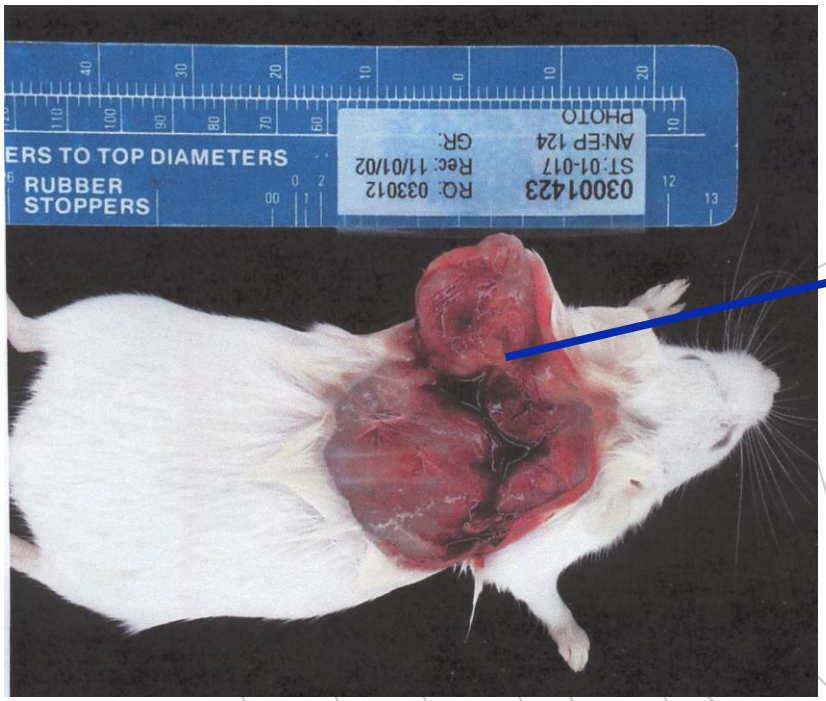
Requirements

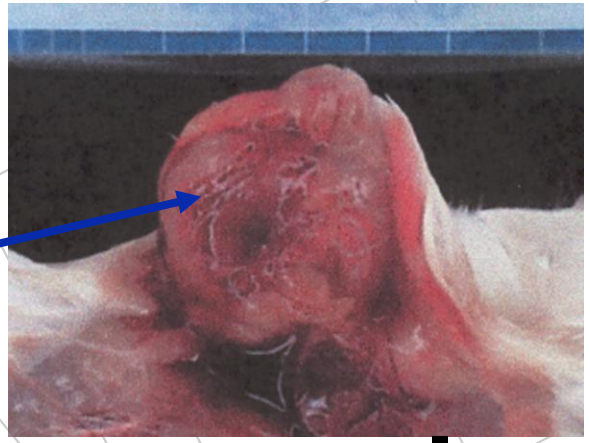
- Choose oncogenes that have been shown to transform efficiently primary cells in culture
- Express these oncogenes under promoters known to function efficiently and for prolonged periods in mice

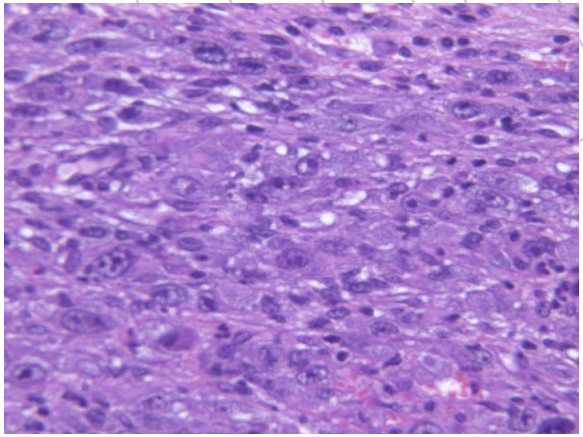
Structure of pMSV-T24-H-*ras* and pMSV-c-*myc*



ras-myc Tumor in NIH Swiss Mouse

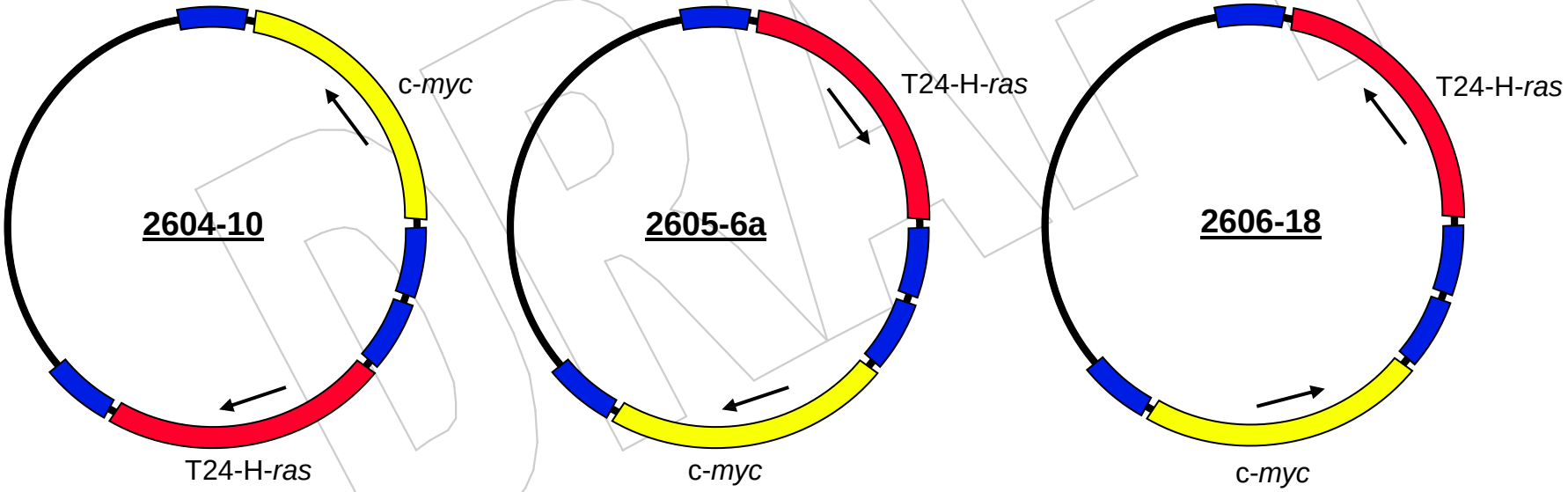






Plasmids Expressing Both *ras* and *myc* Oncogenes

Dual Expression Plasmids for Human T24-H-*ras* and Mouse c-*myc*



Summary of DNA Oncogenicity

- Dominant oncogenes can induce tumors in normal mice
- Both *ras* and *myc* are required
- Newborns are more sensitive than adults
- Dual expression plasmid is more active (1 µg)

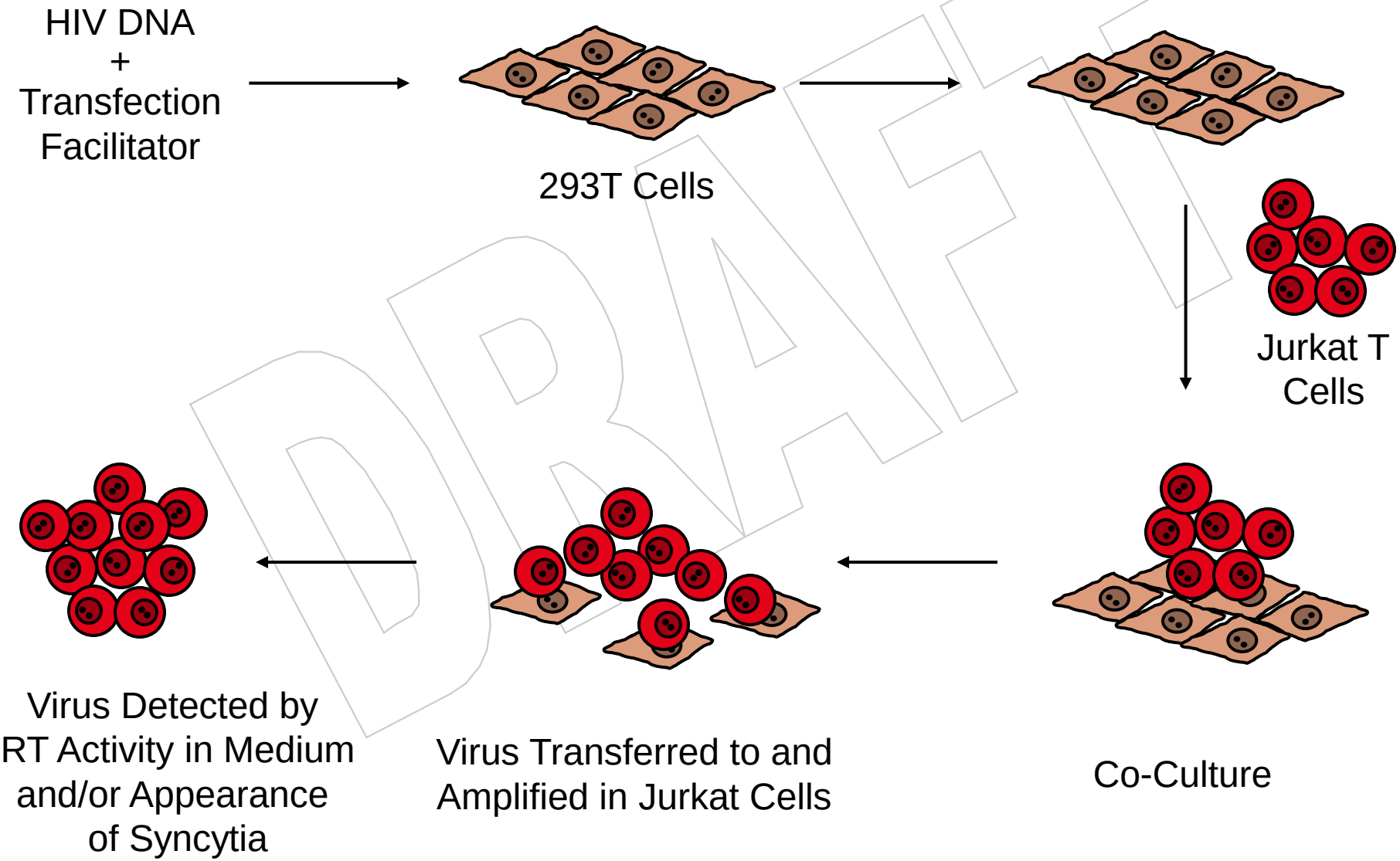
Therefore, models to evaluate DNA oncogenicity are being established

Development of an *in vitro* Assay to Assess Infectivity of DNA

Rationale for Assessing DNA Infectivity

- Infectivity risk may be more important than DNA oncogenicity (VRBPAC)
- DNA infectivity has been incompletely studied
- Assay will allow other aspects of DNA activity to be studied

Transfection/Co-Culture Assay for the Detection of Retroviral DNA



Summary of Results Obtained From *in vitro* DNA Infectivity Assay

- 1 pg of retroviral DNA can be detected

  This corresponds to ~ 1 x 10^5 molecules

- 1 µg of cellular DNA from HIV-infected cells is infectious (not shown)

DNA Inactivation Methods

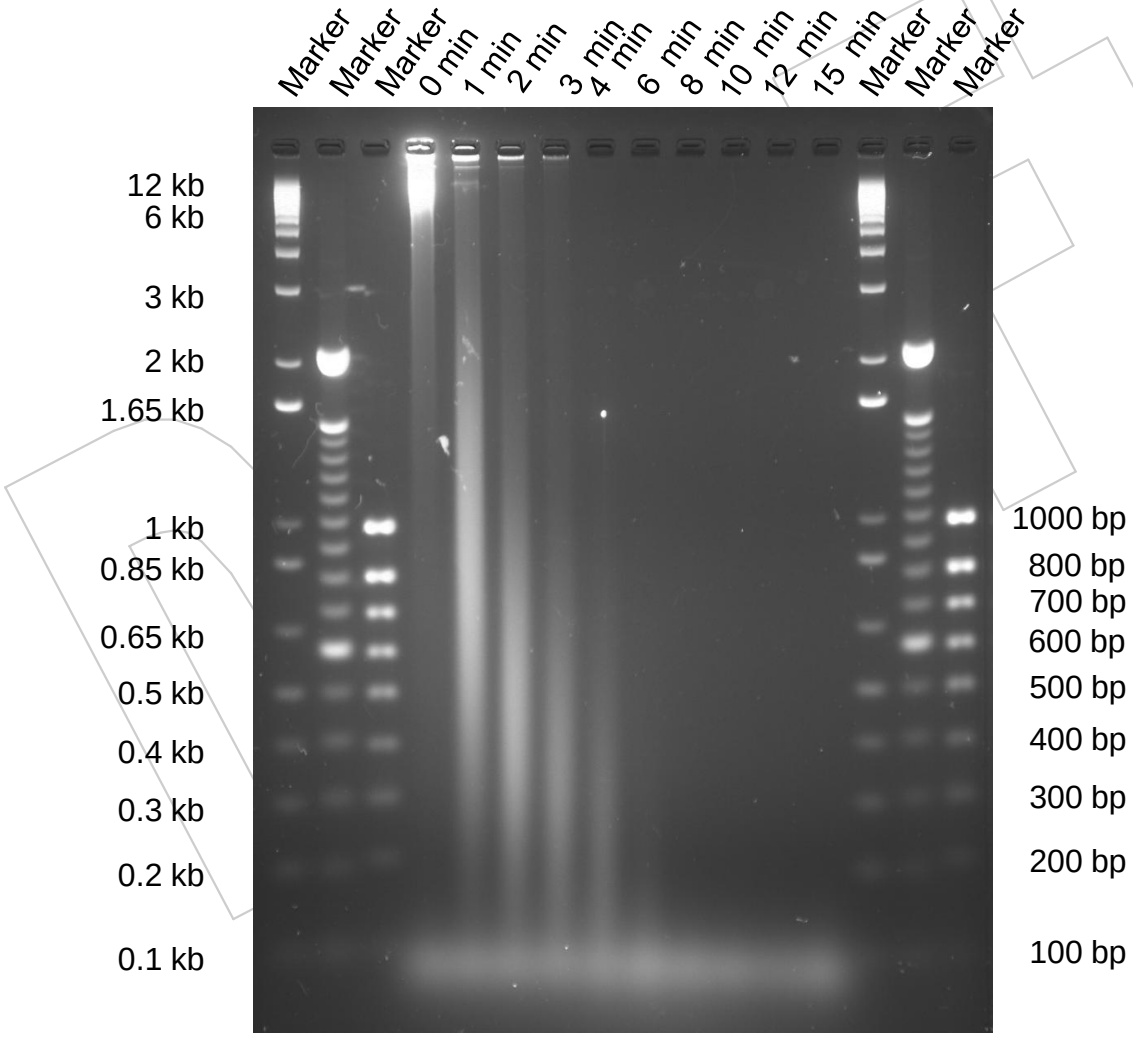
- Live Virus Vaccines

  Nuclease digestion (Benzonase)

- Inactivated Virus Vaccines

  β-propiolactone treatment

Elimination of HIV DNA Infectivity With Benzonase



Assumptions for DNA Activity

- For a given DNA, the level of the response of a cell to that DNA is proportional to the amount of that DNA
- The activity of a gene/viral genome integrated in chromosomal DNA or as part of plasmid vector is equivalent
- The amount of uptake and expression of a gene/viral genome by a cell is related to the concentration of the gene/virus in the DNA
- The activity of a gene/viral genome inoculated as chromatin is the same as when the same gene/viral genome is inoculated as free DNA -- not yet known

Definition of Safety/Clearance Factor

The multiplicative factor by which the biological activity of DNA is reduced

- This reduction can occur by lowering the amount of DNA and/or by inactivating the DNA
- It is analogous to the “clearance” of adventitious agents
  - Safety factors of 10^7 or more would provide substantially additional safety

Safety Factors Based on DNA Infectivity
Digestion with Benzonase

Digestion of DNA to a mean size of 650 bp resulted in the loss of biological activity of 0.15 µg of cloned viral DNA

Based on the proportion of a retroviral genome in the cell, 150 ng of viral DNA corresponds to:

$150 \div (1.67 \times 10^{-6})$ ng of cellular DNA

$= 90 \times 10^{6}$ ng

= 90 mg

Therefore, for 10 ng of cellular DNA with a single provirus, the safety factor is:

9×10^{6}

Calculations of Safety From DNA Infectivity Studies

Based on the proportion of cellular DNA represented by a single copy retroviral genome, for 10 ng of cellular DNA, safety factors can be estimated:

- From cloned HIV DNA, safety factor: 60 (not shown)
- From BPL treatment, safety factor: 3×10^7 (not shown)
- From benzonase digestion, safety factor: 9×10^6

Calculations of Safety Factors From DNA Oncogenicity Studies

10 µg of two plasmids each expressing an oncogene induces a tumor

Oncogene represents 10^{-5} to 10^{-6} of the mammalian genome

That is, 10^{6} to 10^{7} µg of cellular DNA would be required to induce an oncogenic event

For 10 ng cellular DNA, then, the Safety Factor is 10^{8} to 10^{9}

This Safety Factor excludes:

- That two oncogenes in the same cell are required, and thus the probability of tumor induction is further lowered
- Additional safety from size reduction of DNA (~ 1.5 x 10^{5})

How Safety Factors Can Assist in the Regulatory Process: A Hypothetical Example -1

A tumorigenic cell substrate is proposed for the manufacture of an inactivated vaccine

The manufacturing process reduces the amount of DNA to ≤ 2 ng per dose

The inactivation procedure reduces the size of the DNA to below 200 bp

How Safety Factors Can Assist in the Regulatory Process: A Hypothetical Example - 2

Oncogenic Risk

From a consideration of DNA quantities alone, our current data suggest that the safety factor for an oncogenic risk from 2 ng of residual DNA is 5×10^8 to 5×10^9

Number excludes:

- The additional safety factor of derived from DNA size reduction (*i.e.*, increased to ~ 7.5×10^{13} to 7.5×10^{14})
- Reduction due to the number of potential dominant oncogenes (~ 200)

How Safety Factors Can Assist in the Regulatory Process: A Hypothetical Example - 3

Infectivity Risk

From a consideration of DNA quantities alone, our current data suggest that the infectivity risk from 2 ng of residual DNA is 300

Because reducing the size of the DNA to below 650 bp provides a Safety Factor of 9×10^6 for 10 ng of DNA, this value becomes 4.5×10^7 for 2 ng of DNA

How Safety Factors Can Assist in the Regulatory Process: A Hypothetical Example - 4

Conclude that, for this inactivated vaccine, the manufacturing process adequately deals with the safety issues with respect to residual cell-substrate DNA

Additional Considerations For DNA Oncogenicity

- The multi-stage nature of human carcinogenesis makes it unlikely that a single dominant oncogene will induce cancer
- The possibility of “initiating” a cell, however, remains a potential concern, but there are no known assays to assess this

Additional Considerations From *in vivo* DNA Infectivity Studies

Amounts of viral DNA to establish infection:

- Polyoma viral DNA infectivity: 50 pg
  (9×10^6 genomes)
- Retroviral DNA infectivity: 15 to 30 µg
  (1.1 to 2.2×10^{12} genomes)

Therefore, safety factors could be increased by:

50 fold (for polyoma virus DNA) to
3×10^7 fold (for retrovirus DNA)

Conclusions

- Development of quantitative *in vivo* DNA oncogenicity assays and *in vitro* DNA infectivity assays are feasible
- Because these assays are highly sensitive, they represent a “worst case”
- Data from these assays will likely assist in resolving safety concerns associated with residual cell-substrate DNA and permit the introduction of new cell substrates

OVRR Recommendations
Addressing Potential Safety Concerns With Residual DNA From Tumorigenic Cell Substrates

- Clearance of DNA

  Reducing the amount of DNA to ≤10 ng DNA per dose
  Reducing the size of the DNA to below 200 bp
  Safety Factors of $>10^7$ fold can be obtained

- Inoculating Cellular DNA into Animals

  ≥ 100 µg cell substrate DNA
  - Newborn hamsters
  - Newborn rats
  - Newborn nude mice

  Animals are monitored for 5 months for tumor formation (and general health)
  Assay has undefined sensitivity

Issues that Remain to be Addressed

- Biological activity of chromatin
- Routes of inoculation
  - Oral (~10,000 less efficient than IM for DNA uptake)
  - Nasal (unknown)
- Whether DNA can induce an initiation event
- Whether hereditable epigenetic effects can induce initiation events in vaccine recipients and whether these could pose a safety concern