Environmental Health Perspectives Supplements
101 (Suppl. 2): 293–310 (1993)

Transformation of BALB/c-3T3 Cells: II. Investigation of Experimental Parameters that Influence Detection of Benzo[*a*]pyrene-Induced Transformation

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Benzo[*a*]pyrene (BaP) induced significant morphological transformation of clone A31-1-13 BALB/c-3T3 cells without exogenous activation. Therefore, BaP was selected as a model to determine the internal consistency of detection of chemical-induced transformation. BaP induced a continuum of type I–III foci of different sizes, and the ratio of type I–III to type III foci/vessel was usually about 2-fold. The major finding was that BaP induced highly significant transformation responses, and the magnitude of these responses were inversely correlated with the cytotoxicity of the treatment doses. Thus, the induction of BaP-induced transformation behaved as though it was caused by a mutational event. Variability among responses were shown to depend on the serum lot and the cryopreserved ampule of cells. In addition, experiments with low spontaneous transformation responses had an impaired ability to detect BaP; however, experiments with high or normal spontaneous responses had a normal ability to detect BaP. Because the expression of BaP-induced transformation depended on both the cytotoxicity of the treatment and the cumulative number of mitoses, the frequency of BaP-induced transformation should be reported as the number of foci/vessel, but not expressed as the number of foci/viable cell surviving the chemical treatment. These conclusions are important because the same 110 experiments described in this report were also used to evaluate the transformation responses of many different carcinogenic and noncarcinogenic chemicals. These data are being reported separately.

Introduction

There have been a number of reports describing chemical-induced morphological transformation of BALB/c-3T3 cells (*1–4*), and these investigations invariably used 3-methylcholanthrene (MCA) as the positive control. In addition, government agencies (*5*) and scientific committees (*6*) have recommended MCA be routinely used in this assay. Nevertheless, there has not been a study that systematically investigated the effects of different experimental parameters on MCA-induced transformation responses in BALB/c-3T3 cells. Likewise, the kinetics of MCA-induced cytotoxic and transformation responses have not been systematically compared with the activities of other chemicals in the assay.

Preliminary investigations in this laboratory revealed that the kinetics of induction of MCA cytotoxic and transformation responses in BALB/c-3T3 cells were different from most test chemicals tested in the assay. For example, MCA induced very high transformation responses over a two log range in concentrations from 0.1 to 10 μg/ml, whereas most test chemicals, such as *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine (MNNG), induced transformation over a limited range of treatment doses (i.e., 0.5–2.0 μg/mL MNNG; data not presented). In addition, 48 or 72-hr MCA treatments induced cytotoxic responses that changed very gradually with concentration over a three-log range, whereas most chemicals under similar treatment conditions induced sharp dose-related changes in cytotoxicity. Thus, MCA was not a good choice to investigate the effect of different experimental parameters on chemical-induced cytotoxic and transformation responses.

Therefore, this laboratory investigated four chemicals to be substituted for MCA as a possible positive control for the BALB/c-3T3 cell transformation assay: benzo[*a*]-pyrene (BaP), 5-bromo-2'-deoxyuridine (BUdR), cytosine arabinoside, and N-methyl-*N'*-nitro-nitrosoguanidine (MNNG). All four chemicals induced highly significant transformation responses (data not reported). In contrast to MCA, all four chemicals induced dose-related increases in both cytotoxic and transformation responses over a relatively short range of treatment doses. Previously, BaP and MNNG have also been demonstrated to be active in this assay (*1–4*). BaP was selected as the positive control for the BALB/c-3T3 transformation assay over the other

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three chemicals, because many carcinogens in rodent bioassay are thought to require cellular metabolism to become carcinogenic and are not considered direct-acting mutagenic chemicals (*7,8*). Thus, BaP was selected because it acts as a mutagen only after the parent chemical has been metabolized by cells, or with an exogenous activation system (*9*). This report summarizes data obtained in 110 transformation experiments conducted over a 2-year period. BaP was used as the positive control and tested at two treatment doses. To eliminate experimental parameters known to influence detection of transformation responses, all experiments were conducted using only two lots of fetal bovine serum (FBS), two frozen pools of BaP, and a single cryopreserved pool of wild type (WT) cells. Furthermore, since WT BALB/c-3T3 cells expressed a continuum of type I–III foci of different sizes (*10*), the significance of chemical-induced responses could possibly be influenced by the inherent subjectivity of scoring morphological variants. Therefore, all of the foci observed in these experiments were recorded and analyzed.

Materials and Methods

Cell Culture

The investigations in this report used the 1-13 clone of A31 BALB/c-3T3 cells (*11,12*). The materials and methods used to culture the cells have been previously reported in detail (*13*) and are summarized in part I of these investigations (*10*).

Standard Clonal Survival Assay

The standard clonal survival assay, using low-density cultures of BALB/c-3T3 cells, was conducted according to our modification (*13*) of the method described by Kakunaga (*14*). Briefly, 200 WT cells were seeded in either 60-mm culture dishes (Corning, Corning, NY) or 25-cm^2 culture flasks (Corning). BaP (Sigma, St. Louis, MO) treatment doses were applied to triplicate cultures for 48 hr beginning 2 days after seeding. BaP treatments were terminated by removal of the treatment medium, washing the culture vessels twice with Hank's balanced salt solution (HBSS; Quality Biologicals, Gaithersburg, MD), and fed with culture medium. After a total culture period of 8 days, the vessels were washed, fixed in methanol, stained with Giemsa, and colonies of cells were hand tabulated according to the procedure described in part IV of these investigations (*15*).

Co-Culture Clonal Survival Assay and Transformation Assay

The procedure used for the co-culture clonal survival assay has been previously reported in detail (*13,16*) and is summarized in part III of this series (*17*).

BaP-induced transformation of BALB/c-3T3 cells was evaluated in a standard transformation assay protocol that has been reported in detail (*13*) and is summarized in these investigations in part IV (*15*). Briefly, each transformation assay contained three components: a standard clonal survival assay (*13,15*), a co-culture clonal survival assay (*13,17*), and a transformation assay (*13,15*). In each experiment, BaP-induced transformation was detected in the positive control, which consisted of 20 vessels seeded with 3.2×10^4 cells/vessel. BaP doses were applied to cell cultures for 48 hr, day 2–4, using standard procedures (*13,15*).

Evaluation of Transformed Foci

The method used to evaluate transformed foci of BALB/c-3T3 cells has been reported in detail (*13*) and is summarized in Part IV of this series (*15*) of these investigations. Briefly, the number of type I–III transformed foci of BALB/c-3T3 cells were identified microscopically using published criteria (*5–6,14,18–19*), and type III foci had three phenotypic properties: piling and overlapping cells, disorientation of cells at the periphery of the focus, and invasion of transformed cells into a contact-inhibited monolayer of WT cells. Type I and II foci also appeared in many different sizes, but they lacked one or more of the three phenotypic properties of the type III transformed focus.

Handling of Test Chemical

Two large batches of BaP were prepared and used during this investigation. Experiments 1–84 used the first batch of BaP, and experiments 84–110 used the second batch. Both batches of BaP were tested in the same experiment, and they induced comparable cytotoxic and transformation responses (unpublished data). The BaP was dissolved in dimethyl sulfoxide (DMSO) at 5 mg/mL and diluted with DMSO to prepare 100 μg/mL aliquots of BaP. The ampules containing 100 μg/mL BaP were frozen and stored until use in experiments. The 100 μg/mL BaP stock was diluted 100-fold with culture medium to a 5-fold concentrated dosing solution of 1.0 μg/mL, and 1 mL of this was rapidly administered to culture vessels containing 4 mL of culture medium.

Statistical Methods

The method used to determine the distribution of spontaneous transformed foci of BALB/c-3T3 cells has been previously reported (*13,20–21*) and is described in detail in part I of these investigations (*10*). Briefly, after examining several mathematical transformations (*22*), the data were found to fit a logarithmic ($\log_{10}$) transformation (*10,13,20*).

The significance of different sets of transformation responses was determined in three steps using SAS software (*23*). First, an analysis of variance was performed on $\log_{10}$ data using the *F*-test. Second, the significance of responses was calculated using modifications of the Student's *t*-test, one assuming equal variance (EV) between the control and comparison set and the other assuming unequal variance (UV). The correct *t*-statistic was distinguished by a *F*-test confidence level of 5% (i.e., $p<0.05$). Third, the probability of individual sets of data having a significant activity was determined using the appropriate EV or UV *t*-statistic.

Rank-ordering of BaP-induced Transformation Responses. BaP-induced highly significant transformation responses in 109/110 experiments and was inactive only in experiment 62. The magnitude of the BaP responses was variable and could be rank-ordered on the basis of the statistical significance of the response. A rank-order method using t-statistics was selected because the magnitude of the t-statistic was proportional to the significance of the BaP response and was independent of the magnitude of the spontaneous response. The significance of each BaP response, or average t-statistic, was calculated by averaging the t-statistics of the two treatment doses. Negative t-statistics were arbitrarily assigned the value of zero (Table A1). The large range of spontaneous responses in the 110 experiments [refer to part I of these investigations (*10*)] precluded the use of the mean BaP response to rank-order BaP responses. A similar method of combining significance levels has been employed using z-statistics from nonparametric statistical tests (*24*).

Statistical Sensitivity versus Frequency of Spontaneous Transformation. The magnitude of the frequencies of spontaneous transformation in the BALB/c-3T3 cell transformation assay was variable among the 110 experiments, and the experimental parameters affecting spontaneous frequencies have been reported (*10*). Variability among spontaneous frequencies directly affected the ability of a transformation assays to discriminate a significant test chemical-induced response. Experiments with a spontaneous frequency lower than the median frequency had a lower statistical sensitivity to detect test chemical transformation responses. Conversely, experiments with a spontaneous frequency higher than the median frequency had a higher statistical sensitivity to detect chemical responses.

The statistical sensitivity can be determined by calculating the ratio of the t-statistic of the experimental (i.e., t^{exp}) divided by the t-statistic of the median experiment (i.e., t^{med}). Thus, the statistical sensitivity is equal to the ratio of X^{exp}/SE^{exp} divided by X^{med}/SE^{med}. The X^{exp} and X^{med} are the mean experimental and median spontaneous frequencies, and the SE^{exp} and SE^{med} are the standard errors of the mean experimental and median spontaneous frequencies. The statistical sensitivity ratio was calculated for each of the 110 experiments and used to rank-order the experiments from the highest to the lowest in terms of statistical sensitivity (Table A2).

Detection Sensitivity versus Benzo(a)pyrene Transformation Response. A mathematical model was developed to compare the BaP transformation responses in different experiments. This model compared the transformation responses of the two BaP treatment doses to the median historical response of the assay.

In some experiments the two BaP treatment doses had responses that were both higher than the median responses, and these experiments had a high detection sensitivity for BaP. Conversely, some experiments had both two BaP transformation responses that were lower than the median responses, and these experiments had low detection sensitivity for BaP. After separating experiments according to FBS lot used (Table 1), the magnitude of the BaP transformation responses detected at two treatment doses per experiment were compared statistically to the median activity. The t-statistics from the two doses were averaged by adding the two t-statistics and dividing this number by the square root of n (i.e., square root of 2 or 1.414).

In contrast, a portion of the experiments had two BaP responses in which one BaP response was larger then the median response and the other was smaller than the median response. These experiments had responses with t-statistics with opposite signs relative to the median BaP responses; therefore, the detection sensitivities of these experiments were indistinguishable from the median BaP responses.

Results

Effect of Experimental Parameters on the Magnitude of BaP-Induced Cytotoxic and Transformation Responses

The effect of varying four different experimental parameters on the magnitude of BaP-induced transformation of BALB/c-3T3 cells was investigated in 110 experiments using a standard assay protocol (see Materials and Methods). The four experimental parameters were *a*) the use of two lots of FBS, *b*) 18 ampules of cryopreserved cells from a single cell pool, *c*) p4–p22 passage levels of cells, and *d*) 110 different spontaneous transformation responses. Experiments 1–61 used FBS lot A and ampules of cells 1A–1L; experiments 62–110 used FBS lot B and ampules 1L–1R. BaP was tested in all experiments using treatment doses 0.20 and 0.063 μg/mL.

FBS Lot. The data summarizing the effect of FBS on the BaP-induced transformation and cytotoxic responses are presented in Table 1, and experimental data from individual experiments are provided in detail in Table A1. The data presented in Table 1 show that the median spontaneous transformation response detected in experiments using FBS lot A was less than the median response detected for experiments using serum lot B, i.e., 0.43 versus 0.61 type III foci/vessel, respectively. In contrast, the median BaP-induced transformation responses detected for 0.20 and 0.063 μg/mL treatment doses were higher for experiments using FBS lot A versus FBS lot B. For example, the BaP treatment dose of 0.20 μg/mL induced a median response of 7.76 type III foci/vessel in experiments using FBS lot A and only 4.64 type III foci/vessel in experiments using FBS lot B. Statistical analyses of the data revealed that the BaP transformation response for 0.200 μg/mL treatment dose was significantly ($p<0.001$) higher for FBS lot A versus FBS lot B, if the mean spontaneous transformation responses for these experiments was first subtracted from the corresponding mean BaP transformation responses. Similarly, the mean BaP transformation response detected for the 0.0633 μg/mL treatment dose was significantly ($p<0.001$) higher for FBS lot A versus FBS lot B.

The explanation for the elevated BaP transformation responses in experiments using FBS lot A was shown to correlate directly with the BaP-induced cytotoxic

Table 1. Effect of lot of FBS on the median and mean of BaP-induced cytotoxic and transformation responses.

Experimental parameter[a]			Cytotoxic response,[b] % RCE		Transformation response,[c]
FBS lot	Exp. no.	BaP, μg/mL	Standard assay	Co-culture assay	type III foci/vessel
Median experimental responses					
A	1- 61	0.200	2	38	7.76
B	62-110	0.200	8	75	4.64
A	1- 61	0.0633	7	65	3.93
B	62-110	0.0633	21	85	2.88
A	1- 61	0	100	100	0.43
B	62-110	0	100	100	0.61
Mean experimental responses, mean ± SE[d]					
A	1- 61	0.200	2.91 ± 0.40 (59)	40.6 ± 2.28 (60)	7.67 ± 0.37 (62)
B	62-110	0.200	12.9 ± 1.82 (47)***	63.2 ± 2.28 (48)***	5.97 ± 0.62 (49)**
B	62–76, 83–110[e]	0.200	14.4 ± 2.66 (41)***	63.8 ± 2.29 (42)***	4.75 ± 0.30 (43)***
A	1- 61	0.0633	7.89 ± 0.74 (59)	74.8 ± 2.04 (48)	4.28 ± 0.29 (62)
B	62-110	0.0633	26.7 ± 2.20 (47)***	83.7 ± 1.72 (48)***	3.83 ± 0.48 (49)*
B	62–76, 83–110[e]	0.0633	26.7 ± 2.69 (41)***	84.5 ± 1.78 (42)***	2.80 ± 0.20 (43)***
A	1- 61	0	100 (59)	100 (60)	0.66 ± 0.08 (62)
B	62-110	0	100 (47) (NS)	100 (48) (NS)	1.37 ± 0.26 (49) (NS)
B	62–76, 83–110[e]	0	100 (41) (NS)	100 (42) (NS)	0.92 ± 0.14 (43) (NS)

Abbreviations: BaP, benzo[*a*]pyrene; exp. no., experiment number; FBS lot, fetal bovine serum number; %RCE, percent relative cloning efficiency; NS, not significant ($p > 0.05$).

[a]Experiments numbered 1–61 used FBS lot A, and experiments numbered 62–110 used FBS lot B (see Materials and Methods for sources of FBS). The BaP treatment doses were prepared from frozen stocks of BaP dissolved in dimethylsulfoxide.

[b]The cytotoxic responses of BaP in the 110 experiments was measured in either a standard or a co-culture clonal survival assay. The cytotoxic response represents the percent %RCE of the BaP-treated cell cultures relative to the untreated cell cultures. The median cytotoxic responses were determined by rank-ordering the responses from experiments that used the two different FBS lots. The responses are expressed in terms of the mean ± SE; numbers in parentheses refer to the number of experiment in the subgroup.

[c]Transformation response: The BaP-induced transformation responses were calculated using a three-step procedure involving the $\log_{10}$ mathematical transformed data. The arithmetic value of the transformation response, or foci/vessel, represents the anti-log of the $\log_{10}$ mean transformation response minus one.

[d]The significance of BaP transformation responses detected in experiments using FBS lot A versus FBS lot B were calculated using the FBS lot A responses as the control. Before these comparisons were made statistically, the mean frequency of spontaneous transformation was subtracted from the BaP transformation response in each group. This was necessary due to the approximately 2-fold difference in the spontaneous frequencies detected for the two FBS lots.

[e]Experiments 77–82 using FBS lot B and cells from ampule 1N had very high spontaneous frequencies (see Table A1) and were outliers in this investigation (*15*). Thus, the BaP responses detected in FBS lot B experiments were calculated for significance in the presence and the absence of experiments 77–82.

*Significant transformation response, $0.01 < p \leq 0.05$.

**Significant transformation response, $0.001 < p \leq 0.01$.

***Significant transformation response, $p \leq 0.001$.

responses detected in experiments using the two different FBS lots. The standard clonal survival assay assessed the relative cloning efficiency (RCE) of cells in low cell density cultures; the co-culture assay measured RCE of cells in high-density cell cultures (see Materials and Methods). The median and mean cytotoxic responses detected in experiments using FBS lot A were consistently higher than responses in experiments using FBS lot B. For example, in experiments using FBS lot A, 0.200 μg/mL BaP induced a median 2% RCE in the standard clonal survival assay and 38% RCE in the co-culture clonal survival assay. In contrast, in experiments using FBS lot B, the same treatment dose induced a median of 8% RCE in the standard assay and 75% RCE in the co-culture assay. Analogous differences for the cytotoxic responses in experiments using the two FBS lots were observed for the 0.063 μg/mL BaP treatment dose. In conclusion, experiments performed with FBS lots A and B both demonstrated dose-related increases in cytotoxic and transformation responses; however, the magnitude of both responses was FBS-dependent for the same concentration of BaP.

Ampule of Cryopreserved Cells. The spontaneous and BaP-induced transformation responses detected in 110 experiments using 18 different ampules of cryopreserved cells are summarized in Table 2. The data were rank-ordered according to the significance of the average BaP transformation response detected in experiments using cells from a single ampule. The significance of the response is directly proportional to the magnitude of the *t*-statistic. In addition, the data were separated for experiments conducted in the two FBS lots.

The data demonstrated that variability among the BaP-induced transformation responses was highly correlated with the use of different ampules of cryopreserved cells. For example, cells from ampule 1B using FBS lot A had the

Table 2. Spontaneous transformation frequencies and BaP-induced transformation responses detected in cultures derived from 18 ampules of cells.

Amp no.[a]	Cytotoxic response, mean %RCE[b]				Transformation response,[c] Type III foci/vessel, mean ± SE			Significance,[d] *t*-statistic; mean ± SE		
	Standard assay		Co-culture assay							
	0.063	0.200	0.063	0.200	Control	0.0633	0.200	0.0633	0.200	Average
Serum lot A										
1B (4)	10	4	61	30	0.26 ± 0.11	3.24 ± 0.78	7.48 ± 1.35	12.4 ± 2.72	20.6 ± 2.38	16.5 ± 2.29
1H (9)	10	3	74	37	0.72 ± 0.13	6.80 ± 0.89	10.8 ± 1.49	13.0 ± 1.56	18.0 ± 1.62	15.5 ± 1.23
1D (4)	4	1	69	26	0.19 ± 0.11	3.23 ± 0.31	5.74 ± 1.33	12.1 ± 2.00	14.1 ± 0.62	13.1 ± 1.04
1I (5)	10	3	87	44	0.46 ± 0.04	3.57 ± 0.25	7.79 ± 0.54	8.69 ± 0.77	17.0 ± 1.78	12.8 ± 1.66
1E (8)	4	1	79	54	0.50 ± 0.08	3.68 ± 0.28	7.30 ± 0.98	9.30 ± 0.54	15.6 ± 1.77	12.4 ± 1.21
1F (5)	7	2	63	23	0.54 ± 0.15	3.76 ± 0.73	8.24 ± 1.16	8.03 ± 1.10	16.0 ± 1.56	12.0 ± 1.61
1K (7)	6	3	66	31	0.31 ± 0.08	2.29 ± 0.45	6.43 ± 0.48	7.02 ± 1.16	16.7 ± 1.30	11.8 ± 1.58
1C (4)	7	2	66	28	0.67 ± 0.51	4.18 ± 2.54	6.34 ± 1.45	7.46 ± 1.29	13.3 ± 2.37	10.4 ± 1.67
1G (8)	7	4	82	51	1.28 ± 0.27	5.57 ± 0.29	7.77 ± 0.65	8.39 ± 0.53	11.6 ± 0.95	9.98 ± 0.67
1A (4)	14	10	71	64	0.98 ± 0.30	3.92 ± 0.79	7.93 ± 0.71	6.09 ± 1.01	12.9 ± 1.40	9.52 ± 1.52
1L (1)	10	0	70	33	0.22	1.92	4.28	8.76	14.3	8.76
1J (3)	9	3	77	48	1.51 ± 0.64	5.92 ± 1.55	6.26 ± 1.30	7.87 ± 0.16	8.51 ± 0.48	8.19 ± 2.48
Serum lot B										
1N (6)[e]	30	20	74	68	4.63 ± 1.11	11.3 ± 1.63	14.7 ± 2.67	9.20 ± 4.68	11.4 ± 1.71	10.3 ± 0.91
1P (4)	24	8	90	56	1.36 ± 0.55	4.21 ± 0.53	5.95 ± 0.70	6.76 ± 1.90	10.5 ± 2.23	8.62 ± 1.53
1Q (9)	34	22	91	72	0.58 ± 0.08	2.63 ± 0.19	4.54 ± 0.51	6.64 ± 0.58	10.7 ± 1.01	8.49 ± 0.52
1L (8)	15	5	81	54	0.47 ± 0.25	1.58 ± 0.30	4.04 ± 0.51	4.26 ± 0.61	11.5 ± 1.20	7.89 ± 1.20
1R (5)	34	25	78	63	1.72 ± 0.44	4.53 ± 0.30	6.16 ± 0.46	5.65 ± 0.81	8.73 ± 3.90	7.19 ± 0.99
1M (7)	15	4	83	61	0.78 ± 0.20	2.78 ± 0.29	4.84 ± 1.16	4.96 ± 0.74	8.37 ± 1.34	6.66 ± 0.87
1O (10)	37	20	85	69	0.58 ± 0.16	2.17 ± 0.37	3.83 ± 0.58	4.99 ± 0.65	7.64 ± 0.90	6.31 ± 0.62
Median responses for serum lots A and B										
Median A	8	3	71	35	0.52	3.72	7.39	8.54	15.8	11.9
Median B	30	20	83	63	0.78	2.78	4.84	5.65	10.5	7.89

Abbreviations: Amp. No., ampule number; BaP, benzo[*a*]pyrene; % RCE, percent relative cloning efficiency.

[a]The experimental parameter ampule number refers to the aliquot of cryopreserved cells used in a sequence of experiments. The numbers in parentheses represent the number of experiments that used the same ampule of cells.

[b]The cytotoxic response of BaP at the levels indicated in the 110 experiments was measured in either a standard or a co-culture clonal survival assay (see Materials and Methods). The cytotoxic response represents the %RCE of the BaP-treated cell cultures relative to the untreated cell cultures. The median cytotoxic responses were determined by rank-ordering the responses from experiments that used the two different FBS lots.

[c]The BaP-induced transformation responses at the levels indicated were calculated using a three-step procedure involving the $\log_{10}$ mathematical transformed data (see Materials and Methods). The arithmetic value of the transformation response, or foci/vessel, represents the anti-log of the $\log_{10}$ mean transformation response minus one.

[d]The significance of groups of BaP-induced transformation responses was calculated using SAS software (*23*), as described in the text. The *t*-statistic according to the *F*-test was used to calculate the statistics in this table. The *t*-statistics of each treatment dose were averaged to determine the average *t*-statistic.

[e]Outlier ampule of cells. The cells from ampule 1N had a very high average spontaneous transformation frequency that has been reported to be an outlier relative to the other 17 ampules of cells used in this investigation (*15*). These frequencies resulted in significantly high statistical sensitivity.

most significant BaP transformation responses, with a mean *t*-statistic of 16.5. This mean *t*-statistic was obtained by averaging the mean *t*-statistics of 12.4 and 20.6 for the 0.0633 and 0.200 μg/mL BaP treatment doses, respectively. Conversely, ampule 1O using FBS lot B had the least significant BaP transformation response, with an average *t*-statistic of 6.31.

The median significance of the experiments using FBS lot A and ampule of cells 1A–1L was a *t*-statistic of 11.9 (Table 2). In contrast, the median significance of the experiments using FBS lot B and ampule of cells 1L–1R was much lower and had a *t*-statistic of 7.89. The overwhelming majority of experiments using FBS lot A had more significant transformation responses than those detected in experiments using FBS lot B, if one excluded experiments conducted with cells from ampule 1N. Experiments using cells from ampule 1N had an unusually high spontaneous transformation response, and this ampule has been reported to be a statistical outlier to the remaining 17 ampules (*15*). The significance of the range of experiments using FBS lot A was 8.19–16.5 versus the range of experiments using FBS lot B of 6.31–8.62. Therefore, the use of different lots of FBS not only affected the absolute magnitude of the BaP transformation response (Table 1), but it also had a corresponding effect on the relative significance of the BaP transformation response. Therefore, the data presented in Table 2 demonstrated that much of the variability associated with BaP-induced transformation responses was correlated with the use of different FBS lots and different ampules of cryopreserved cells. Conversely, if the BaP-induced responses were compared for experiments using the same FBS lot and ampule of cells, then the average BaP responses were remarkably similar in magnitude.

Culture Passage Level. The laboratory cultures used in these experiments included passage p4–p22; thus, it was possible that cells from different passage levels could have had different detection sensitivities for BaP. The data

summarizing the effect of passage level on BaP transformation responses are presented in Table A1. The experiments with the different BaP detection sensitivities were rank-ordered from the experiment with the highest to the lowest sensitivities for each ampule of cells. Because the experiments had increasing passage number for increasing experiment numbers, an effect of passage on the significance of the BaP transformation response would result in a sequencing the experiments for each ampule of cells. These data clearly showed that increasing passage level of laboratory cultures had no effect on the detection sensitivity for BaP. The same conclusion was reached if the 0.20 μg/mL BaP treatment alone or the 0.063 μg/mL BaP treatment alone were used to rank-order the data.

Frequency of Spontaneous Transformation. The frequencies of spontaneous transformation and BaP-induced transformation responses detected in the 110 experiments both included activities that were high and low relative to the median activities. If the two types of activities were mechanistically related, then an elevated frequency of spontaneous transformation could have automatically resulted in an elevated BaP response. However, the data presented in Table 2 demonstrated that an elevated BaP-induced transformation response did not automatically correlate with an elevated spontaneous transformation frequency. For example, the highest BaP responses were detected in experiments using FBS lot A and ampule 1H, in which cells had a spontaneous frequency of 0.72 type III foci/vessel. The type III transformation responses for the 0.200 and the 0.0633 μg/mL treatment doses were 10.8 foci/ vessel and 6.08 foci/vessel, respectively. These responses were far higher than those detected for experiments that used FBS lot A and ampule 1J cells that had a relatively high spontaneous transformation frequency of 1.51 type III foci/vessel. The BaP-induced type III transformation responses for these experiments were 6.26 and 5.92 type III foci/vessel for the 0.20 and 0.0633 μg/mL BaP treatment doses. Likewise, experiments using ampules of cells 1G, 1J, 1N, 1P, and 1R all had relatively high average spontaneous frequencies > 1.00 type III foci/vessel. However, the significance of average BaP-induced transformation responses in experiments using 1G, 1J, and 1R ampules of cells included experiments with relatively low average *t*-statistics, and ampules 1N and 1P had relatively high average *t*-statistics. Thus, the occurrence of type III foci, whether spontaneous or due to BaP-induction, appeared independently of one another.

Effect of Statistical Sensitivity on Detection Sensitivity for BaP

Frequencies of spontaneous transformation in this investigation in individual experiments ranged from 0.035 (ampule 1B, experiment 5) to 8.01 (ampule 1N, experiment 82) type III foci/vessel (Table A1). Experiments with high spontaneous frequencies had high statistical sensitivity and required a relatively small increase in the BaP-induced response to be statistically significant. Experiments with low spontaneous frequencies had low statistical sensitivities and required a relatively large increase in the BaP-induced response to be statistically significant. (The procedure for calculating the statistical sensitivity for individual experiments is explained in the Materials and Methods.)

The spontaneous transformation statistical sensitivities measured in 110 experiments are summarized in Table 3 and compared in detail in Table A2. The statistical sensitivities were divided into 4 groups: group S1, 12 experiments with significantly high sensitivity; group S2, 36 experiments with above average sensitivity; group S3, 53 experiments with below average sensitivity; and group S4, 9 experiments had significantly low sensitivity. Therefore, a total of 89/110 or 80.9% (Groups S2 + S3) of the experiments had statistical sensitivities that were not significantly different from the statistical sensitivity of the median experiment. In contrast, 9/110 or 8.2% (group S4) of the experiments had a significantly low statistical sensitivity, and a spontaneous frequency that was less than approximately 0.20 type III foci/vessel. Experiment 5 with a spontaneous frequency of 0.035 type III foci/vessel was

Table 3. Comparison of experiments with different statistical sensitivities with their detection sensitivities for BaP.

	Spontaneous transformation statistical sensitivity[a]		BaP transformation detection sensitivity[b]	
	Level	% of experiments	Level	% of experiments
Experiments with significantly high sensitivity to detect BaP (group B1)				
Group S1	Significantly high	100 (12/12)	High	33.3 (4/12)
Group S2	Above average	100 (36/36)	High	41.7 (15/36)
Group S3	Below average	100 (53/53)	High	17.0 (9/53)
Group S4	Significantly low	100 (9/9)	High	0 (0/9)
		Total (110/110)		Total (28/110)
Experiments with significantly low sensitivity to detect BaP (group B3)				
Group S1	Significantly high	100 (12/12)	Low	16.7 (2/12)
Group S2	Above average	100 (36/36)	Low	16.7 (6/36)
Group S3	Below average	100 (53/53)	Low	28.3 (15/53)
Group S4	Significantly low	100 (9/9)	Low	66.7 (6/9)
		Total (110/110)		Total (29/110)

[a]Variable spontaneous frequencies resulted in experiments with different statistical sensitivities to detect chemical-induced transformation (see Materials and Methods). The spontaneous frequencies and the corresponding statistical sensitivities of 110 experiments are listed in Table A2.

[b]Variable experimental BaP-induced transformation responses results in experiments were due to experiments with different sensitivities to detect BaP-induced transformation (see Materials and Methods). The relative detection sensitivities for BaP of 110 experiments are listed in Table A2.

5.3-fold less sensitive than the median experiment. Conversely, 12/110 or 10.9% (group S1) of the experiments had a significantly high statistical sensitivity, and a spontaneous frequency greater than approximately 2.5 foci/vessel. Experiment 82 with a spontaneous frequency of 8.01 type III foci/vessel and was 6.3-fold more sensitive than the median experiment.

BaP-induced transformation responses in individual experiments ranged from a t-statistic of 22.7 in experiment 43 (ampule 1H) to a t-statistic of 1.18 in experiment 62 (ampule 1L). Experiments with BaP transformation responses higher than the median response had a higher detection sensitivity to detect BaP, and conversely, experiments with BaP transformation responses lower than the median response had a lower detection sensitivity to detect BaP. (The method for calculating the detection sensitivity for BaP is described in Materials and Methods.) The detection sensitivity for BaP for the 110 experiments was divided into three groups: group B1, 28 experiments had a significantly high ($p<0.05$) sensitivity for BaP; group B2, 29 experiments had a significantly low ($p<0.05$) sensitivity for BaP, and group B3, 53 experiments with sensitivities indistinguishable from the median experiment.

Taken together, the data in Table 3 show that low statistical sensitivities of experiments directly effected the experimental detection sensitivity for BaP. The nine experiments with significantly low statistical sensitivities had no experiments (0/9) with significantly high detection sensitivity. In contrast, 66.7% (6/9) of the experiments with significantly low statistical sensitivity had significantly low detection sensitivity for BaP. Thus, a significantly low spontaneous transformation frequency in a experiment was highly correlated with a significantly low BaP transformation response.

In contrast to the experiments with significantly low statistical sensitivity, experiments with high statistical sensitivities or high spontaneous transformation frequencies have no apparent effect on the detection sensitivity for BaP. The 11 experiments with significantly high statistical sensitivity had 36.4% (4/11) experiments with significantly high detection sensitivity. The 36 experiments with above average statistical sensitivity had a comparable 41.7% (15/36) experiments with significantly high detection sensitivity. Likewise, the 11 experiments with significantly high statistical sensitivity had 18.2% (2/11) experiments with significantly low detection sensitivity for BaP. The 36 experiments with above average statistical sensitivity had a comparable 16.7% (6/36) experiments with significantly low detection sensitivity.

Comparison of BaP Transformation Responses Scored for Type III versus Type I–III Foci

Spontaneous and BaP-induced transformed foci occurred as a continuum of different sizes and morphological types; thus, BaP transformation responses could be expressed at type I, II, III, I–II, II–III or I–III foci/vessel. Table 4 presents a summary of experiments in which both type III and type I–III spontaneous and BaP-induced transformation activities were simultaneously scored and recorded. The transforming activity data from individual experiments are provided in detail in Table A1. The selection of type III and I–III transformation activities was arbitrary, and the type I + II transformation response can be calculated directly by subtracting the type III response from the type I–III response.

The median transformation response for experiments using FBS lot A and 0.200 μg/mL BaP was 20.1 type I–III foci/vessel and 7.39 type III/foci/vessel. Thus, the type I–III BaP-induced response was 2.86-fold higher (i.e., 20.1/7.39 = 2.86) than the type III response. Furthermore, the median BaP transformation response contained 12.71 type I + II foci/vessel (i.e., 20.1 − 7.39 = 12.71). A comparable calculation revealed that the median 0.0633 BaP type I–III response was 2.29-fold higher than the type III response. Likewise, the median spontaneous transformation type I–III frequency was 2.26-fold higher than the type III response. Therefore, the ratio of type I–III foci to the type III foci was approximately 2-fold and was roughly equal for the spontaneous transformation frequency and the BaP-induced transformation responses detected in experiments using FBS lot A. The same 2-fold ratio of type I–III foci to type III was observed in experiments using FBS lot B. Taken together, these data showed that BaP induced the same ratio of type III and type I + II foci that appeared spontaneously in the culture vessels. Furthermore, an approximately 2-fold ratio of type I–III to type III foci was observed in experiments using different ampules of cells and FBS lots.

Because BaP did not alter the ratio of type III versus type I–III foci/vessel in most experiments, than the experiments scored for type III versus type I–III foci should have had approximately the same detection sensitivities for BaP. The data in Table 4 verify this prediction. The median average t-statistic for experiments using FBS lot A was 11.9 for the type III transformation response and 14.1 for the type I–III response. Taken together, a 2.6-fold increase (2.29 + 2.86/2 = 2.60) in the type I–III foci versus the type III foci resulted in only a 1.2-fold increase in the average t-statistic for experiments using FBS lot A. The average t-statistic for experiments using FBS lot B were only 1.14-fold higher for the type I–III versus the type III response. Furthermore, the average t-statistics for type III versus type I–III BaP-induced transformation responses were roughly equal for experiments that used different ampules of cells.

Discussion

The purpose of this investigation was to determine experimental parameters that correlated with the variable detection of chemical-induced transformation of BALB/c-3T3 cells. BaP was selected for this investigation because it induced reproducible, significant transformation responses. Furthermore, 48-hr BaP treatments induced cytotoxic and transformation responses that both exhibited dose-related increases in activity (Table 1). Likewise, BaP has been reported to be mutagenic only when the

Table 4. Comparison of the magnitude and the significance of type III versus type I-III BaP-induced transformation responses.

Amp. no.[a]	Transformation responses, foci/vessel[b] BaP, μg/mL			Significance (*t*-statistic)[c] BaP, μg/mL		
	Control	0.0633	0.200	0.0633	0.200	Average
Median responses detected using FBS lots A and B						
Median A	0.52/1.45	3.72/8.41	7.39/20.01	8.54/9.66	15.8/19.0	11.9/14.1
Median B	0.78/1.52	2.78/5.19	4.84/9.86	5.65/6.35	10.5/10.5	7.89/11.4
Responses detected using FBS lot A						
1B (4)	0.26/ND[d]	3.24/ND	7.48/ND	12.4/ND	20.6/ND	16.5/ND
1H (9)	0.72/1.46	6.80/15.4	10.8/28.1	13.0/13.6	18.0/20.6	15.5/17.1
1D (4)	0.19/0.36	3.23/7.13	5.74/14.9	12.1/15.4	14.1/21.2	13.1/18.3
1I (5)	0.46/1.04	3.57/8.12	7.79/23.1	8.69/9.26	17.0/18.8	12.8/14.0
1E (8)	0.50/1.13	3.68/8.36	7.30/19.1	9.30/9.84	15.6/19.2	12.4/14.5
1F (5)	0.54/5.72	3.76/16.1	8.24/26.0	8.03/9.09	16.0/17.5	12.0/13.3
1K (7)	0.31/0.70	2.29/5.90	6.43/19.5	7.02/9.99	16.7/22.8	11.8/16.4
1C (4)	0.67/1.43	4.18/8.46	6.34/14.1	7.46/9.48	13.3/18.7	10.4/14.1
1G (8)	1.28/3.02	5.57/14.2	7.77/21.3	8.39/9.95	11.6/15.9	9.98/12.9
1A (4)	0.98/ND	3.92/ND	7.93/ND	6.09/ND	12.9/ND	9.52/ND
1L (1)	0.22/0.38	1.92/4.43	4.28/13.8	8.76/7.77	14.3/20.5	8.76/14.1
1J (3)	1.51/4.73	5.92/18.1	6.26/20.6	7.87/8.17	8.51/9.59	8.19/8.88
Responses detected using FBS lot B						
1N (6)[e]	4.63/9.16	11.3/25.4	14.7/35.1	9.20/10.0	11.4/13.3	10.3/11.7
1P (4)	1.36/3.05	4.21/9.00	5.95/12.7	6.76/7.60	10.5/10.5	8.62/9.05
1Q (9)	0.58/1.03	2.63/4.92	4.54/8.82	6.64/7.49	10.7/11.5	8.49/9.50
1L (8)	0.47/1.00	1.58/3.31	4.04/8.92	4.26/5.19	11.5/12.8	7.89/9.00
1R (5)	1.72/5.25	4.53/10.8	6.16/15.7	5.65/6.35	8.73/10.5	7.19/8.43
1M (7)	0.78/1.52	2.78/5.19	4.84/9.86	4.96/5.98	8.37/9.69	6.66/7.84
1O (10)	0.58/0.87	2.17/3.67	3.83/5.17	4.99/5.42	7.64/8.15	6.31/6.79

Abbreviations: Amp. no., ampule number; BaP, benzo[*a*]pyrene; ND, not determined.

[a]The experimental parameter ampule number refers to the aliquot of cryopreserved cells used in a sequence of experiments. The numbers in parentheses represent the number of experiments that used the same ampule of cells.

[b]The BaP-induced transformation responses were calculated using a three-step procedure involving the $\log_{10}$ mathematical transformed data (see Materials and Methods). The arithmetic value of the transformation response, or foci/vessel, represents the anti-log of the $\log_{10}$ mean transformation response minus one.

[c]The significance of groups of BaP-induced transformation responses was calculated using SAS software (*23*), as described in the text. The *t*-statistic according to the *F*-test was used to calculate the statistics in this table. The *t*-statistics of each treatment dose were averaged to determine the average *t*-statistic.

[d]Because type I–II foci were not scored in experiments 1–6 using ampules 1A and 1B, the type I–III foci response could not be determined.

[e]Outlier ampule of cells. The cells from ampule 1N had a very high average spontaneous transformation frequency that has been reported to be an outlier relative to the other 17 ampules of cells used in this investigation (*15*). These frequencies resulted in significantly high statistical sensitivity.

parent form of the chemical was metabolized into an electrophilic form (*9*), and BALB/c-3T3 cells have been reported to metabolize BaP (*4,25–26*). Therefore, experimental factors affecting cellular metabolism, as well as the expression of the transformed phenotype, could affect the induction of BaP cytotoxic and transformation responses.

This investigation determined that three different experimental parameters can result in variable detection of the BaP-induced transformation response. First, despite our attempts to minimize the known effect of serum on the expression of transformation (*4,13,27–29*) through screening of FBS lots (*10*), the induction of BaP-induced transformation were still correlated with the use of different serum lots. The transformation responses and cytotoxic responses were demonstrated to be significantly higher for FBS lot A verses FBS lot B (Table 1). The mechanism by which FBS affected the BaP-induced cytotoxic and transformation responses is not known, but it could be related to the metabolism of BaP by the BALB/c-3T3 cells (*25,26*). Different FBS lots may alter either the kinetics of the uptake of BaP, or the metabolism of BaP, and this could modulate the cytotoxicity of the BaP. An experimental parameter that changed the BaP-induced cytotoxic response could simultaneously change the BaP-induced transformation response as well. These investigations did eliminate a number of potential explanations for the FBS-dependent BaP transformation responses. Because all experiments were conducted with a single source of BaP, and aliquots of BaP were obtained from frozen vials of BaP, the FBS-dependent variable responses were not related to the BaP used in the experiments. In addition, these experiments used only one cryopreserved pool of cells; thus, the origin of the cells used in the experiments could not explain the results. Although the different ampules of cells from the same cryopreserved pool were shown to affect the BaP transformation response (Table 2), the average BaP transformation responses of most ampules of cells were separated from one another on the basis on the FBS lot used.

Fortunately, the FBS effect on BaP-induced transformation response demonstrated in this investigation was not an insurmountable technical problem for the BALB/c-3T3 cell transformation assay. Because the FBS-dependent transformation response is clearly related to

the cytotoxic response of BaP, comparable BaP transformation responses could be obtained by merely adjusting the concentration of BaP so that experiments conducted with different FBS lots had comparable cytotoxic responses for the BaP. For example, in this investigation the 0.0633 μg/mL BaP treatment dose with FBS lot A had nearly the same cytotoxic and transformation responses as the 0.200 μg/mL treatment dose for FBS lot B. Similarly, the transformation response of 0.633 μg/mL BaP in an experiment using FBS lot B was comparable to that obtained with 0.200 μg/mL BaP using FBS lot A (unpublished data).

The second experimental parameter to affect the detection of BaP transformation was correlated with the use of different ampule of cells to initiate laboratory cultures (Table 2). Thus, the lowest variability among BaP-induced transformation and cytotoxic responses was observed when experiments were conducted with a single FBS lot and a single ampule of cells. In contrast, variability among BaP-induced transformation and cytotoxic responses were not correlated with increasing passage levels of laboratory stock cultures (Table A1). This observation conflicts with data presented in a recent report by Sheu et al. (*30*), who showed that the magnitude of MCA-induced transformation increased with serial passage of WT A31-1-1 BALB/c-3T3 cells.

There are two possible explanations for conflicting results regarding the effect of passage level of cultures on chemical-induced transformation responses obtained in these two investigations. First, this investigation used the 1-13 clone of BALB/c-3T3 cells, and Sheu et al. (*30*) used the 1-1 clone. The phenotypic stability of the 1-1 clone may be less than that of the 1-13 clone, and may exhibit an increased probability of transformation with passage. Second, the frequency of this phenomenon may vary between the two clones of cells. While passage-related increases in the significance of the average *t*-statistic BaP transformation response (i.e., two-treatment dose response) was not observed for any of the ampules of cells, passage-related increases in the foci/vessel BaP-induced transformation response were observed for the 0.20 μg/mL BaP treatment dose. These passage-related increases were observed in only 4 of the 18 ampules of cells (i.e., 1I, 1J, 1D, 1K; refer to Table A1); thus, 14 of 18 ampules did not exhibit a passage-related increase in the focus/vessel activity. At the lower BaP treatment dose 0.063 μg/mL, the passage-related increase in foci/vessel was only observed for cells from ampule 1J. Thus, a passage-dependent increase in the foci/vessel, chemical-induced transformation response was a rare event in this investigation and observed for only 1 of 18 ampules of cells.

The third experimental parameter shown to affect the BaP transformation response was the magnitude of the frequency of spontaneous transformation. A total of 10.9% (12/110) of the experiments in this investigation had significantly high statistical sensitivities, and 8.2% (9/110) of the experiments had significantly low statistical sensitivities (Table A2). Thus, 19.1% of the experiments in this investigation had spontaneous transformation frequencies that resulted in significantly high or low statistical sensitivity. Rather than arbitrarily excluding these experiments from the investigation, the impact of high or low statistical sensitivity was examined on the probability of detection either high or low BaP-induced transformation responses (Table 3). These analyses revealed that the detection sensitivity for BaP was demonstrated to be diminished in experiments that used cells that had low spontaneous transformation frequencies and had significantly low statistical sensitivity. In contrast, the detection sensitivity for BaP was not changed in experiments that used cells that had high spontaneous transformation frequencies and had significantly high statistical sensitivity.

This is an important observation because it shows that each transformation experiment has its own unique statistical sensitivity, as well as its own detection sensitivity for BaP. Furthermore, this implies that each experiment could have had a different statistical sensitivity and detection sensitivity for other test chemicals. Thus, experiments with spontaneous transformation frequencies lower than about 0.20 type III/foci vessel have a significant diminished capability to detect chemical-induced transformation of BALB/c-3T3 cells. However, experiments with high spontaneous transformation frequencies and significantly high statistical sensitivities have no altered sensitivity to detect BaP. Comparable experimental variability among statistical sensitivities and chemical detection sensitivities would be predicted for other *in vitro* mammalian cell assays that have detected chemical-induced genotoxic activity in a large series of experiments.

Although the mechanism by which BaP-induced transformation of the 1-13 clone of BALB/c-3T3 cells was not the objective of this investigation, this investigation presents three lines of evidence that are consistent the hypothesis that BaP-induced a mutation(s) in the WT cells and this resulted in the induction of the transformed phenotype (*31*). According to this hypothesis, the WT cells were genetically altered by the BaP, and the transformed cells acquired the phenotypic capacity to grow within the contact-inhibited monolayer of WT cells. In support of this hypothesis, spontaneous and chemical-induced transformed phenotype of the 1-13 clone of BALB/c-3T3 cells have both been reported to be stable through many population doublings (*10,14,20*). In addition, recent evidence reported from this laboratory support the hypothesis that spontaneous transformation was a mutation of the contact-inhibited phenotype of WT BALB/c-3T3 cells (*10*). In a related cell transformation system, Grisham has reported that MNNG-induced transformation of C3H10T1/2 cells was caused by a mutation of the WT cells (*32,33*).

The first line of evidence was related to the direct dependence of BaP-induced transformation responses of BALB/c-3T3 cells on the BaP-induced cytotoxic responses. In experiments using FBS lot A, 0.20 μg/mL BaP induced a median response of 7.76 type III foci/vessel and a median 38% RCE (Table 1). At the lower treatment dose of 0.063 μg/mL, BaP induced a reduced median response of 3.93 type III foci/vessel and a median 75% RCE. Thus, the absolute magnitude of the BaP transformation response was inversely related to the %RCE and directly related to the cytotoxic response of the chemical treatment. Compar-

able observations have been reached for chemical-induced mutations at the HGPRT locus in Chinese hamster ovary cells (*34*) and the $TK^{+/-}$ locus in L5178Y cells (*35*).

The second line of evidence comes from analyses of transformation responses detected in cultures initiated at different seeding densities. Spontaneous transformation frequencies have been shown to be expressed at the same frequency in cultures seeded at different densities (*10*). In contrast, we have shown that the frequency of chemical-induced transformation detected in MNNG-treated BALB/c-3T3 cells increased proportionally over a range of seeding densities of 1–10 $\times$ 10^4 cells/vessel (unpublished data). In addition, BaP induced highly significant transformation at seeding densities of 3.2 $\times$ 10^4 cells/vessel, but it was inactive at 0.32 $\times$ 10^4 cells/vessel (unpublished data). In these experiments the cytotoxic activities of individual MNNG and BaP treatment doses were identical at the different seeding densities. Therefore, the increases in MNNG and BaP-induced transformation responses with increasing seeding density were most likely due to an increased number of genetically damaged cells at the higher seeding densities.

The third line of evidence comes from consideration of the metabolism of BaP by BALB/c-3T3 cells (*25,26*). The electrophilic or mutagenic form of BaP is a metabolite of BaP and not the parent compound (*9*). The direct-acting mutagenic form of BaP, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-diolepoxide-anti, has a comparable cytotoxic activity as the parent BaP, and induced comparable transformation of the BALB/c-3T3 cells (unpublished data). In contrast, in other mammalian cell systems in which the parent BaP is inactive as a mutagen, and no evidence of metabolism by the WT cells has been reported, BaP is observed to be relatively noncytotoxic to the target cells. Furthermore, if BaP was acting as a mutagen in these experiments, then the magnitude of BaP-induced transformation should have been correlated to experimental parameters that affected metabolism of BaP by BALB/c-3T3 cells, such as the duration of the treatment and the cytotoxic activity of the treatments. In support of this conclusion, we have observed that the cytotoxic response of BaP is time dependent, and the LD_{50} for treatment times of 4 and 48-hr are about 2.0 and 0.10 μg/mL BaP.

This study also compared the frequency of spontaneous transformation and BaP-induced transformation responses in individual experiments. The data show that the two types of transformation responses are clearly dissociated from one another. The data presented in Table 2, as well as in Table A1, demonstrate that the average BaP-induced response was normally distributed for the 18 ampules, and responses ranged approximately 3-fold. For example, ampule 1L cells exhibited consistently low responses, and ampule 1H cells exhibited consistently high responses. A comparable effect of cell ampule on the spontaneous transformation frequency has been reported for the same experiments (*10*); however, high and low spontaneous frequencies occurred in different ampules of cells from those that exhibited high and low BaP-induced transformation responses. Therefore, BaP-induced transformation responses did not increase in proportion to changes in spontaneous transformation of WT cells. If the two frequencies had been proportionally linked in their expression, then BaP might have caused transformation of the BALB/c-3T3 cells by a nonmutational mechanism. For example, BaP might have enhanced the expression of spontaneous transformed cells that were suppressed by WT cells. In support of this possible mechanism of chemical action, it has been reported that phorbol esters blocked the suppression of SV40 virus-transformed BALB/c-3T3 cells by WT cells (*36*).

While the magnitude of the BaP-induced transformation response did not correlate with the frequency of spontaneous transformation in most experiments, there may have been one interesting exception to this observation. The frequency of spontaneous transformation is theoretically composed of both preexisting (*10*) variants and cells that spontaneously transformed during the experiment. In contrast to transformants that arise spontaneously during the course of the experiment, preexisting variants represent transformed cells that arise spontaneously in laboratory cultures that supply cells for transformation experiments. Although we found little evidence in this investigation for the presence of preexisting variants in most experiments, we noted that preexisting variants may have been present in an outlier experiment, no. 62 (*10*). If the frequency of preexisting variants is high relative to the background level of spontaneous transformation, they could make it difficult to detect the BaP-induced transformation response. This prediction was verified, and experiment 62 was the only experiment among 110 experiments in which BaP did not induce significant transformation.

The data from this study were also used to determine the appropriate units to use for the BaP-induced transformation response. In an earlier study we determined that the frequency of spontaneous transformation was directly related to the cumulative number of cell mitoses, and this frequency could be expressed at the number of foci/cell that survived and proliferated to confluence (*10*). The frequency of spontaneous transformation has been reported to be about 0.71 $\times$ 10^{-6} for type III foci and 1.55 $\times$ 10^{-6} for the combined total of type I–III foci (*10*). These frequencies were calculated based on the absolute number of foci detected in a contact-inhibited cell monolayer containing an estimated 8 $\times$ 10^5 cells/60-mm dish. This response corresponded to the absolute number of type III foci detected and median response of 0.57 foci/vessel (*10*). It was also demonstrated that the detection of spontaneous transformation was independent of the initial seeding density of WT cells over a range of 0.1–3.2 $\times$ 10^4 cells/vessel. Furthermore, the frequency of spontaneous transformation was not dependent on the cytotoxicity of any chemical treatment because all solvent vehicles were used at noncytotoxic concentrations (*17*).

Thus, the choice of the proper method for expressing the BaP-induced transformation response was a more complex decision than that for expressing the frequency of spontaneous transformation. In contrast to the frequency of spontaneous transformation, the BaP-induced transformation was dependent on the initial seeding density of the

cell cultures and the cytotoxicity of the treatment dose. In this respect, the activity of BaP was comparable to that of a chemical being tested for mutagenic activity. However, the expression of the transformation phenotype also depends on the cumulative number of mitoses (*10*). Thus, the BaP transformation response is directly influenced by three separate experimental parameters: cell seeding density, cytotoxicity of the treatment dose, and the cumulative number of cell mitoses. Taken together, the most appropriate method for expressing the BaP transformation response is in terms of foci/vessel.

Finally, the large shift in BaP-induced cytotoxic responses detected in the two types of clonal survival assays used in this investigation is similar to that reported for other test chemicals (*16,17*). The LD_{50} detected in the standard clonal survival assay using 200 cells was 10-fold lower than the LD_{50} detected in the modified clonal survival assay using 3.2×10^4 WT and 200 OUA^r cells. In other words, the BaP was much less cytotoxic to the relatively high cell densities used in transformation assays than to the low density cultures of 200 cells. The mechanism for this difference in cytotoxic activities is unknown, but it has been detected for several chemicals (*16,17*).

The opinions expressed in this paper are solely those of the author and do not necessarily reflect the positions of the U.S. Food and Drug Administration.

Experimental work was supported by funds from the National Institute of Environmental Health Science (NIEHS) on Contract No. NO1-ES-65150. The investigations were performed at Litton Bionetics, Inc. and Hazleton Laboratories America, Inc.

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Appendix A.

Table A1. Evaluation of BaP-induced cytotoxic and transformation responses in 110 experiments.

Exp. no.[a]	Cytotoxic Response[b] (% RCE) Stand. A Co-culture A BaP (μg/ml) .063	.20	.063	.20	Transforming activity[c] (focus type) BaP (μg/ml) Cont. III/I-III (n)	0.633 III/I-III (n)	.200 III/I-III (n)	Transformation response[d] (foci/vessel) BaP (μg/ml) Cont. III/I-III	0.633 III/I-III	.200 III/I-III	Significance[e] (t-Statistic) BaP (μg/ml) .0633 III/I-III	.200 III/I-III	AVG. III/I-III
							Serum Lot A						
Ampule 1B [16.5 ± 2.29]													
6A	14	2	86	31	18/ ND(36)	101/ ND(18)	175/ ND(18)	.35/ ND	5.42/ ND	9.16/ ND	19.8 / ND	18.5 / ND	19.2 / NA
7	3	2	57	48	7/ 13(36)	41/ 71(20)	212/337(19)	.14/ .22	1.81/3.07	10.0 /15.9	7.07/8.58	26.6 /24.7	16.8 /16.6
6B	8	3	86	31	24/ ND(38)	69/ ND(20)	141/ ND(20)	.51/ ND	3.14/ ND	6.80/ ND	10.1 / ND	21.9 / ND	16.0 / NA
5	13	8	13	9	2/ ND(40)	57/ ND(20)	83/ ND(19)	.04/ ND	2.58/ ND	3.97/ ND	12.5 / ND	15.5 / ND	14.0 / NA
AVG.	*10*	*4*	*61*	*30*				*.26/ ND*	*3.24/ ND*	*7.48/ ND*	*12.4 / ND*	*20.6 / ND*	*16.5/ NA*
Ampule 1H 15.5 ± 1.23]													
43	5	1	78	53	44/109(35)	270/600(20)	382/777(20)	1.05/1.82	13.0 /27.4	18.9 /38.6	19.0 /16.9	26.3 /23.3	22.7 /20.1
41	6	1	78	34	13/ 19(36)	123/235(18)	189/529(18)	.27/ .41	6.37/12.1	10.2 /28.1	17.1 /19.7	23.1 /28.4	20.1 /24.1
38	9	2	75	28	27/ 52(40)	162/342(20)	174/416(20)	.50/1.08	7.75/15.8	8.17/19.8	18.0 /17.5	15.6 /20.2	16.8 /18.9
39	4	1	65	24	27/ 70(40)	145/457(20)	172/519(20)	.43/ .96	6.84/21.0	8.04/22.3	15.8 /17.3	15.9 /14.3	15.9 /15.8
42	6	2	65	36	52/ 79(40)	161/393(19)	280/784(20)	.86/1.32	7.44/17.5	13.7 /37.8	9.55/11.9	20.2 /23.9	14.9 /17.9
45	28	9	86	42	7/ 25(59)	77/173(20)	186/589(20)	.72/1.57	3.42/7.94	8.98/28.1	7.04/8.61	19.5 /23.4	13.3 /16.0
40	9	1	79	40	28/ 59(40)	101/213(18)	182/470(19)	.53/ .75	4.86/10.2	8.71/23.5	9.93/11.7	14.9 /22.5	12.4 /17.1
44	14	5	67	26	77/197(40)	137/352(20)	335/885(20)	1.52/3.70	6.15/13.7	15.8 /42.2	7.33/6.61	16.3 /18.0	11.8 /12.3
37	7	2	77	46	32/ 75(39)	113/295(20)	101/300(20)	.63/1.50	5.38/13.3	4.59/12.6	13.5 /12.3	9.94/11.4	11.7 /11.9
AVG.	*10*	*3*	*74*	*37*				*.72/1.46*	*6.80/15.4*	*10.8 /28.1*	*13.0 /13.6*	*18.0 /20.6*	*15.5 /17.1*
Ampule 1D [13.1 ± 1.04]													
15	4	1	57	12	10/ 17(39)	72/147(20)	209/474(20)	.19/ .31	3.40/6.85	8.95/22,4	14.9 /16.7	15.8 /27.3	15.4 /22.0
13	2	1	75	31	11/ 18(40)	66/133(20)	95/248(20)	.20/ .34	3.14/6.42	4.38/11.8	15.7 /17.6	14.2 /22.1	15.0 /19.9
12	78*91*		99*114*		8/ 18(40)	81/183(17)	61/160(19)	.16/ .31	3.94/8.68	2.90/8.24	10.5 /16.8	13.1 /15.6	11.8 /16.2
14	5	2	75	36	12/ 20(40)	61/178(20)	177/402(20)	.21/ .46	2.45/6.55	6.73/17.1	7.12/10.3	13.3 /19.8	10.2 /15.1
*SA & CCA accidentally not dosed with BaP													
AVG.	*4*	*1*	*69*	*26*				*.19/ .36*	*3.23/7.13*	*5.74/14.9*	*12.1 /15.4*	*14.1 /21.2*	*13.1 /18.3*
Ampule 1I [12.8 ± 1.66]													
50	8	4	97	51	20/ 38(39)	108/269(20)	172/554(20)	.38/ .76	4.01/8.65	8.37/25.6	8.01/8.45	22.9 /21.4	15.5 /14.9
49	3	0	88	58	22/ 40(40)	87/235(20)	188/565(19)	.43/ .81	3.94/9.68	9.26/28.0	11.3 /13.5	18.5 /23.5	14.9 /18.5
47	8	0	76	28	31/101(39)	88/195(20)	173/456(20)	.58/1.23	3.89/8.87	8.10/21.0	8.73/9.50	14.6 /14.8	11.7 /12.2
48	10	2	91	48	29/ 63(40)	63/163(20)	148/442(20)	.54/1.30	2.68/6.32	7.06/20.0	6.53/7.60	16.5 /17.0	11.5 /12.3
46	22	6	81	36	24/ 83(40)	75/166(20)	127/423(20)	.38/1.09	3.32/7.07	6.14/20.8	8.90/7.23	12.5 /17.5	10.7 /12.4
AVG.	*10*	*3*	*87*	*44*				*.46/1.04*	*3.57/8.12*	*7.79/23.1*	*8.69/9.26*	*17.0 /18.8*	*12.8 /14.0*
Ampule 1E [12.4 ± 1.21]													
20	2	0	77	39	21/ 35(40)	99/215(20)	268/711(20)	.37/ .66	4.00/8.95	13.0 /34.2	9.22/12.5	26.3 /30.7	17.8 /21.6
21	1	0	76	46	19/ 47(40)	63/140(19)	133/323(18)	.35/ .79	3.11/6.72	6.92/17.0	10.5 /10.0	15.8 /19.3	13.2 /14.7
22	7	2	81	53	45/111(40)	116/229(20)	187/457(20)	.89/2.35	5.25/10.8	8.86/22.1	9.33/10.3	16.0 /20.4	12.7 /15.4
16	5	1	77	48	17/ 35(36)	58/126(17)	139/281(17)	.34/ .73	2.77/6.55	7.96/16.2	7.77/10.2	17.5 /23.5	12.6 /16.9
17	4	0	79	53	18/ 64(40)	86/195(20)	94/248(20)	.33/ .83	3.91/9.09	4.43/11.9	11.6 /12.3	13.5 /15.0	12.6 /13.7
19	2	0	63	89	18/ 65(38)	100/293(20)	99/336(20)	.36/ .70	4.02/11.7	4.61/16.5	10.6 /10.9	13.4 /18.6	12.0 /14.8
18	9	2	79	46	33/ 85(40)	86/195(20)	125/367(20)	.66/1.41	3.36/6.91	5.91/17.8	7.28/6.80	13.0 /17.4	10.1 /12.1
23	5	0	100	61	23/ 96(27)	57/115(18)	157/378(18)	.66/1.56	3.04/6.12	6.71/16.8	8.07/5.75	9.25/8.30	8.66/7.03
AVG.	*4*	*1*	*79*	*54*				*.50/1.13*	*3.68/8.36*	*7.30/19.1*	*9.30/9.84*	*15.6 /19.2*	*12.5 /14.5*
Ampule 1F [12.0 ± 1.61]													
26	1	0	56	14	46/378(40)	144/643(20)	204/722(20)	.91/8.90	6.58/31.3	9.88/35.7	10.5 /14.7	17.8 /21.8	14.2 /18.3
25	ND	ND	73	21	5/238(36)	59/212(18)	192/472(18)	.10/5.57	2.55/10.0	9.74/25.6	7.52/3.36	19.9/15.0	13.7 /9.18
28	7	3	68	29	41/124(40)	62/185(20)	189/552(20)	.82/2.86	2.78/ 8.34	9.02/26.2	5.73/8.51	16.9 /21.3	11.3 /14.9
24	6	0	70	19	18/119(40)	86/211(20)	83/341(20)	.31/2.63	3.73/15.9	3.65/9.81	10.7 /13.3	10.7 /9.47	10.7 /11.4
27	13	4	47	34	31/327(36)	73/285(18)	170/601(18)	.56/8.66	3.18/14.9	8.90/32.9	5.71/5.56	14.8 /19.9	10.3 /12.7
AVG.	*7*	*2*	*63*	*23*				*.54/5.72*	*3.76/16.1*	*8.24/26.0*	*8.03/9.09*	*16.0 /17.5*	*12.0 /13.3*
Ampule 1K [11.8 ± 1.58]													
54	0	1	61	18	15/ 53(40)	100/322(20)	105/350(20)	.27/ .46	4.52/12.4	4.75/16.3	13.5 /12.1	13.8 /18.4	13.7 /15.3
58	15	4	61	34	10/ 21(40)	71/132(20)	128/454(20)	.19/ .39	1.83/4.32	6.17/22.0	4.78/8.48	22.5 /32.4	13.6 /20.4
57	5	4	70	30	15/ 43(40)	37/100(20)	162/562(20)	.28/ .56	1.63/4.57	7.55/23.8	6.91/8.80	18.7 /17.5	12.8 /13.2
56	2	3	61	33	13/ 25(39)	33/105(20)	122/347(20)	.26/ .51	1.47/3.95	5.54/15.9	6.97/9.04	16.5 /21.8	11.7 /15.4
55	4	2	62	24	7/ 12(40)	48/104(20)	133/344(20)	.13/ .22	1.88/4.58	5.49/16.0	7.12/14.7	15.2 /28.5	11.2 /21.6
59	7	1	74	37	15/ 27(40)	33/ 72(19)	164/483(20)	.30/ .53	1.34/3.37	7.31/22.4	4.28/8.83	17.9 /24.8	11.1 /16.8
60	12	5	75	44	54/148(40)	77/175(20)	178/422(20)	.77/2.25	3.40/8.14	8.22/20.4	5.55/7.96	12.1 /16.1	8.83/12.0
AVG.	*6*	*3*	*66*	*31*				*.31/ .70*	*2.29/5.90*	*6.43/19.5*	*7.02/9.99*	*16.7 /22.8*	*11.9 /16.4*

(Continued on next page)

Table A1. *Continued.*

Exp. no.[a]	Cytotoxic Response[b] (% RCE) Stand. A BaP (μg/ml) .063	.20	Co-culture A (μg/ml) .063	.20	Transforming activity[c] (focus type) BaP (μg/ml) Cont. III/I-III (n)	0.633 III/I-III (n)	.200 III/I-III (n)	Transformation response[d] (foci/vessel) BaP (μg/ml) Cont. III/I-III	0.633 III/I-III	.200 III/I-III	Significance[e] (t-Statistic) BaP (μg/ml) .0633 III/I-III	.200 III/I-III	AVG. III/I-III
Ampule 1L [11.5 ± ND]													
61	10	0	70	33	12/ 20(40)	43/126(20)	95/313(20)	.22/ .38	1.92/4.43	4.28/13.8	8.76/7.77	14.3 /20.5	11.5 /14.1
AVG.	*10*	*0*	*70*	*33*				*.22/ .38*	*1.92/4.43*	*4.28/13.8*	*8.76/7.77*	*14.3 /20.5*	*11.5 /14.1*
Ampule 1C [10.4 ± 1.67]													
10	9	2	92	31	3/ 6(40)	34/ 62(20)	105/198(20)	.05/ .10	1.37/2.54	4.79/9.41	6.23/8.72	18.3 /29.4	12.3 /19.1
9	9	3	87	38	8/ 15(40)	47/ 68(20)	108/205(20)	.15/ .26	1.92/2.85	4.93/9.70	7.04/8.90	16.2 /22.2	11.6 /15.6
8	4	1	52	6	110/246(40)	254/517(20)	244/494(19)	2.19/4.94	11.8 /25.0	10.7 /23.8	11.2 /14.1	8.19/9.76	9.70/11.9
11	7	1	34	36	21/ 34(40)	37/118(20)	128/347(20)	.30/ .41	1.62/3.43	4.94/13.3	5.38/6.19	10.5 /13.6	7.94/9.90
AVG.	*7*	*2*	*66*	*28*				*.67/1.43*	*4.18/8.46*	*6.34/14.1*	*7.46/9.48*	*13.3 /18.7*	*10.4 /14.1*
Ampule 1G [9.98 ± .67]													
29	2	1	79	57	36/ 87(40)	142/306(20)	122/293(20)	.61/1.02	6.19/14.6	5.79/14.0	10.3 /13.2	13.1 /14.5	11.7 /13.9
34	19	7	85	59	108/256(40)	138/345(20)	167/523(20)	2.51/5.76	6.39/15.1	8.23/25.7	7.61/7.56	14.5 /18.1	11.1 /12.8
30	3	1	101	60	40/ 85(40)	98/220(19)	158/346(20)	.79/1.68	4.25/10.2	7.23/16.2	8.08/9.75	12.3 /15.3	10.2 /12.5
33	6	3	51	2	54/182(37)	130/443(20)	214/614(20)	1.04/3.31	5.86/18.1	10.0 /29.6	7.74/7.90	13.6 /15.2	10.7 /11.6
36	8	3	78	40	20/ 41(36)	88/236(18)	73/203(18)	.42/ .86	4.37/11.0	7.27/21.9	11.0 /12.4	8.91/25.2	9.96/18.8
31	5	2	100	69	43/ 75(36)	126/255(18)	136/301(15)	.93/1.70	6.06/12.4	8.63/19.1	8.51/10.1	11.1 /13.5	9.81/11.8
32	6	10	77	52	91/219(38)	115/297(20)	197/482(19)	1.99/4.54	5.46/13.5	10.1 /24.6	6.94/7.15	12.6 /13.2	9.77/10.2
35	12	5	88	66	94/232(40)	133/394(20)	103/405(20)	1.97/5.26	6.01/18.7	4.91/19.5	6.93/11.5	6.41/12.1	6.77/11.8
AVG.	*7*	*4*	*82*	*51*				*1.28/3.02*	*5.57/14.2*	*7.77/21.3*	*8.39/9.95*	*11.6 /15.9*	*10.0 /12.9*
Ampule 1A [9.52 ± 1.52]													
2	3	1	69	36	34/ ND(40)	110/ ND(20)	187/ ND (20)	.66/ ND	4.52/ ND	8.89/ ND	8.43/ ND	15.5 / ND	12.0 / ND
3	13	11	47	82	17/ ND(40)	39/ ND(20)	127/ ND (20)	.29/ ND	1.61/ ND	5.84/ ND	5.63/ ND	14.8 / ND	10.2 / ND
1	16	10	91	56	73/ ND(40)	114/ ND(20)	171/ ND (20)	1.44/ ND	5.18/ ND	8.16/ ND	6.68/ ND	12.1 / ND	9.39/ ND
4	24	16	75	83	118/ ND(40)	116/ ND(20)	184/ ND (20)	1.51/ ND	4.38/ ND	8.81/ ND	3.62/ ND	9.38/ ND	6.50/ ND
AVG.	*14*	*10*	*71*	*64*				*.98/ ND*	*3.92/ ND*	*7.93/ ND*	*6.09/ ND*	*12.9 / ND*	9.50/ *ND*
Ampule 1J [8.19 ± 2.48]													
52	11	4	75	40	55/191(38)	126/408(20)	150/532(20)	1.09/2.82	5.61/16.6	6.63/22.7	8.18/7.53	9.35/9.09	8.77/8.31
51	7	1	78	46	34/ 77(40)	77/212(20)	81/287(19)	.67/1.65	3.41/9.67	3.85/14.0	7.68/11.2	8.49/13.9	8.09/12.6
53	8	5	77	59	128/592(40)	195/637(20)	182/544(20)	2.78/9.73	8.74/28.0	8.30/25.2	7.74/5.78	7.68/5.79	7.71/5.79
AVG.	*9*	*3*	*77*	*48*				*1.51/4.73*	*5.92/18.1*	*6.26/20.6*	*7.87/8.17*	*8.51/9.59*	*8.19/8.88*
Serum Lot B													
Ampule 1N 10.3 ± .91]													
81	28	10	68	64	583/1287(72)	280/698(18)	378/969(18)	7.36/15.3	15.3 /38.1	20.8 /52.7	10.5 /11.6	15.5 /15.4	13.0 /13.5
77	17	6	79	70	94/246(78)	114/259(19)	179/366(20)	.97/2.32	5.53/12.3	8.33/16.4	10.0 /11.3	13.6 /10.3	11.8 /10.8
79	39	23	80	94	430/780(72)	241/436(18)	279/479(13)	5.12/9.40	12.9 /22.9	20.8 /36.1	9.34/8.55	13.9 /15.0	11.6 /11.8
80	36	17	87	65	317/572(80)	185/493(20)	261/680(20)	3.02/5.83	8.53/22.9	12.9 /33.0	7.65/9.50	13.0 /17.7	10.3 /13.6
82	47	56	47	52	649/1303(72)	288/684(18)	371/1046(18)	8.01/15.6	15.5 /36.9	19.4 /56.2	7.97/10.2	7.45/14.7	7.71/12.5
78	15	8	84	61	296/701(72)	184/353(18)	116/305(18)	3.28/6.49	9.84/19.0	6.11/16.0	9.74/8.98	4.93/6.70	7.34/7.84
AVG.	*30*	*20*	*74*	*68*				*4.63/9.16*	*11.3 /25.4*	*14.7 /35.1*	*9.20/10.0*	*11.4 /13.3*	*10.3 /11.7*
Ampule 1P [8.62 ± 1.53]													
93	8	3	80	47	43/ 70(79)	115/178(20)	138/244(20)	.42/ .66	4.80/7.07	6.48/11.2	12.0 /12.0	16.8 /16.6	14.4 /14.3
96	39	11	75	44	62/159(70)	62/171(20)	86/186(20)	.66/1.74	2.88/6.51	4.03/8.35	7.12/6.96	9.40/8.87	8.26/7.92
95	33	16	ND	ND	263/652(77)	115/317(20)	152/359(20)	2.84/6.83	5.27/15.0	7.35/17.2	3.82/7.35	9.48/9.28	6.65/8.32
94	17	0	114	76	150/314(71)	81/162(18)	122/311(18)	1.52/2.96	3.88/7.40	5.92/14.0	4.08/4.10	6.26/7.22	5.17/5.66
AVG.	*24*	*8*	*90*	*56*				*1.36/3.05*	*4.21/9.00*	*5.95/12.7*	*6.76/7.60*	*10.5 /10.5*	*8.63/9.05*
Ampule 1Q [8.49 ± .52]													
101	ND	ND	85	65	27/ 55(78)	48/ 92(20)	108/231(20)	.26/ .58	2.11/4.27	4.63/8.96	9.73/12.4	12.7 /13.9	11.2 /13.2
99	32	19	85	68	65/165(80)	94/191(20)	160/315(20)	.59/1.04	3.59/7.42	6.67/14.1	7.95/8.15	12.4 /16.0	10.2 /12.1
97	17	5	104	78	47/118(80)	52/126(20)	118/248(20)	.41/1.07	2.26/5.14	5.02/9.95	7.26/7.49	12.5 /11.3	9.88/9.60
103	24	9	92	77	89/164(79)	75/178(20)	95/179(20)	.87/1.67	2.86/5.80	4.59/8.36	5.37/4.94	13.7 /12.2	9.70/8.57
100	81	90	94	78	28/ 44(72)	62/ 92(18)	65/127(18)	.27/ .42	2.85/4.46	3.30/6.21	7.75/10.7	11.7 /13.1	9.60/11.9
98	29	8	91	80	39/ 76(45)	75/131(18)	132/228(18)	.62/1.07	3.38/5.41	6.82/11.4	6.81/6.56	11.8 /11.3	9.31/8.93
102	19	10	93	70	64/105(72)	45/ 93(18)	99/196(18)	.70/1.17	2.22/4.61	4.65/9.42	5.22/7.07	9.48/11.7	7.35/9.39
105	18	6	87	63	58/108(77)	40/ 79(19)	59/111(20)	.58/ .88	1.93/3.67	2.43/5.58	5.54/6.22	6.70/7.49	6.12/6.86
104	51	25	90	65	83/128(71)	63/ 93(18)	62/157(18)	.88/1.38	2.47/3.49	2.79/5.44	4.10/3.92	4.87/6.26	4.49/5.09
AVG.	*34*	*22*	*91*	*72*				*.58/1.03*	*2.63/4.92*	*4.54/8.82*	*6.64/7.49*	*10.7 /11.5*	*8.67/9.50*

(Continued on next page)

Table A1. *Continued.*

Exp. no.[a]	Cytotoxic Response[b] (% RCE) Stand. A BaP (μg/ml) .063	.20	Co-culture A (μg/ml) .063	.20	Transforming activity[c] (focus type) BaP (μg/ml) Cont. III/I-III (n)	0.633 III/I-III (n)	.200 III/I-III (n)	Transformation response[d] (foci/vessel) BaP (μg/ml) Cont. III/I-III	0.633 III/I-III	.200 III/I-III	Significance[e] (t-Statistic) BaP (μg/ml) .0633 III/I-III	.200 III/I-III	AVG. III/I-III
Ampule 1L [7.89 ± 1.20]													
65	19	7	90	61	14/ 28(40)	43/ 78(20)	85/253(18)	.24/ .51	1.80/3.54	4.38/11.7	6.94/9.08	13.9 /15.2	10.4 /12.1
68	5	4	77	52	11/ 16(36)	37/ 57(18)	91/131(18)	.23/ .34	1.73/2.65	4.63/6.72	5.18/5.82	15.0 /16.1	10.1 /11.0
64	17	6	69	27	17/ 27(40)	32/ 82(20)	98/284(20)	.29/ .48	1.37/3.01	4.47/12.7	5.13/6.70	12.7 /16.8	8.92/11.8
66	6	2	98	52	3/ 10(38)	24/ 57(20)	90/203(20)	.06/ .19	.80/1.63	3.92/8.24	3.69/4.10	14.1 /14.5	8.90/9.30
69	11	2	68	50	15/ 32(40)	33/ 75(20)	63/146(20)	.29/ .58	1.17/2.22	2.67/5.61	3.53/4.47	9.34/9.93	6.44/7.20
67	21	6	64	34	5/ 11(39)	39/111(20)	48/ 90(20)	.09/ .19	.97/1.95	2.07/3.80	3.32/3.53	8.71/9.64	6.02/6.59
63	25	4	93	81	84/175(39)	93/229(20)	141/329(20)	1.92/4.00	3.20/8.15	6.13/13.7	2.02/2.63	6.87/7.77	4.45/5.20
62*	19	5	85	73	261/689(40)	105/222(20)	188/527(20)	6.02/15.4	5.59/11.3	8.32/22.3	.00/ .00	2.35/2.62	1.18/1.31
AVG.	*15*	*5*	*81*	*54*				*.47/1.00*	*1.58/3.31*	*4.04/ 8.92*	*4.26/5.19*	*11.5 /12.8*	*7.88/9.00*
Ampule 1R [7.19 ± .99]													
108	30	16	66	31	108/242(70)	72/161(13)	139/307(18)	1.17/2.78	4.77/11.7	7.38/16.5	5.96/7.83	13.9 /11.6	9.93/9.72
110	12	27	61	88	65/211(75)	75/168(18)	116/328(18)	.61/2.29	3.89/8.98	5.78/17.5	8.52/12.3	10.7 /18.9	9.61/15.6
106	41	25	78	57	74/150(43)	91/162(18)	134/262(18)	1.30/2.73	4.53/8.06	6.88/13.7	5.56/5.26	8.00/10.9	6.78/8.08
107	21	6	76	47	274/929(80)	122/330(20)	131/402(20)	2.95/9.56	5.53/16.0	6.09/19.5	4.05/6.00	5.74/8.11	4.90/7.06
109	66	52	108	94	237/796(80)	81/190(20)	99/228(20)	2.55/8.96	3.91/9.22	4.69/11.2	4.16/ .34	5.31/3.02	4.74/1.68
AVG.	*34*	*25*	*78*	*63*				*1.72/5.26*	*4.53/10.8*	*6.16/15.7*	*5.65/6.35*	*8.73/10.5*	*7.19/8.43*
Ampule 1M [6.66 ± .87]													
71	18	4	69	51	110/212(75)	77/179(20)	251/553(20)	1.06/2.15	3.50/8.17	11.0 /23.6	7.12/8.95	12.1 /13.2	9.61/11.1
72	7	3	82	57	29/ 58(72)	84/123(18)	85/165(18)	.29/ .62	3.04/5.24	4.11/8.31	6.20/10.3	12.7 /14.7	9.45/12.5
75	28	7	85	67	89/225(78)	67/147(20)	149/344(20)	.88/1.98	3.10/6.30	6.35/13.5	6.56/6.00	10.9 /10.1	8.73/8.05
73	7	2	89	72	45/ 99(79)	48/ 90(20)	61/132(19)	.27/ .61	1.58/3.20	2.60/6.38	4.37/5.92	8.09/13.1	6.23/9.51
70	12	1	81	49	36/ 75(54)	11/ 35(5)	19/ 20(3)	.53/1.07	3.62/6.30	3.38/6.11	5.73/4.23	5.04/3.90	5.38/4.07
76	23	6	97	63	152/256(71)	54/ 91(18)	87/144(18)	1.79/3.03	2.72/4.65	4.41/7.28	2.26/2.54	5.23/5.39	3.75/3.97
74	10	3	78	69	65/130(71)	27/ 49(18)	43/ 74(18)	.66/1.17	1.29/2.47	2.02/3.86	2.49/3.95	4.51/7.47	3.50/5.71
AVG.	*15*	*4*	*83*	*61*				*.78/1.52*	*2.78/5.19*	*4.84/9.86*	*4.96/5.98*	*8.37/9.69*	*6.67/7.84*
Ampule 10 [6.31 ± .62]													
83	14	3	79	73	48/ 78(80)	64/121(20)	141/295(20)	.35/ .55	2.93/5.59	6.14/12.0	9.12/10.7	13.5 /14.8	11.3 /12.8
89	32	8	89	69	57/ 90(79)	63/134(20)	119/176(20)	.49/ .71	2.33/3.96	4.77/ 7.05	5.84/4.68	10.3 /10.6	8.07/7.64
86	48	19	88	63	47/ 79(72)	42/ 69(18)	64/ 99(18)	.46/ .76	2.02/3.35	3.32/ 5.23	5.74/6.09	9.07/9.04	7.41/7.57
88	36	16	83	74	37/ 52(67)	24/ 30(15)	54/ 79(15)	.41/ .58	1.50/1.90	3.36/ 4.58	4.90/6.64	9.58/9.50	7.24/8.07
84	NA	50	72	58	50/ 73(72)	57/ 99(18)	71/174(18)	.51/ .72	2.44/3.78	3.27/ 6.53	6.14/5.39	6.82/7.42	6.48/6.41
87	42	25	80	77	43/ 72(80)	34/ 91(20)	59/112(20)	.35/ .60	1.45/2.91	2.26/ 3.67	5.24/6.14	5.84/5.62	5.54/5.88
85	29	19	92	56	38/ 70(80)	66/110(19)	133/291(20)	.31/ .50	2.10/3.31	3.43/ 7.47	4.56/5.30	5.10/6.74	4.85/6.02
90	52	29	96	76	219/397(71)	111/241(18)	157/249(18)	1.95/2.93	4.91/8.97	7.60/11.3	3.71/4.13	5.89/5.32	4.80/4.81
92	19	18	78	72	62/100(71)	32/ 40(17)	44/ 60(18)	.60/ .91	1.52/2.04	2.14/ 3.15	3.32/2.99	5.20/7.57	4.26/5.28
**		0		45			69/101(18)			3.54/ 5.32		8.31/11.4	
91	58	29	90	74	31/ 46(75)	14/ 23(20)	60/128(20)	.32/ .46	.50/ .87	2.00/ 3.09	1.31/2.16	5.11/4.92	3.21/3.54
AVG.	*37*	*20*	*85*	*69*				*.58/ 87*	*2.17/3.67*	*3.83/ 5.17*	*4.99/ 5.42*	*7.64/8.15*	*6.32/6.79*

Abbreviations: BaP, benzo(*a*)pyrene; % RCE, percent relative cloning efficiency; CC.A., co-culture clonal survival assay; SA, standard clonal survival assay; ND, not determined.

[a]Exp. No.: The 110 experiments in this table were sequentially numbered as they were performed over a two year period. Experiments 6A and 6B were performed on the same day and used the same laboratory cultures of cells; however, experiment 6A was conducted in culture dishes and experiment 6B was conducted in culture flasks.

[b]Cytotoxic response: The cytotoxic responses of BaP in the 110 experiments was measured in either a standard or a co-culture clonal survival assay (refer to Materials and Methods). The cytotoxic response represents the %RCE of the BaP-treated cell cultures relative to the untreated cell cultures. The median cytotoxic responses were determined by rank-ordering the responses from experiments that used the two different FBS lots.

[c]Transforming activity: The number of type I, II, III foci/vessel were scored as described in the Materials and Methods. The transforming activity was arbitrarily listed in this table as either the type III foci/vessel, or as the type I–III foci/vessel. The type I–III activity included all type I, II, and III foci recorded per vessel; thus the number of type I + II foci can be calculated by subtracting the type III activity from the type I–III activity.

[d]Transformation response: The BaP-induced transformation responses were calculated using a three step procedure involving the $\log_{10}$ mathematical transformed data (refer to Materials and Methods). The arithmetic value of the transformation response, or foci/vessel, represents the anti-log of the $\log_{10}$ mean transformation response minus one.

[e]Significance: The significance of groups of BaP-induced transformation responses was calculated by computer using the SAS statistical software, and the method is described in detail in Materials and Methods. The correct *t*-statistic according to the F-test was used to calculate the *t*-statistics presented in this table. The *t*-statistics of each treatment dose of BaP were averaged to determine the average *t*-statistic for BaP.

Table A2. Rank-ordered comparison of the historical median spontaneous transformation frequency with individual experiment frequencies in terms of the statistical sensitivities and their detection sensitivities for benzo(a)pyrene.

Exp. No.[a]	Spontaneous Transformation Frequency[b] (Foci/Vessel) Mean (X) antilog	Frequency $\log_{10}X \pm SE$	(Statistical Sensitivity[c] $t_{exp.} = \frac{X_{exp.}}{SE_{exp.}}$ $t_{med.} = \frac{X_{med.}}{SE_{med.}}$	Benzo(a)pyrene Detection Sensitivity[d] High t-statistic B(a)P Conc. .200	.0633	Avg.	Low t-statistic B(a)P Conc. .200	.0633	Avg.
Group S1. 12 Experiments with Significantly High Statistical Sensitivity[e]									
1.	82	8.01	0.954 ± .022	+ 6.32***	+ 8.07	+10.7	+13.3 ***		
2.	81	7.36	0.922 ± .021	+ 6.11***	+11.0	+11.8	+16.1 ***		
3.[g]	*62*	*6.02*	*0.846 ± .027*	*+ 4.36****			NS		
4.	79	5.12	0.652 ± .027	+ 3.36**	+11.6	+10.8	+15.9 ***		
5.	107	2.95	0.596 ± .025	+ 3.33**			NS		
6.	109	2.55	0.550 ± .024	+ 3.19*				- 4.88	- 2.77 - 5.41***
7.	34	2.51	0.545 ± .024	+ 3.16*			NS		
8.	95	2.84	0.584 ± .026	+ 3.12*			NS		
9.	78	3.28	0.631 ± .030	+ 2.93*			NS		
10.	80	3.02	0.605 ± .031	+ 2.71*	+ 7.28	+ 7.35	+10.4 ***		
11.	53	2.78	0.577 ± .030	+ 2.68*			NS		
12.	76	1.79	0.445 ± .026	+ 2.38*				- 2.95	- 4.96 - 5.59***
Group S2 36 Experiments with above Normal Statistical Sensitivity									
13.	63	1.92	0.465 ± .030	+ 2.16			NS		
14.	35	1.97	0.473 ± .036	+ 1.83			NS		
15.	90	1.95	0.469 ± .037	+ 1.76	+ 2.17	+ 2.24	+ 3.12 **		
16.	8	2.19	0.504 ± .040	+ 1.75	+ 1.58	+ 7.66	+ 6.53 ***		
17.	77	.972	0.295 ± .024	+ 1.71	+ 4.69	+ 5.80	+ 7.42 ***		
18.	94	1.52	0.402 ± .033	+ 1.69			NS		
19.	32	1.99	0.475 ± .039	+ 1.69			NS		
20.	108	1.17	0.337 ± .028	+ 1.67	+ 2.75	+ 2.36	+ 3.61 **		
21.	75	.882	0.275 ± .025	+ 1.53	+ 2.13	+ 1.03	+ 2.23 *		
22.	103	.874	0.273 ± .025	+ 1.52			NS		
23.	44	1.52	0.402 ± .038	+ 1.47	+ 8.99	+ 2.05	+ 7.81 ***		
24.	71	1.06	0.314 ± .030	+ 1.46	+ 6.16	+ 1.66	+ 5.53 ***		
25.	1	1.44	0.387 ± .038	+ 1.42			NS		
26.	104	.877	0.274 ± .028	+ 1.36				- 4.24	- .99 - 3.70***
27.	102	.697	0.230 ± .024	+ 1.33			NS		
28.	106	1.30	0.362 ± .040	+ 1.26	+ 2.44	+ 3.30	+ 4.06 ***		
29.	105	.581	0.199 ± .022	+ 1.26				- 4.98	-2.57 - 5.34***
30.	43	1.05	0.311 ± .035	+ 1.24	+10.9	+10.2	+14.8 ***		
31.	96	.660	0.220 ± .026	+ 1.18			NS		
32.	74	.657	0.219 ± .026	+ 1.17				- 6.54	- 7.13 - 9.67***
33.	99	.586	0.200 ± .024	+ 1.16	+ 2.70	+ 2.39	+ 3.60 **		
34.	52	1.09	0.321 ± .039	+ 1.14			NS		
35.	45	.732	0.238 ± .029	+ 1.14			NS		
Group S3 53 Experiments with Below Normal Statistical Sensitivity									
36.	22	.893	0.277 ± .034	+ 1.13	+ 1.54	+ 1.65	+ 2.26 *		
37.	26	.907	0.280 ± .035	+ 1.11	+ 2.88	+ 3.61	+ 4.59 ***		
38.	110	.609	0.207 ± .026	+ 1.10	+ 1.80	+ 3.80	+ 3.96 ***		
39.	28	.818	0.260 ± .033	+ 1.10			NS		
40.	30	.787	0.252 ± .032	+ 1.10			NS		
41.	92	.597	0.203 ± .026	+ 1.09				- 2.13	- 1.41 - 2.50*
42.	31	.930	0.286 ± .037	+ 1.08	+ 1.13	+ 2.37	+ 2.48 *		
43.	93	.416	0.151 ± .020	+ 1.05	+ 3.30	+ 4.94	+ 5.83 ***		
44.	84	.511	0.179 ± .024	+ 1.04			NS		
45.	6	.431	0.156 ± .021	+ 1.03			NS		
46.	18	.663	0.221 ± .030	+ 1.02				- 2.27	- 1.51 - 2.67*
47.	70	.526	0.184 ± .025	+ 1.02			NS		
48.	51	.672	0.223 ± .031	+ 1.00				- 5.76	- 1.59 - 5.20***
49.	33	1.04	0.309 ± .044	+ .977	+ 2.61	+ 2.27	+ 3.45 **		
50.	4	1.51	0.399 ± .057	+ .974			NS		
51.	89	.492	0.174 ± .025	+ .968			NS		
52.	86	.464	0.166 ± .024	+ .962				- 2.42	- .64 - 2.16*
53.	2	.660	0.220 ± .032	+ .956			NS		
54.	97	.414	0.151 ± .022	+ .955			NS		
55.	42	.861	0.270 ± .040	+ .939	+ 6.86	+ 4.00	+ 7.68 +++		
56.	91	.322	0.121 ± .018	+ .935				- 4.59	-15.0 -13.8***

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Table A2. *Continued.*

Exp. No.[a]	Spontaneous Transformation Frequency[b] (Statistical Sensitivity[c]) (Foci/Vessel) Mean (X) antilog	Frequency $\log_{10}X \pm SE$	$t^{exp.} = \frac{X^{exp.}}{SE^{exp.}}$ / $t^{med.} = \frac{X^{med.}}{SE^{med.}}$	Benzo(a)pyrene Detection Sensitivity[d] High t-statistic B(a)P Conc. .200	.0633	Avg.	Low t-statistic B(a)P Conc. .200	.0633	Avg.
57.	88	.406	0.148 ± .023 + .895				- 2.14	- 4.05	- 4.38***
58.	37	.631	0.212 ± .033 + .894			NS			
59.	98	.618	0.209 ± .033 + .881	+ 3.30	+ 2.10	+ 3.82 ***			
60.	40	.533	0.186 ± .031 + .835	+ 1.51	+ 1.52	+ 2.14 *			
61.	47	.579	0.198 ± .033 + .835			NS			
62.	101	.260	0.100 ± .017 + .818			NS			
63.	87	.346	0.129 ± .022 + .816				- 4.09	- 3.71	- 5.52***
64.	48	.537	0.187 ± .032 + .813				- .28	- 3.08	- 2.38*
65.	49	.433	0.156 ± .027 + .804			NS			
66.	23	.661	0.220 ± .039 + .785				- .60	- 3.06	- 2.59*
67.	85	.313	0.118 ± .021 + .782			NS			
68.	29	.606	0.206 ± .037 + .774			NS			
69.	38	.496	0.175 ± .032 + .761	+ .73	+ 6.22	+ 4.92 ***			
70.	72	.29_	0.110 ± .020 + .765			NS			
71.	83	.35_	0.130 ± .024 + .753	+ 2.45	+ 2.05	+ 3.18 **			
72.	60	.766	0.247 ± .046 + .747			NS			
73.	100	.268	0.103 ± .020 + .716			NS			
74.	36	.424	0.153 ± .030 + .709			NS			
75.	59	.297	0.113 ± .023 + .683			NS			
76.	50	.381	0.140 ± .029 + .672			NS			
77.	19	.357	0.133 ± .028 + .661			NS			
78.	27	.555	0.192 ± .041 + .651			NS			
79.	21	.347	0.129 ± .028 + .641			NS			
80.	73	.274	0.105 ± .023 + .635				- 3.08	- 2.06	- 3.64**
81.	20	.368	0.136 ± .030 + .631	+ 6.27	+ .27	+ 4.63 ***			
82.	39	.427	0.154 ± .035 + .612	+ 1.07	+ 4.82	+ 4.17 ***			
83.	17	.327	0.123 ± .028 + .611			NS			
84.	56	.260	0.100 ± .023 + .605				- 1.89	- 7.69	- 6.78***
85.	69	.29_	0.110 ± .024 + .638				- 3.28	- 4.38	- 5.42***
86.	57	.278	0.107 ± .025 + .595			NS			
87.	16	.344	0.128 ± .030 + .593			NS			
88.	46	.384	0.141 ± .034 + .577			NS			
89.	24	.308	0.117 ± .029 + .561				- 5.31	- 1.59	- 4.88***
90.	41	.274	0.105 ± .026 + .562	+ 3.74	+ 4.20	+ 5.62 ***			
91.	54	.265	0.102 ± .026 + .546			NS			
92.	64	.291	0.111 ± .029 + .532			NS			
93.	61	.222	0.087 ± .023 + .526				- 3.88	- 5.10	- 6.35***
94.	3	.285	0.109 ± .029 + .523				- 1.54	- 4.56	- 4.31***
95.	65	.244	0.095 ± .026 + .508			NS			
96.	13	.201	0.080 ± .022 + .506				- 3.96	- 1.31	- 3.73***
97.	58	.189	0.075 ± .021 + .497				- 1.12	- 3.66	- 3.38**
98.	68	.226	0.089 ± .025 + .495			NS			
99.	14	.213	0.084 ± .024 + .487			NS			
100.	15	.186	0.074 ± .022 + .468			NS			
101.	11	.301	0.114 ± .034 + .466				- 2.35	- 6.69	- 6.39***
Group S4	*9 Experiments with Significantly Low Statistical Sensitivity* [f]								
102.	9	.149	0.060 ± .019 + .439*				-2.72	- 4.12	- 4.84 ***
103.	12	.160	0.065 ± .021 + .431*			NS			
104.	55	.129	0.053 ± .018 + .410*				- 1.65	- 4.19	- 4.13 ***
105.	7	.135	0.055 ± .021 + .364*			NS			
106.	25	.101	0.042 ± .018 + .325*			NS			
107.	67	.085	0.035 ± .017 + .286**				- 4.46	- 3.75	- 5.81***
108.	66	.056	0.024 ± .013 + .257**				- .16	- 5.32	- 3.88***
109.	10	.053	0.023 ± .013 + .246**				- 2.87	- 5.87	- 6.18***
110.	5	.035	0.015 ± .011 + .190***				- 4.11	- 2.15	- 4.43***

Abbreviations: AVG., average; B(a)P conc., benzo(a)pyrene concentration; Exp. No., experiment number; NS, not significant; $SE^{exp.}$ and $SE^{med.}$ are the standard errors of the mean experimental and median spontaneous frequencies; $t^{exp.}$, *t*-statistic of the experimental; $t^{med.}$, *t*-statistic of the median experiment; $X^{exp.}$ and $X^{med.}$, the mean experimental and median spontaneous frequencies; X, mean

(Continued on next page)

Table A2. *Continued.*

[a]Exp. No.: The table summarizes the "Statistical Sensitivities" and the "Benzo(a)pyrene Detection Sensitivities" of 110 experiments. The experiments with different experiment numbers are rank-ordered in terms of their statistical sensitivities from the 12 experiments with significantly high statistical sensitivity to the 9 experiments with significantly low statistical sensitivity. Explanation for the calculation of statistical sensitivity and B(a)P detection sensitivity are provided below.

[b]Frequency of spontaneous transformation: The frequency of spontaneous transformation is presented in terms of the antilog$_{10}$ of the mean number of foci/vessel for each experiment. The experimental design of the assay and the method of calculating the antilog$_{10}$ frequency are described in Materials and Methods.

[c]Statistical sensitivity: The method for calculation of statistical sensitivity is provided in the Materials and Methods. The median spontaneous transformation frequency for 110 experiments (*i.e.*, $X_{med.}$) was 0.190 log$_{10}$ foci/vessel [arithmetic equivalent to 0.549 foci/vessel]. The median standard deviation (SD) of the mean (X) spontaneous frequency was 0.196 log$_{10}$ foci/vessel [arithmetic equivalent of 0.570 foci/vessel]. The median standard error of mean (*i.e.*, $SE_{med.}$) was 0.026429 foci/vessel. Using the ratio of the experimental and median *t*-statistics, the 110 independent experiments were rank ordered from the highest to the lowest in terms of statistical sensitivity. An example calculation is provided below.

$$\text{Statistical Sensitivity of Experiment \#81} = \frac{.922/.021}{.190/.026429} = \frac{43.905}{7.1891} = +6.106$$

[d]Benzo(a)pyrene Detection Sensitivity: The method for calculating the detection sensitivity of B(a)P was described in the Materials and Methods. The experiments which had detection sensitivity for B(a)P that was indistinguishable from the median experiment responses were evaluated as not significant.

[e]High statistical sensitivity: A total of 12/110 (10.9%) of the experiments had a "significantly high statistical sensitivity". A significantly high statistical sensitivity was calculated by determining the variance the statistical sensitivities of the 110 experiments. This calculation is shown below and it revealed that a 2.208-, 3.220-, and 4.232-fold increase in the statistical sensitivity compared to the median statistical sensitivity resulted in significant high statistical sensitivities at the $p<.0.5$, $p<.01$ and $p<.001$ confidence levels. Note, before this calculation could be made the experiments with below average statistical sensitivity had their rank *t*-statistic converted from a positive fraction less than one to a whole negative rank *t*-statistic by dividing it into one.

Mean Rank, *t*-statistic for Statistical Sensitivity = 1.196 ± 1.012 (*110*); therefore, at

.05 CONFIDENCE LEVEL = mean + 1SD = 1.196 + 1.012 = 2.208
.01 CONFIDENCE LEVEL = mean + 2SD = 1.196 + 2.024 = 3.220
.001 CONFIDENCE LEVEL = mean + 3SD = 1.196 + 3.036 = 4.232

[f]Low statistical sensitivity: The historical range of experiments revealed that 8.2% (9/110) of the experiments had a "significantly low statistical sensitivity". The method for determining the experiments with a significantly low statistical sensitivity is identical to that used to determine experiments with a significantly high statistical sensitivity (refer to footnote e above).

[g]Experiment #62 was the only experiment among the 110 experiments in which the positive control B(a)P did not induce a highly significant transformation response, and this experiment was evaluated as being unacceptable.

*Significantly high or low spontaneous or B(a)P transformation response, $0.01 < p \leq 0.05$.

**Significantly high or low spontaneous or B(a)P transformation response, $0.001 < p \leq 0.01$.

***Significantly high or low spontaneous or B(a)P transformation response, $p < 0.001$.