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¹ Spotts, C. R., and R. Y. Stanier, *Nature*, **192**, 633 (1961).

² Flaks, J. G., E. C. Cox, and J. R. White, *Biochem. Biophys. Res. Commun.*, **7**, 385 (1962).

³ Speyer, J. F., P. Lengyel, and C. Basilio, these PROCEEDINGS, **48**, 684 (1962).

⁴ Cox, E. C., J. R. White, and J. G. Flaks, these PROCEEDINGS, **51**, 703 (1964).

⁵ Davies, J., these PROCEEDINGS, **51**, 659 (1964).

⁶ Davies, J., W. Gilbert, and L. Gorini, these PROCEEDINGS, **51**, 883 (1964).

⁷ Stern, J. L., and S. S. Cohen, these PROCEEDINGS, **51**, 859 (1964).

⁸ Paranchych, W., and A. G. Graham, *J. Cellular Comp. Physiol.*, **60**, 199 (1962).

⁹ Paranchych, W., *Biochem. Biophys. Res. Commun.*, **11**, 28 (1963).

¹⁰ Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).

¹¹ Stent, G. S., and S. Brenner, these PROCEEDINGS, **47**, 2005 (1961).

¹² Brock, T. D., *Biochem. Biophys. Res. Commun.*, **9**, 184 (1962).

¹³ Loeb, T., and N. D. Zinder, these PROCEEDINGS, **47**, 282 (1961).

BIOCHEMICAL STUDIES ON ADENOVIRUS MULTIPLICATION, VII. HOMOLOGY BETWEEN DNA'S OF TUMORIGENIC AND NONTUMORIGENIC HUMAN ADENOVIRUSES*

By SISTER S. LACY† AND MAURICE GREEN‡

INSTITUTE FOR MOLECULAR VIROLOGY AND DEPARTMENT OF MICROBIOLOGY,
SAINT LOUIS UNIVERSITY SCHOOL OF MEDICINE

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The finding that adenovirus types 12 and 18 induce tumors in newborn hamsters¹⁻⁴ has generated a great deal of interest in these viruses. Since this is the first case of a "human" virus known to possess carcinogenic properties, it was of obvious importance to determine the molecular basis of this potentiality. Green and Piña^{3, 4} have chemically analyzed and characterized the DNA's of purified tumorigenic adenoviruses by means of sedimentation velocity, thermal denaturation, and buoyant density measurements. These studies have revealed striking similarities between the DNA's of tumorigenic adenovirus types 12 and 18, while similar studies on the DNA's of nontumorigenic adenovirus types 2 and 4 have revealed remarkable differences between these DNA's and those of the tumorigenic adenoviruses (e.g., the DNA's of the latter contain 48-49% guanine-cytosine, while those of the nontumorigenic adenoviruses contain 56-57% guanine-cytosine).

The similar base composition and the carcinogenic activity of adenovirus types 12 and 18 suggest that they may be closely related genetically. The DNA-agar technique of Bolton and McCarthy⁵⁻¹¹ has been employed successfully to measure the degree of genetic relatedness among the enterobacteria,⁶ among several mammalian and other vertebrate types,⁷ and to demonstrate homology between bacteriophage lambda and the *E. coli* chromosome.⁸ Using this technique in the studies reported here, the ability of the DNA's of adenovirus types 12 and 18 to hybridize with each other *in vitro* was measured. The degree of genetic relatedness as reflected by nucleotide sequence homology was estimated by quantitative comparison of the heterologous reaction (e.g., type 12 DNA × type 18 DNA) with the homol-

ogous reaction (e.g., type 12 DNA $\times$ type 12 DNA). Nucleotide sequences held in common are not only indicative of common genes, but may provide evidence that the carcinogenic potential of these viruses lies in the possession of a specific polynucleotide sequence or sequences. This technique was used also to study the extent of genetic homology among nontumorigenic adenovirus types 2 and 4, and to measure the degree to which these tumorigenic and nontumorigenic adenoviruses are related.

Materials and Methods.—Preparation of viral DNA: Radioactive virus was prepared by addition of H^3 -labeled thymidine to adenovirus-infected KB cells at appropriate times after infection. Subsequent to harvesting and purification,¹² the DNA was extracted by the method described by Green and Piña⁴ with the following modifications: after dialysis against 0.01 *M* Tris, pH 8.1, the virus was treated with papain in 0.1 *M* phosphate buffer, pH 6.0 containing 0.1 *M* cysteine and 0.005 *M* EDTA. Following centrifugation of the phenol-treated preparation, the phenol layer was removed, and the remaining interface and aqueous layers were treated twice more with phenol. The final aqueous layer was then dialyzed against 0.1 $\times$ SSC (SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate) as described. Final DNA yields here were 80–95% as determined by comparative diphenylamine reactions on DNA prepared from radioactive or unlabeled virus and on the original virus preparations.

DNA-agar preparations were made by the procedure of Bolton and McCarthy.⁶ The amount of denatured viral DNA trapped in the agar was determined from its ultraviolet absorption after dissolving a portion in boiling 5 *M* NaClO₄.⁶ Generally, 60–85 % of the DNA was trapped in the agar using this procedure. Where the optical density values were too low to be reliable (less than 28 μ g of viral DNA trapped per gram of agar), 60 % trapping was assumed. A knowledge of the absolute amount of DNA trapped is not critical here, since in nearly every case the ratios of embedded to added DNA were much in excess of those required for optimal hybridization and since homologous reactions were run with each experiment.

Preparation of radioactive DNA fragments: Labeled DNA in 0.1 $\times$ SSC was fragmented in a Raytheon 10-kc sonic oscillator for 10 min at a setting of 1.25 amps. Fragments prepared in this manner have a molecular weight of 6.9×10^5 as calculated from their sedimentation coefficients utilizing the relationship described by Burgi and Hershey.¹³ Sedimentation coefficients were determined by centrifugation in density gradients of sucrose.¹³

Hybrid formation: The procedure employed was a modification of that first described by Bolton and McCarthy⁶ and later used by these and other workers.^{6–11} This technique as utilized in our studies consisted of immobilizing, in agar, strands of denatured DNA of a given adenovirus type and incubating this DNA-agar at 60° with an equal volume of 2 $\times$ SSC containing radioactive, denatured fragments of DNA of the same adenovirus type (homologous reaction) or of another adenovirus type (heterologous reaction). After 16 hr of incubation, the DNA-agar preparations were washed in 15-ml centrifuge tubes 5–10 times (depending on the amount of radioactivity employed) with 10-ml amounts of 2 $\times$ SSC at 60° for 5 min; during this time, the contents of each tube (DNA-agar and its “wash”) were mixed three times by means of a Vortex Jr. Mixer. The eluates were collected after centrifugation at 2000 rpm for 5 min in an International Centrifuge, model PR-2; this procedure removes unbound radioactive DNA. The DNA-agar preparations were then washed 5–10 times with 10-ml amounts of 0.01 $\times$ SSC at 75° for 5 min, mixed, and centrifuged each time as described above; this procedure elutes the hybridized radioactive DNA.

Determination of radioactivity and amount of hybrid formed: Herring sperm DNA (100 μ g) was added as carrier to each of the 10-ml fractions collected after centrifugation. The DNA content of each tube was then precipitated in the cold with 2 ml of cold 50% trichloroacetic acid, and dried on Schleicher and Schuell membrane filters (27-mm diameter, coarse) and counted in 10 ml of scintillation fluid (4 gm PPO and 100 mg POPOP per liter of toluene) in a Packard TriCarb liquid scintillation counter.

Results and Discussion.—The results of a typical experiment are shown in Figure 1. Here, all unbound material was eluted prior to fraction 8. Essentially, all hybridized material was removed subsequently with the first two 10-ml washes of

FIG. 1.—Extent of homology between tumorigenic adenovirus type 12 and nontumorigenic adenovirus type 2. Here, two 0.4-ml portions of $2 \times \text{SSC}$, one containing $1.9 \mu\text{g}$ of H^3 -labeled type 12 DNA (specific activity = $11,800 \text{ cpm}/\mu\text{g}$) (a), the other containing $2.0 \mu\text{g}$ of H^3 -labeled type 2 DNA (specific activity = $6300 \text{ cpm}/\mu\text{g}$) (b), were added to two 0.4-gm portions of DNA-agar, each containing $10.6 \mu\text{g}$ adenovirus type 12 DNA. After incubation the preparations were eluted (8 washes with $2 \times \text{SSC}$ and 7 with $0.01 \times \text{SSC}$), and radioactivity determined as described in *Materials and Methods*. Here, the per cent of the total radioactivity of labeled DNA eluted with each wash is plotted against the fraction number of the eluate. Total counts per minute of unbound and of hybridized material are indicated.

$0.01 \times \text{SSC}$ in the homologous reaction (Fig. 1a) and with the first 10-ml wash in the heterologous reaction (Fig. 1b).

Control experiments were performed to determine the extent of nonspecific binding of viral DNA fragments. When DNA-agar preparations containing $220 \mu\text{g}$ of calf thymus DNA were incubated with $4.3 \mu\text{g}$ of adenovirus type 2 DNA ($27,000 \text{ cpm}$) or with $1.1 \mu\text{g}$ of adenovirus type 12 DNA ($50,000 \text{ cpm}$), only 0.3 per cent of the viral DNA was bound. Likewise, when DNA-agar preparations containing $450 \mu\text{g}$ of hamster tumor DNA were incubated with $25 \mu\text{g}$ of adenovirus type 2 DNA ($74,000 \text{ cpm}$) or with $4.9 \mu\text{g}$ of adenovirus type 12 DNA ($68,000 \text{ cpm}$), again only 0.3–0.4 per cent of the viral DNA was bound. Therefore, the contribution of nonspecific binding to the hybridization values reported here is negligible.

The time course of hybrid formation was studied to determine the optimum experimental conditions. The results seen in Figure 2 indicate that the reaction is essentially complete at eight hr. However, a 16-hr incubation period was chosen for convenience.

Because of the dependence of the degree of hybrid formation upon the relative concentrations of embedded DNA and free DNA (labeled),¹⁰ a study was made to determine that embedded:free DNA ratio which would facilitate maximum hybridization. The results of this study are shown in Figure 3. It may be noted that values as high as 80 per cent hybridization are achieved here. While extensive hybrid formation is achieved at ratios as low as 2:1, maximum proportions of added radioactive DNA are bound when ratios of 20:1 to 100:1 are employed. With few exceptions, and these can be noted from the appropriate tables, ratios in this range were employed in our experiments.

In all of the experiments described here, homologous, heterologous, and reciprocal (e.g., type 2 DNA in agar $\times$ added type 12 DNA, and vice versa) reactions were set up so that all of the 16 possible combinations of the DNA's of the tumorigenic

FIG. 2.—Time course of hybrid formation. The per cent of the added radioactive DNA bound is plotted as a function of the number of hr the reaction mixture was incubated at 60°. Here, each of eight 0.5-gm portions of DNA-agar containing 15 μ g of adenovirus type 2 DNA was incubated with 0.5 ml of 2 $\times$ SSC containing 1.2 μ g of H³-labeled adenovirus type 2 DNA (3000 cpm/ μ g). Following incubation, each tube was washed and radioactivity of the washes determined as described in *Materials and Methods*.

FIG. 3.—Relationship between the ratio of DNA in agar (type 2) to added H³-labeled type 2 DNA and the extent of hybridization. The per cent of added radioactive DNA fragments bound is plotted as a function of the ratio (plotted on logarithmic axis) of the concentration of embedded adenovirus type 2 DNA to added H³-labeled adenovirus type 2 DNA fragments. The data plotted here are taken from two experiments. The values from the first are represented by open circles. Here, four 0.6-gm portions of DNA-agar, each containing 58 μ g, were incubated with 30.6, 17.4, 8.2, and 4.2 μ g of labeled DNA (1900 cpm/ μ g), respectively. In the second experiment, nine 0.2-gm aliquots of DNA-agar, each containing 4.8 μ g DNA, were incubated with 0.5, 0.2, 0.08, 0.048, and 0.01 μ g of DNA (6300 cpm/ μ g), respectively, the first four being done in duplicate. Following incubation, the tubes were washed, and radioactivity was determined as indicated in *Materials and Methods*.

adenovirus types 12 and 18 and the nontumorigenic adenovirus types 2 and 4, taken two at a time, were tested for ability to hybridize with each other. The results of the hybridization experiments carried out with the tumorigenic adenoviruses can be seen in Table 1. The high degree of homology (79%) observed between these viruses provides strong evidence for their close genetic relatedness. Furthermore, it is highly suggestive that the potential for tumorigenicity resides in nucleotide sequences which these viruses have in common. Adenovirus types 12 and 18 share several other features in addition to oncogenicity, including the inability to agglutinate certain erythrocytes and the possession of a common group specific antigen and other cross-reacting components.¹⁴ Therefore, it seems likely that the high

TABLE 1

EXTENT OF HYBRIDIZATION BETWEEN THE DNA'S OF TUMORIGENIC ADENOVIRUS TYPES 12 AND 18*

Expt. no.	Source of DNA in agar (type no.)	Concentration of DNA ($\mu\text{g}/\text{gm}$ of agar)†	Source of labeled DNA (type no.)	Amount of labeled DNA added (μg)	% Labeled DNA bound	% Labeled DNA bound relative to homologous reaction‡
1	12	12	18	0.24	58.8	75.4
2	12	18	18	0.15	57.1	80.9
3	12	18	18	0.15	56.2	79.6
4	12	17	18	0.15	56.7	81.0
5	12	17	18	0.15	55.9	79.9
6	18	27	12	0.48	72.1	76.9
7	18	28	12	0.29	57.9	78.5
Avg.					78.9 (± 2.1)§	

* Seed cultures of adenovirus types 12 (prototype strain) and 18 were obtained from Dr. Robert J. Huebner. These viruses were then cultivated, isolated, purified, and their DNA's extracted as noted in *Materials and Methods*.

† Here, 1.0 gm DNA-agar was employed in expts. 1 and 6, and 0.2 gm in expts. 2-5, and 7. These were incubated with equal volumes of $2 \times \text{SSC}$ containing indicated amounts of H^3 -labeled adenovirus DNA. Specific activities of 3300 cpm/ μg were used in expts. 1-5, and 11,800 cpm/ μg were used in expts. 6 and 7.

‡ In each experiment, homologous reactions were run in parallel. Values for homologous hybridization reactions ranged from 70 to 90%.

§ Standard deviation.

TABLE 2

EXTENT OF HYBRIDIZATION BETWEEN THE DNA'S OF NONTUMORIGENIC ADENOVIRUS TYPES 2 AND 4*

Expt. no.	Source of DNA in agar (type no.)	Concentration of DNA ($\mu\text{g}/\text{gm}$ of agar)†	Source of labeled DNA (type no.)	Amount of labeled DNA added (μg)	% Labeled DNA bound	% Labeled DNA bound relative to homologous reaction‡
1	2	67	4	5.0	20.7	38.7
2	2	67	4	3.5	27.8	32.8
3	4	109	2	3.8	19.0	32.0
4	4	109	2	1.6	22.9	31.4
5	4	28	2	0.32	29.8	39.6
Avg.					34.9 (± 3.9)§	

* Seed cultures of adenovirus type 2 and type 4 (#68578) were obtained from Drs. Robert J. Huebner and Robert Chanock, respectively. These viruses were then cultivated, isolated, purified, and their DNA's extracted as noted in *Materials and Methods*.

† The following portions of DNA-agar were employed here: 0.5 gm in expts. 1, 3, and 4; 0.7 gm in expt. 2; and 0.2 gm in expt. 5. These were incubated with equal volumes of $2 \times \text{SSC}$ containing the indicated amounts of H^3 -labeled adenovirus DNA. The specific activity (cpm/ μg DNA) was 1100 in expts. 1 and 2, and 6300 cpm in expts. 3-5.

‡ In each experiment, homologous reactions were run in parallel. Values for homologous hybridization reactions ranged from 50 to 85%.

§ Standard deviation.

degree of genetic relatedness observed here is due to the possession of similar nucleotide sequences associated not only with tumor production but also with other common characteristics of these two viruses.

It is evident from the data presented in Table 2 that the nontumorigenic adenovirus types 2 and 4 are not as closely related to each other as are the tumorigenic adenoviruses. The degree of genetic homology observed here (35%) is a little less than half that obtained with the tumorigenic viruses.

The results of hybridization experiments between the DNA's of tumorigenic and nontumorigenic adenoviruses (type 2 $\times$ type 12, type 4 $\times$ type 12, type 2 $\times$ type 18, type 4 $\times$ type 18) indicate an average value of only 22 per cent (18-26%) homology (Table 3, Fig. 1). Thus, there is less homology observed between the DNA's of tumorigenic and nontumorigenic adenoviruses than is seen between the nontumorigenic adenovirus types 2 and 4.

The results of the experiments described in this paper suggest that the extensive homology (79%) observed between tumorigenic adenovirus types 12 and 18 may reflect complementarity of nucleotide sequences concerned with tumor production.

TABLE 3
EXTENT OF HYBRIDIZATION AMONG THE DNA'S OF TUMORIGENIC AND NONTUMORIGENIC
ADENOVIRUSES

Expt. no.	Source of DNA in agar (type no.)	Concentration of DNA ($\mu\text{g}/\text{gm}$ of agar)*	Source of labeled DNA (type no.)	Amount of labeled DNA added (μg)	% Labeled DNA bound	% Labeled DNA bound relative to homologous reaction†
1	2	24	12	1.5	10.7	16.4
2	2	67	12	2.2	11.4	13.4
3	2	23	12	0.45	15.6	18.6
4	2	54	12	0.26	8.9	12.1
5	2	54	12	0.26	11.2	15.3
6	2	54	12	0.30	12.9	17.5
7	12	20	2	1.5	21.7	31.0
8	12	27	2	1.7	10.3	21.1
9	12	12	2	0.41	19.5	25.0
10	12	18	2	0.21	12.8	21.8
11	12	18	2	0.21	12.8	21.8
12	12	17	2	0.21	11.9	22.8
Avg.						19.7 (± 5.3)‡
13	2	23	18	0.27	15.3	18.3
14	18	27	2	0.41	20.9	22.3
15	18	28	2	0.32	21.1	28.6
Avg.						23.1 (± 5.2)
16	12	16	4	1.2	13.7	24.4
17	12	27	4	2.6	9.3	19.0
18	4	28	12	0.29	10.9	14.5
19	4	28	12	0.29	12.0	15.9
Avg.						18.4 (± 4.4)
20	4	28	18	0.14	20.0	26.6
21	4	28	18	0.14	18.1	24.1
22	4	54	18	0.21	21.2	26.6
23	4	54	18	0.21	23.7	29.7
24	4	54	18	0.23	17.5	21.9
25	4	54	18	0.23	16.7	21.0
26	18	28	4	0.34	21.5	29.1
27	18	28	4	0.34	20.9	28.3
Avg.						25.9 (± 3.3)

* Here, 1.0 gm DNA-agar was used in expts. 1, 3, 7, 9, and 13; 0.7 gm in expt. 2; 0.2 gm in expts. 4-6, 10-12, 14, 15, 18-27; 0.4 gm. in expts. 8 and 17; and 1.2 gm in expt. 16. These portions were incubated with equal volumes of $2 \times \text{SSC}$ containing the indicated amounts of H^3 -labeled adenovirus DNA. The range of specific activity in these experiments was 1,100-31,300 cpm/ μg DNA.

† In each experiment, homologous reactions were run in parallel. Values for homologous hybridization reactions in these experiments ranged from 49 to 94%.

‡ Standard deviation.

These results give impetus to further studies to isolate and characterize those DNA regions shared exclusively by tumorigenic adenoviruses. The degree of relatedness indicated by hybrid formation between nontumorigenic adenovirus types 2 and 4 (35%) suggests that within the adenovirus group one may see genetic diversity as extensive as that encountered within larger taxonomic groupings;^{6,7} however, further generalization is unwarranted until the genetic relatedness of the 24 other human adenovirus types is established. Finally, only 18-26 per cent hybrid formation was observed between tumorigenic and nontumorigenic adenoviruses; this homology is considered due to a similarity of nucleotide sequences in those cistrons concerned with the production of the group antigen and of those other features shared by all adenoviruses.¹⁵

Summary.—The degree of genetic relatedness among tumorigenic human adenovirus types 12 and 18 and nontumorigenic adenovirus types 2 and 4 was estimated by measurements of DNA-DNA homology. The DNA-agar technique was employed to determine the interactions among all 16 possible pairs (homologous, het-

erologous, and reciprocal) of the four viral DNA's. The DNA's of tumorigenic adenovirus types 12 and 18 hybridized with each other to the extent of 79 per cent, indicating a high degree of genetic homology; those of adenovirus types 2 and 4 hybridized 35 per cent indicating that they are about half as related to each other as are the tumorigenic types. The DNA's of tumorigenic adenovirus types hybridized with those of the nontumorigenic adenovirus types only to the extent of 18-26 per cent, suggesting that the nucleotide sequences in most of their cistrons are dissimilar.

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¹ Huebner, R. J., W. P. Rowe, and W. T. Lane, these PROCEEDINGS, **48**, 2051 (1962).

² Trentin, J. J., Y. Yabe, and G. Taylor, *Science*, **137**, 835 (1962).

³ Green, M. and M. Piña, these PROCEEDINGS, **50**, 44 (1963).

⁴ *Ibid.*, **51**, 1251 (1964).

⁵ Bolton, E. T., and B. J. McCarthy, these PROCEEDINGS, **48**, 1390 (1962).

⁶ McCarthy, B. J., and E. T. Bolton, these PROCEEDINGS, **50**, 156 (1963).

⁷ Hoyer, B. H., B. J. McCarthy, and E. T. Bolton, *Science*, **144**, 959 (1964).

⁸ Cowie, D. B., and B. J. McCarthy, these PROCEEDINGS, **50**, 537 (1963).

⁹ Bolton, E. T. and B. J. McCarthy, *J. Mol. Biol.*, **8**, 201 (1964).

¹⁰ McCarthy, B. J., and E. T. Bolton, *J. Mol. Biol.*, **8**, 184 (1964).

¹¹ Hoyer, B. H., B. J. McCarthy, and E. T. Bolton, *Science*, **140**, 1408 (1963).

¹² Green, M., and M. Piña, *Virology*, **20**, 199 (1963).

¹³ Burgi, E., and A. Hershey, *Biophys. J.*, **3**, 309 (1963).

¹⁴ Huebner, R. J., W. P. Rowe, H. C. Turner, and W. T. Lane, these PROCEEDINGS, **50**, 379 (1963).

¹⁵ Pereira, H. G., R. J. Huebner, H. S. Ginsberg, and J. Van Der Veen, *Virology*, **20**, 613 (1963).

THE BINDING OF ESTRADIOL IN THE UTERUS: A MECHANISM FOR DEREPRESSION OF RNA SYNTHESIS

BY G. P. TALWAR,* S. J. SEGAL, A. EVANS, AND O. W. DAVIDSON

LABORATORIES OF THE POPULATION COUNCIL, ROCKEFELLER INSTITUTE

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Several hormones stimulate the synthesis of RNA in specific target organs.¹⁻¹³ Studies on the effect of growth hormone on liver,¹ and estradiol on the uterus,^{2, 3} show that the activation of RNA synthesis is an early metabolic event which precedes the synthesis of proteins (enzymes) and the increment in cytoplasmic mass. Short pulse labeling and fractionation studies^{5,6} reveal that the stimulatory process originates in the nucleus. The system is sensitive to inhibition by actinomycin D. Moreover, the morphological manifestations of the hormone effect are dependent on the initial stimulation of RNA synthesis and are prevented by inhibition of DNA-dependent RNA synthesis.⁴

The manner in which hormones select target sites and activate the synthesis of