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Reprinted from JOURNAL OF CELLULAR PHYSIOLOGY
Vol. 68, No. 1, August 1966

Purine Pyrophosphorylase as a Selective Genetic Marker in a Mouse Lymphoma, P388, in Cell Culture

HARVEY L. OZER [1]
Department of Genetics, Stanford University School of Medicine, Stanford, California

ABSTRACT It is possible to use the purine pyrophosphorylase in mammalian cell culture systems as a genetic marker in selecting small numbers of enzyme positive cells from large populations of pyrophosphorylase negative cells of the mouse lymphoma line P388 in medium containing amethopterin, hypoxanthine, glycine and thymidine. Conversely, it is readily possible to obtain pyrophosphorylase-deficient cells by treatment with 8-Azaguanine. We were unsuccessful in demonstrating DNA-mediated transformation using DNA from enzyme positive cells incubated with cells which were enzyme negative.

Mammalian cell lines in culture have been shown to be able to synthesize purine nucleotides by either of two pathways, as summarized in figure 1. One pathway, termed *de novo*, involves stepwise enzymatic synthesis starting with PRPP and glutamine. The second or "salvage" pathway required PRPP, free purine base, and a single pyrophosphorylase in both the bacterial and the mammalian systems studied (Szybalski and Szybalska, '62). *De novo* synthesis of purines (and thymidine) can be inhibited by treatment with AMT, (Aranow, '59) making the cell dependent on the integrity of the "salvage pathway" for continued purine synthesis. Szybalski and Szybalska ('62) showed that incubation of a human cell line Detroit 98 in medium containing AMT supplemented with hypoxanthine and thymidine (HAT) growth continued whereas no growth was observed in AMT alone. Conversely, a variant cell line, selected for resistance to AZH and shown to be deficient in pyrophosphorylase activity, was unable to grow in HAT medium. Szybalska and Szybalski ('62) later reported that, following incubation with DNA extracted from the parent line, it was possible to obtain clones from the AZH resistant population which grew in HAT medium and which they proposed to be "DNA-mediated transformation" similar to that observed in bacterial systems.

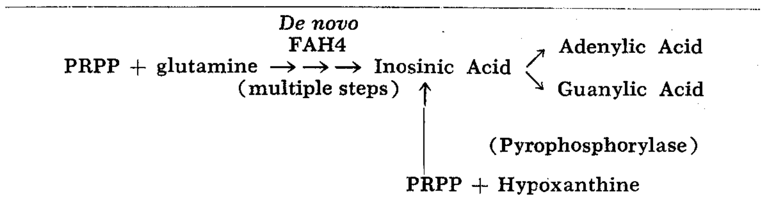

Fig. 1 Biosynthesis of purines.

The following abbreviations are used:
- FAH4, Tetrahydrofolic acid
- AMT, Amethopterin
- AZG, 8-Azaguanine
- AZH, 8-Azahypoxanthine
- 6MP, 6-Mercaptopurine
- TMP, thymidylic acid
- CMP, cytidylic acid
- dCMP, deoxy-cytidylic acid
- dC, deoxycytidine
- dU, deoxyuridine
- PRPP, α-5-phosphoribosyl-pyrophosphate
- DNA, deoxyribonucleic acid
- RNA, ribonucleic acid
- BSA, bovine serum albumin

Received May 1, '65. Accepted Feb. 14, '66.

[1] Present address: LBV, NIAID, Bldg. 5, Rm. 315, Department of Health, Education, and Welfare, National Institutes of Health, Bethesda, Maryland 20014.

The present study was undertaken to determine whether a similar phenomenon could be demonstrated using the mouse lymphoma culture line P388 and its pyrophosphorylase-deficient, AZG-resistant subline. In model experiments, it was found that pyrophosphorylase-positive clones could be isolated from mixtures of P388 and resistant cells but no genetic transformants were detected under the conditions studied.

MATERIALS AND METHODS

Cultured cell lines

P388 has been grown continuously in culture since its establishment in 1956 from a methylcholanthrene induced lymphoma of a DBA/2 mouse. Growth and culture conditions have been previously described (Herzenberg and Roosa, '60). Two sublines have been used (2B-2 and CR-2) which were indistinguishable under the selection conditions used. They differ in respect to sensitivity to thymidine (table 3) and cytotoxicity with isoantibody and complement.

P388/AG clone 18a was isolated at 1×10^{-4} M AZG as described by Roosa et al., ('62). Brockman et al. ('62) demonstrated resistance to be due to loss of the pyrophosphorylase for conversion of guanine or hypoxanthine to the ribonucleotide. Adenine to AMP conversion was not affected.

Media

Normal growth medium composed of Eagle's medium (Eagle, '59) supplemented with 6% calf serum, 10^{-3} M sodium pyruvate, and 2×10^{-4} M L-serine under an atmosphere of 5% CO_2 and 95% air at 37°C.

Selection medium AGHT for pyrophosphorylase activity composed of AMT (5×10^{-8} M), glycine (1×10^{-4} M), hypoxanthine (5×10^{-5} M) and thymidine (1×10^{-5} M) in normal growth medium.

Incubation medium for transformation experiments composed of phosphate-buffered saline and 1% glucose, pH 7.2 (PBS) (Szybalska and Szybalski, '62). In some tubes 1.7 μg/ml spermine was added and the pH readjusted to 7.2 (S–PBS).

Incubation conditions

Cell mixtures or DNA and cells were incubated in 2 ml of various media for 30 minutes at 37° in siliconized tubes as roller cultures. After incubation, 0.4 ml was inoculated into each of four 1 oz. prescription bottles containing 4 ml of AGHT. Fresh AGHT was added on day five in some experiments. Cultures were observed for a minimum of two weeks for growth. A diluted aliquot of the incubation mixture was also added to normal medium to test viability of the inoculum.

Growth measurement

Population growth was measured by cell count with an electronic cell counter (Coulter Model B) and viable cells by colony count after 8 to 10 days growth using staining with crystal violet as previously described (Cann and Herzenberg, '63). Cloning efficiency in normal growth medium averaged 30%.

DNA extraction and characterization

DNA was extracted from P388 by the method used by Szybalski and Szybalska, ('62) for Detroit 98 cells which is a modification of the method used with bacteria. For some preparations RNA was removed by treatment with RN'ase (20 μg/ml at 37° for 60 minutes) and/or isopropanol precipitation as described by Marmur ('61). As a control for efficacy of the DNA isolation procedure, DNA was prepared from Bacillus subtilis by the method of Marmur ('61) and assayed for transforming activity using an indole negative strain. The transforming activity compared satisfactorily with standard preparations (Nester and Lederberg, '61).

DNA content was determined by the diphenylamine method of Dische with calf thymus DNA as standard (Dische, '55a), RNA by Dische and Schwartz using orcinol with adenosine as standard (Dische, '55b), and protein by Lowry et al. ('51) with BSA (Armour) as standard.

Equilibrium sedimentation determination was done using 10 μg/ml DNA (RNA 2.5%, protein less than 10%) in CsCl (d. 1.70), pH 8.5, Tris 0.01 M, EDTA 0.001 M at 44,700 rpm for 20 hours at 25°. Analysis was performed by Dr. Ganesan with poly dAT as standard (d. 1.684).

RESULTS

Roosa et al. ('62) reported a 1000 fold difference in sensitivity to AZG between P388 and P388/AG. The data in table 1 show that after prolonged cultivation of the lines in this laboratory, the P388/AG retains its previous level of resistance to AZG. It is also cross-resistant to 6-MP (as previously reported) (Roosa et al., '62) and 8-AZH.

Integrity of the pathways of purine nucleotide synthesis was tested in the two cell lines as shown in table 2 and figure 2. AMT has been shown to inhibit folic acid reductase preventing synthesis of FAH4 required for methyl transfer and secondarily blocking *de novo* purine and thymidine synthesis (Aranow, '59). AMT at low concentrations inhibits P388 and P388/AG which is not reversed by thymidine. Further addition of a purine source (hypoxanthine) reverses the inhibition for P388 demonstrating the pyrophosphorylase necessary for the "salvage pathway" for purine nucleotides. On the other hand, P388/AG is still unable to grow, confirming the absence of pyrophosphorylase activity. To avoid growth due to selection of AMT-resistant cells, an AMT concentration previously determined to be greater than first step resistance was used.

Increasing the concentration of thymidine or hypoxanthine does not reverse the inhibition of AMT for P388/AG. It should be noted, however, that higher concentrations of thymidine inhibit P388 both alone or in combination with AGH (table 3). Several workers (Morris and Fischer, '63), (Reichard et al., '61) have reported the phenomenon of purine and pyrimidine growth inhibition at substrate concentrations. They demonstrated that "thymidine toxicity" is most likely due to inhibition by TMP of the conversion of CMP to dCMP. Growth inhibition could be specifically reversed by exogenous dC. Similarly in the P388 lines, thymidine inhibition is reversible following addition of dC (10^{-6} M) but not dU (10^{-5} or 10^{-6} M).

Cloning experiments were undertaken in AGHT using P388 and P388/AG separately and in combination at different cell concentrations. P388 cells were diluted from exponentially growing cultures and added to P388/AG obtained as exponential or stationary phase cultures. Incubation conditions were as described in METHODS. As summarized in table 4, cloning efficiency of P388 in AGHT in the presence of 10^4 times as many P388/AG cells approximated that of P388 alone except with 10^6 P388/AG cells where no clones were obtained with 25–250 P388 cells. When 1.5

TABLE 1

Sensitivity to purine analogues

Cell line	Normal media	Concentration AZG			
		1×10^{-7} M	1×10^{-6} M	2×10^{-5} M	1×10^{-4} M
P388	100 [1]	17 [2]	0	0	0
P388/AG	100	NT	NT	NT	90–> 100
		Concentration 6–MP			
		1×10^{-6} M	1×10^{-4} M		
P388	100	0	0		
P388/AG	100	64	64		
		Concentration AZH			
		1×10^{-9} M	1×10^{-8} M	1×10^{-6} M	1×10^{-4} M
P388	100	93	75	65	0
P388/AG	100	NT	NT	82	46

[1] 10^4 cells in log phase were inoculated directly into one ounce prescription bottles containing 4 ml of appropriate medium. Growth was determined after five days by cell count. Generation time in control = 20 hours.

[2] Per cent growth calculated as number of cells in test media/number of cells in normal media × 100. All values are average of three bottles/experiment. Zero indicates less than doubling during growth period.

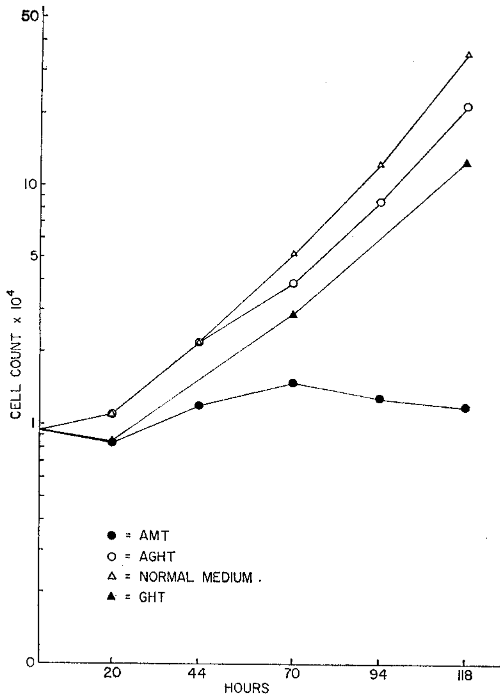

Fig. 2 Cells were inoculated at 1×10^4 per bottle in normal medium. The following day, the medium was aspirated and fresh test medium added. Time refers to hours after addition of test medium. Each point represents the average of three replicate bottles. One standard deviation was less than 15% in most points. ●——●, AMT (5×10^{-8}); ○——○, GHT+AMT (5×10^{-8}); △——△, normal medium; ▲——▲, GHT (Glycine 1×10^{-4} M, hypoxanthine 5×10^{-5} M, thymidine 1×10^{-5} M).

$\times 10^5$ P388/AG cells were mixed with P388, there was no difference with growth phase of P388/AG cells used, incubation medium, or culture conditions. No revertents from 10^4–10^6 P388/AG cells inoculated were obtained in any experiments.

DNA-mediated transformation was investigated using a modification of the procedure reported by Szybalski and Szybalska ('62) for Detroit 98 lines. All experiments were done using a final concentration of 1.5×10^5 P388/AG cells per inoculum. In each experiment the following controls were included: P388/AG alone, P388 and P388/AG ($50{:}1.5 \times 10^5$), and P388 alone.

TABLE 2

Growth in AGHT media

Cell line	Media	Growth [1]
		% [2]
P388	Control	100
	AMT (2×10^{-9} M)	100
	AMT (2×10^{-8} M)	0
	AMT (5×10^{-8} M)	0
	GHT	60
	AGHT	60
P388/AG	Control	100
	AMT (2×10^{-9})	3
	AMT (2×10^{-8})	0
	AMT (5×10^{-8})	0
	GHT	70
	AGHT	0

[1] 10^4 cells in log phase inoculated directly into test media (see table 1).
[2] See table 1, note 2.
GHT, glycine (10^{-4} M), hypoxanthine (5×10^{-5} M), thymidine (1×10^{-5} M).
AGHT, GHT + AMT (5×10^{-8} M).

TABLE 3

Thymidine sensitivity

Cell line	Thymidine concentration (M)							Other additions		
	None	1×10^{-5}	2×10^{-5}	5×10^{-5}	1×10^{-4}	2×10^{-4}	5×10^{-4}	AGH	dU	dC
P388 subline	100 [1]	68	40	22	0	0	NT	−	−	−
CR-2	0	61	50	20	0	NT	NT	+	−	−
P388 clone	100	NT	NT	56	36	NT	0	−	−	−
2B-2	139	NT	NT	153	134	NT	90	−	−	+
	119	NT	NT	60	34	NT	0	−	+	−
P388/AG	100	NT	100	NT	69	NT	23	−	−	−
	0	0	0	0	0	NT	NT	+	−	−
	112	NT	NT	NT	98	NT	53	−	−	+
	100	NT	NT	NT	79	NT	38	−	+	−

[1] Per cent growth after five days (see table 1).
AGH, AMT (5×10^{-8} M), Glycine (1×10^{-4} M), Hypoxanthine (5×10^{-5} M).
dU, deoxyuridine (1×10^{-6} M).
dC, deoxycytidine (1×10^{-6} M).
NT, not tested.

TABLE 4

Reconstruction experiments [1]

Number of experiments	Number of cells added		Average clones	Efficiency
	P388	P388/AG		
				%
2	20	none	2	10
1	25	none	2	8
7	50	none	16	32
2	100	none	24	24
1	250	none	35	14
1	none	1×10^4	0	
11	none	1.5×10^5	0	
3	none	1×10^6	0	
2	20	1×10^5	5	25
1	25	1×10^4	1	4
1	25	1×10^5	5	20
11	50	1.5×10^5	14	29
2	100	1.5×10^5	24	24
1	250	1×10^4	24	10
1	250	1×10^5	35	14
2	25–250	1×10^6	0	

All cells grown in AGHT media and colonies counted as previously stated.

[1] Cells incubated in standard media, PBS + glucose, or PBS + glucose + 1.7 µg/ml spermine for 30′/37°. No difference was noted dependent on these conditions of incubations.

Cells were inoculated into AGHT and normal growth medium as described in METHODS. No growth inhibition was observed in normal medium except when the concentration of spermine greater than 1.7 µg/ml was used during the incubation.

In no experiments was any growth observed in the bottles containing P388/AG alone or after incubation with DNA extracted from P388 cells, although we would expect to detect 20–30 transformants per tube at the efficiency reported by Szybalski and Szybalska ('62). A number of variables were investigated in an effort to obtain transformants.

(1) *Growth phase of recipients.* Since "competence" for transformation in bacterial systems has been found to vary with growth phase of the recipient cells (Hotchkiss, '54), P388/AG cells were used from both exponential and various stages of stationary culture after growth either in normal medium or medium containing 10^{-4} M AZG. In cases of AZG medium, the cells were washed with normal medium as well as PBS before use to ensure elimination of AZG. No significant difference was noted among these conditions in subsequent growth tests.

(2) *Incubation medium.* The incubation medium was prepared as described by Szybalski and Szybalska ('62) for Detroit 98 studies with the exception that appreciably less than 50 µg/ml spermine had to be used due to toxicity to both P388 and P388/AG cells. PBS, S-PBS (1.7 µg/ml spermine), normal medium and normal medium plus 1.7 µg/ml spermine were tested. The medium including calf serum was tested for DN'ase activity by incubation with B. subtilis DNA. No decrease in transforming activity of the DNA was observed (Ganesan and Ozer, unpublished observation).

(3) *DNA preparation.* Final concentrations of DNA ranging from 12.5 to 50 µg/ml with and without prior treatment to remove RNA were used. Although it was not possible to rule out small amounts of denaturation which might be significant in transformation, tests were performed to evaluate the extent of DNA denaturation using a preparation after RN'ase treatment and isopropanol precipitation (RNA 2.5%, protein less than 10%). The percent change in specific viscosity (70%) and absorbency at 260 mµ (36%) upon melting indicated no marked denaturation as compared with biologically active B. subtilis DNA prepared under similar conditions. Equilibrium sedimentation determination in a CsCl gradient showed a typical bi-

modal mouse DNA distribution (d 1.707 and 1.695) as reported by Kit ('61) with the expected shift in density for the thermally denatured sample (final densities 1.725 and 1.704 for the two peaks respectively). Molecular weight determinations were not performed.

(4) *Selection conditions.* In the event that there would be a delay in enzyme expression following incorporation of DNA, cells were plated in normal medium overnight. The following day, the medium was aspirated without detaching the cells and fresh AGHT medium added. In some experiments, the AGHT medium was removed on day five by which time most of the cells had become detached from the glass. Fresh AGHT medium was added to the original bottled and the pooled supernatants for each experimental condition were inoculated into 16 oz. bottled to ensure sufficient surface area for new growth. Although viable cells could be recovered from these supernatants in bottled containing P388 or P388 + P388/AG, none was observed with P388/AG. It does not appear likely, therefore, that phenotypic lag could be responsible for the failure to obtain transformants.

DISCUSSION

These experiments indicate that in the mouse line P388 as well as in the human line Detroit 98 previously described by Szybalski and Szybalska ('62) it is readily possible to select pyrophosphorylase positive cells in the presence of up to 10^4 times as many pyrophosphorylase negative cells. The stability of this marker is indicated by the fact that no revertents were noted in repeated experiments using 10^4 to 10^6 pyrophosphorylase negative cells alone. The few revertents that might have occurred (e.g., while in continuous culture) would have been eliminated by subculturing in 8-AZG, since the revertents would be expected to have become sensitive to AZG as well (Szybalski and Szybalska, '62).

The studies detailed here do not permit us to state the reason for failure to observe transformation. It should be pointed out that there are at least two differences of possible significance between our studies and that of Szybalska and Szybalski ('62), in addition to the cell lines used. They reported an absolute requirement for spermine using 50 μg/ml without determining the minimum concentration needed. The P388 lines were markedly sensitive to this concentration and 1.7 μg/ml was the maximum which could be used. Although requirements may vary for different cell lines, it is also possible that there is an absolute level of spermine required for transformation. Secondly, it should be noted that they used 0.25% "pancreatin" to facilitate detaching their cells from the glass surface. This enzyme may have had an additional role in facilitating "competency" in some undetermined fashion. Other attempts to demonstrate DNA mediated transformation in mammalian cells in culture have been unsuccessful (Mathias and Fischer, '62; Bradley et al., '62; Weisberger, '62).[2] In most cases, "recessive" markers such as drug resistance due to loss of enzyme function were employed. In somatic mammalian cultures where the cells are diploid or more often aneuploid, transfer of "recessive" alleles would be unlikely to be detected. In contrast the acquisition of enzyme function (as purine pyrophorylase) would be expected to be "dominant" or "co-dominant" and easily detectable in a heterozygous state. Furthermore, the appearance of enzyme function is less likely to show phenotypic lag anticipated in cases of genetic loss where previously synthetized recipient enzyme would also have to be lost prior to selection. It is of importance to note that though use of purine pyrophosphorylase for detection of transformation may be limited, it has recently been used in analysis of formation of somatic hybrids *in vitro* as in the work of Littlefield ('64) and Ephrussi (personal communication).

ACKNOWLEDGMENTS

This work was supported by USPHS grants A1-02700 and CA-04681 to the Department of Genetics, Stanford University Medical School, Palo Alto, California. The author is deeply indebted to Dr. L. A. Herzenberg for facilities, encouragement

[2] A recent report by D. J. Podgajetskaya et al., Biochim. Biophys. Acta, *80:* 110 ('64) indicated DNA-mediated transformation can be obtained in rat tumors *in vivo* using sarcolysine-resistance as the selective marker. The mechanism of resistance is unknown.

and advice. I should also like to thank Dr. Ganesan of this Department who performed the CsCl equilibrium sedimentation determinations.

LITERATURE CITED

Aranow, L. 1959 Studies on drug resistance in mammalian cells. I. Amethopterin resistance in mouse fibroblasts. J. Pharm. Exptl. Therapeutics, *127:* 116–121.

Bradley, T. R., R. A. Roosa and L. W. Law 1962 DNA transformation studies with mammalian cells in culture. J. Cell. and Comp. Physiol., *60:* 127–137.

Brockman, R. W., R. A. Roosa, L. W. Law and P. Stutts 1962 Purine ribonucleotide pyrophosphorylase activity and resistance to purine analogs in P388 murine lymphocytic leukemia. J. Cell. and Comp. Physiol., *60:* 65–84.

Cann, H. M., and L. A. Herzenberg 1963 *In vitro* studies of mammalian somatic cell variation. II. Isoimmune cytotoxicity with culture mouse lymphoma and selection of resistant variants. J. Exp. Med., *117:* 267–284.

Dische, Z. 1955a The Nucleic Acids. Ed. by E. Chargoff and J. N. Davidson. Academic Press, Vol. I, 288.

——— 1955b The Nucleic Acids. Ed. by E. Chargoff and J. N. Davidson. Academic Press, Vol. I, 301.

Eagle, H. 1959 Amino acid metabolism in mammalian cell cultures. Science, *130:* 432–437.

Herzenberg, L. A., and R. A. Roosa 1960 Nutritional requirements for growth of a mouse lymphoma in cell culture. Exptl. Cell Res., *21:* 430–438.

Hotchkiss, R. D. 1954 Cyclical behavior in pneumococcal growth and transformability occasioned by environmental changes. Proc. Nat. Acad. Sci., *40:* 49–55.

Kit, S. 1961 Equilibrium sedimentation in density gradients of DNA preparations from animal tissues. J. Mol. Biol., *3:* 711–716.

Littlefield, J. W. 1964 Selection of hybrids from mating of fibroblasts in vitro and their presumed recombinants. Science, *145:* 709–710.

Lowry, O. H., N. S. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the folin phenol reagent. J. Biol. Chem., *193:* 265–275.

Marmur, J. 1961 A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol., *3:* 208–218.

Mathias, A. P., and G. A. Fischer 1962 Transformation experiments with murine lymphoblastic cells (L5178Y) grown in culture. Bioch. Pharm., *11:* 69–78.

Morris, N. R., and G. A. Fischer 1963 Studies concerning the inhibition of cellular reproduction by deoxyribonucleotides. I. Inhibition of the synthesis of deoxycytidine by a phosphorylated derivative of thymidine. Biochim. Biophys. Acta, *68:* 84–92.

Morris, N. R., P. Reichard and G. A. Fischer 1963 Studies concerning the inhibition of cellular reproduction by deoxyribonucleotides. II. Inhibition of the synthesis of deoxycytidine by thymidine, deoxyadenosine and deoxyguanosine. Biochim. Biophys. Acta, *68:* 93–99.

Nester, E. W., and J. Lederberg 1961 Linkage of genetic units of B. subtilis in DNA transformation. Proc. Natl. Acad. Sci., *47:* 52–55.

Reichard, P., Z. N. Canellakis and E. S. Canellakis 1961 Studies on a possible regulatory mechanism for the biosynthesis of DNA. J. Biol. Chem., *236:* 2514–2519.

Roosa, R. A., T. R. Bradley, L. W. Law and L. A. Herzenberg 1962 Characterization of resistance to amethopterin 8-azaguanine and several fluorinated pyrimidines in the murine lymphocytic neoplasm P388. J. Cell. and Comp. Physiol., *60:* 109–126.

Szybalska, E. H., and W. Szybalski 1962 Genetics of human cell lines. IV. DNA-mediated heritable transformation of a biochemical trait. Proc. Nat. Acad. Sci., *48:* 2026–2034.

Szybalski, W., and E. H. Szybalska 1962 Drug sensitivity as a genetic marker for human cell lines. Univ. Mich. Med. Bull., *28:* 277–293.

Weisberger, A. S. 1962 Induction of altered globin synthesis in human immature erythrocytes incubated with ribonucleoprotein. Proc. Nat. Acad. Sci., *48:* 68–80.