Proc. Nat. Acad. Sci. USA
Vol. 73, No. 4, pp. 1092–1096, April 1976
Biochemistry

Deficiency in histone acetylation in nontransforming host range mutants of polyoma virus

(Papova virus mutants/simian virus 40/histone modification/transformation)

BRIAN S. SCHAFFHAUSEN AND THOMAS L. BENJAMIN

Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Communicated by Baruj Benacerraf, January 19, 1976

ABSTRACT **Histones H3 and H4 derived from transforming wild-type polyoma and simian virus 40 particles show extensive acetylation compared to the histones of the host cells. The same histone fractions derived from nontransforming host range mutants of polyoma virus fail to show this high degree of acetylation.**

The presence of histones as internal proteins in polyoma and simian virus 40 (SV40) virus particles has been reported by several laboratories (1–5). Though highly invariant in primary structure, histones acquire considerable heterogeneity through modifications such as acetylation, phosphorylation, and methylation (6, 7). Such is the case for the two arginine-rich histones, H3 and H4, isolated from polyoma and SV40 virions, which have recently been shown to be highly acetylated compared to the same histone fractions from the host cells (5).

The present study reveals that the high degree of acetylation of H3 and H4 histones characteristic of wild-type polyoma virus and SV40 is *not* found in the histones from a particular class of polyoma mutant which has lost the ability to transform cells. These mutants have been isolated by a host range selection that used polyoma-transformed 3T3 cells as a permissive host and normal 3T3 cells as a nonpermissive host (8). The rationale behind this selection is that complementation with the integrated virus would permit the isolation of mutants defective in that portion of the genome which is expressed in transformed cells. Independent mutants selected in this way belong to a single complementation group and present a uniform biological behavior. They fail to induce either abortive or stable transformation of rat and hamster cells *in vitro* (8, 9), and are unable to bring about a cell surface alteration characteristic of transformed cells (10). Mutant *NG-18*, prototype of this class, has also been shown to have lost the ability to induce tumors in newborn hamsters (ref. 11; R. Siegler and T. Benjamin, manuscript in preparation.)

Recent studies have shown that several types of cells, other than polyoma transformants, are capable of growing the mutants as well as polyoma-transformed 3T3 cells (12, 13). Among these are 3T3 cells infected by C-type RNA viruses, primary or secondary (but not higher passage) mouse embryo fibroblasts, and primary epithelial cells from baby mouse kidneys (13). Cellular factors can therefore bypass the defect in the host range mutants. These results are readily explained by assuming that the expression of certain cellular genes is essential in the virus growth cycle, and that the function of the viral gene which has been altered in the host range mutants is to elicit the expression of those cellular genes.

Abbreviations: SV40, simian virus 40; hr-t, host range and transformation mutants; ts, temperature-sensitive mutants; BMK, baby mouse kidney cells.

A mechanism of viral-induced cellular gene expression may well be at the basis of the virus' potential for causing cell transformation. This is supported by the fact that out of 19 host range mutants, all have lost cell transforming ability as an unselected property (ref. 8; R. Staneloni and T. Benjamin, manuscript in preparation). Two biological consequences of mutation in a single viral gene therefore become manifested together: (*1*) a restricted host range, confined to those cell types constitutively expressing permissive factors, and (*2*) an inability to cause cell transformation.

The designation "hr-t" will be used to refer to the viral function altered in this class of mutant, indicating the dual aspects of the role played by this viral gene in determining host range and transformation. To fulfill the predictions based on the biological behavior of these mutants, it will be required to demonstrate a biochemical mechanism which is potentially capable of altering patterns of cellular gene expression and which stems from action of the hr-t viral gene. The deficiency in histone acetylation in hr-t mutants is discussed in terms of the altered biological properties of these mutants, and in terms of possible broader implications of histone metabolism in cell transformation.

MATERIALS AND METHODS

Virus Strains. The standard wild-type polyoma virus used here is a large plaque strain from the California Institute of Technology. Similar results on histone acetylation have been noted with a variety of large and small plaque wild-type isolates (5). Mutants *NG-18*, *NG-23*, *NG-59*, and *HA-33* have been described (8). Mutants *B-2*, *B-4*, and *30'b* were selected by the same procedure. *B-2* and *B-4* were isolated from a stock of virus grown in the presence of 1.5 μg/ml of ICR-191 (gift of Dr. H. Creech), and *30'b* from the light side of the virion band in a cesium chloride density gradient of nonmutagenized wild-type virus. Temperature-sensitive mutants *ts-616*, *ts-3*, and *ts-1260* were obtained from Walter Eckhart, and have been described (14, 15). Wild-type SV40 is strain 777; *ts-209* is an early A group transformation-defective mutant of SV40 isolated by Chou and Martin (16).

Virus Growth and Purification. All polyoma strains were grown on baby mouse kidney cells (BMK) prepared from kidneys of 12- to 15-day-old Swiss mice. The cells were infected 3 days after plating at a multiplicity less than 1 PFU/cell and harvested at the time of extensive cytopathic effect. The virus was precipitated from crude lysates by addition of ammonium sulfate (17). The pellet was resuspended in approximately 1/20 volume of 0.02 M Tris·HCl at pH 9.0, incubated at 45° for 10 min, sonicated briefly, and rewarmed to 45°. This suspension was clarified by centrifugation at 15,000 rpm in a Beckman J21 Centrifuge for 15 min at room temperature with a J-20 rotor. The pellet was reextracted in the same manner. The combined supernatants were layered

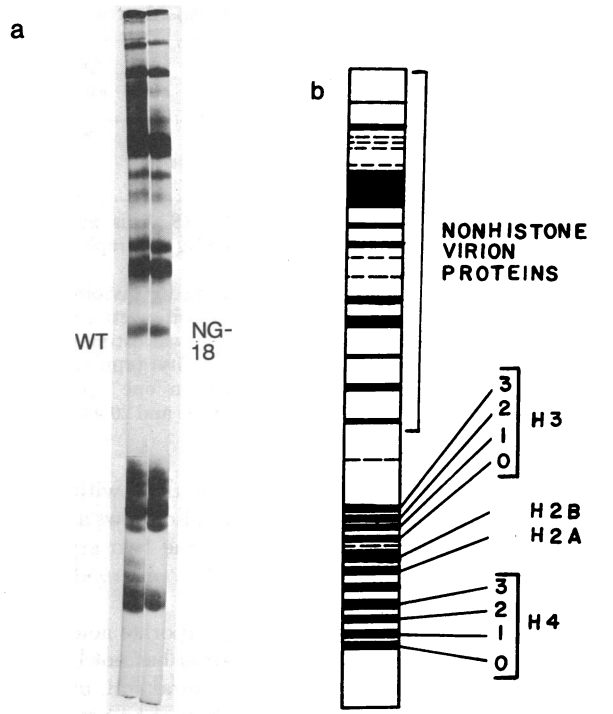

FIG. 1. (a) Acetic acid–urea gels of wild-type virus (WT) and hr-t mutant (*NG-18*). (b) Diagrammatic representation of gels in (a). The numbers in brackets represent the number of internal acetylations. In addition all forms of H4 contain NH_2-terminal acetylserine (7). See *Materials and Methods*.

on a double layer consisting of 10 ml of 20% (wt/vol) sucrose in 0.2 M Tris·HCl at pH 9.0, over 5 ml of cesium chloride (density 1.38 g/ml) in 0.2 M Tris·HCl, pH 9.0. This gradient was centrifuged in an SW27 rotor at 26,000 rpm for 3 hr. The lower band of virions was collected and analyzed for a homogenous A_{260}/A_{280} ratio across the band. A ratio of 1.30-1.33 was considered satisfactory for purification. As needed, samples were further banded by equilibrium density centrifugation in cesium chloride (density 1.30 g/ml) in 0.02 M Tris·HCl at pH 9.0, in an SW50.1 rotor at 36,000 rpm.

SV40 was grown on Vero cells. Virus was pelleted from crude lysates by centrifugation at 28,000 rpm for 2.5 hr in a type 35 rotor. The virus was then purified as described previously (18, 19), employing sucrose–cesium chloride gradients similar to those used for polyoma virus except that neutral pH was used for all solutions.

Analysis of Virion Proteins. The virion proteins were examined by polyacrylamide gel electrophoresis as described previously (5). Approximately 200 μg of viral proteins were dissociated in a solution containing 10 M urea–0.9 M acetic acid–1% (wt/vol), 2-mercaptoethanol–5 mg/ml of protamine sulfate at room temperature for 16 hr. The polypeptides were then resolved on 20 cm gels containing 15% acrylamide–0.9 M acetic acid–2.5 M urea, as originally described by Panyim and Chalkley (20) for the separation of cellular histones. Electrophoresis was carried out for approximately 16 hr at 200 V.

After staining with Coomassie blue for 3 hr, the gels were destained by solvent extraction (5). The stained gels were scanned at 575 nm on an Acta II Spectrophotometer equipped with a gel scanning attachment. These scans were then analyzed on a Dupont 310 Curve Resolver (courtesy of Dr. G. D. Fasman) to determine the relative amounts of the different forms of H3 and H4 histones.

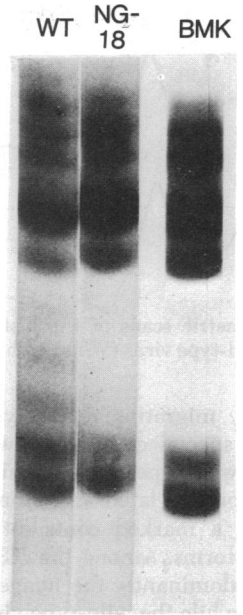

FIG. 2. Close-up of histone regions of gels shown in Fig. 1a and histones isolated from primary baby mouse kidney cells (BMK).

RESULTS

Wild-type virus and hr-t mutants have all been grown on BMK cells as a common host. Fig. 1a shows the polypeptides of wild-type virus and hr-t mutant *NG-18* after dissociation with 10 M urea–0.9 M acetic acid–1% (wt/vol) 2-mercaptoethanol–5 mg/ml of protamine sulfate and electrophoresis on 15% polyacrylamide gels containing 2.5 M urea–0.9 M acetic acid. As reported previously, at least 24 polypeptide species can be resolved from wild-type virions by this technique (5). Many of them (indicated by the upper bracket, Fig. 1b) are of nonhistone origin. There are no substantial differences in the patterns of wild-type and mutant nonhistone proteins. The two viruses are also similar with respect to the relative amounts of histone and nonhistone proteins.

Thirteen of the bands are histones that include at least four different forms of both H3 and H4 histones. These bands consist of acetylated forms, as shown in Fig. 1b. The major evidence supporting the identification of these bands is: (*1*) peptide mapping data confirming the identity of the fractions, (*2*) acetate-labeling experiments showing different specific activities for each of the subspecies, and (*3*) phosphate-labeling experiments ruling out histone phosphorylation as the source of the observed heterogeneity (5). Other laboratories have directly demonstrated that these bands contain *N*-acetyllysine (21). Comparison between mutant and wild type shows no *qualitative differences* in the histone region of the gels; each species observed in the wild type can also be observed in the mutant virions.

A very striking *quantitative difference* can be seen, however, in the relative amounts of the different forms of H3 and H4 histones. The mutant virus shows little of the more highly acetylated forms of these arginine-rich histones. The differences in acetylation are somewhat easier to see in Fig. 2, which gives a close-up of the histone region of the gels of Fig. 1a compared to histones from uninfected host cells. Among the forms of H4 histone, the mutant contains pri-

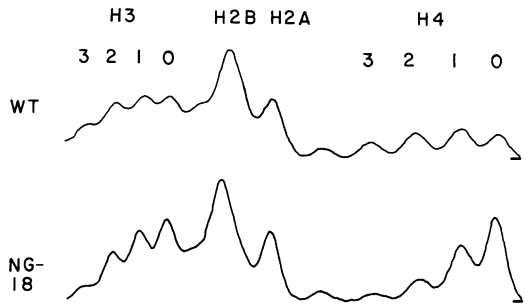

FIG. 3. Densitometric scans of the histone regions of acetic acid–urea gels of wild-type virus (WT) and hr-t mutant (*NG-18*).

marily the rapidly migrating nonacetylated form and has very little of the species containing two or three internal acetylations. The wild-type virus contains a more even distribution of the nonacetylated and monoacetylated species, and also contains a marked contribution from the more highly acetylated forms. Among the H3 species the mutant virus contains predominantly the nonacetylated and monoacetylated forms, while the wild-type virus contains substantial quantities of the di- and tri-acetylated species.

Comparisons of the degree of acetylation can be made from scans of the histone regions of Coomassie-stained gels as shown in Fig. 3. The differences in distribution of the H4 species between the two different viruses are immediately apparent. In *NG-18* the nonacetylated species predominates, while in wild-type virions the mono- and di-acetylated forms are the major species. The mutant also shows less of the highly acetylated forms of H3 histones than does the wild-type virus.

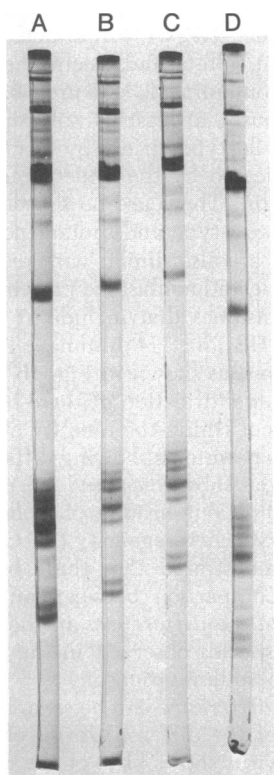

FIG. 4 (A–D, *left*). Acetic acid–urea gels of hr-t mutants (A=*NG-23*, B=*30'b*, and C=*B-2*) and of wild-type virus (D=WT).

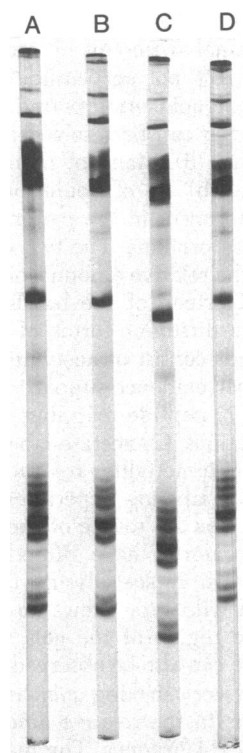

FIG. 5 (A–D, *right*). Acetic acid–urea gels of temperature-sensitive mutants (A=*616*, B=*3*, and C=*1260*) and of wild-type virus (D=WT).

Table 1. Acetylation of major capsid proteins*

Major capsid protein	cpm	Degree of acetylation†
WT	53,942	1.36
NG-18	26,773	1.31

* Equal quantities of wild-type and *NG-18* virus grown in the presence of [^{3}H]acetate (5) were resolved by electrophoresis as described in *Materials and Methods*.

† The degree of acetylation was calculated by dividing the total cpm in the major capsid band by the specific activity of acetate for the corresponding virus. This quantity was estimated for each virus by determining the specific activity (cpm/OD) of the H4(0) band which is known to contain one acetate (NH_2-terminal) per molecule: 39,700 for wild type and 20,400 for *NG-18*.

Three other hr-t mutants are shown along with wild-type virus in Fig. 4. Each of these mutants also shows a characteristic reduced level of acetylation of the two arginine-rich histones. Similar results have also been observed with hr-t mutants *NG-59*, *HA-33*, and *B-4*.

Nonhistone virion proteins also incorporate acid-labile acetate, but the nature of this acetylation has not been determined (B. Schaffhausen and W. T. Murakami, unpublished results). Reduced acetylation in the case of hr-t mutants concerns only histones H3 and H4, and not the capsid proteins which show equal levels of acetylation between mutant and wild type (see Table 1).

Preliminary studies have been carried out to see if the defect in histone acetylation is specific to the hr-t mutants or whether other classes of mutants of polyoma virus might also show reduced acetylation of the virion histones. Fig. 5 compares wild-type virus to three other kinds of polyoma mutants. *Ts-616* is a member of the ts-A class, defective in transformation and T-antigen production at the non-permissive temperature (22, 23). *Ts-3* is a temperature-sensitive mutant apparently blocked in uncoating (24). It also may render some of the cellular properties associated with transformation temperature-sensitive, such as lectin agglutinability (25). *Ts-1260* is a late mutant, unaffected in transforming ability and most likely defective in one of the capsid proteins (14). Each of these mutants shows an acetylation pattern more characteristic of wild-type virus than of the hr-t class, although the levels of acetylation may be slightly reduced (see Table 2). It should be noted also in Table 2 that in a wild-type stock grown at 31°, the levels of acetylation are somewhat less than in stocks grown at 37.5°. Fig. 6 shows that both wild-type SV40 and a *ts-A* class mutant, *ts-209*, show high levels of acetylation of the viral histones H3 and H4. The level of acetylation of H3 and H4 in chromatin of normal monkey cells is markedly less than the levels shown here for SV40 (data not shown). Since *ts* mutants are necessarily grown at low temperature where they show wild-type phenotype, these results are not surprising. Experiments on subviral complexes isolated after infection and shift to the nonpermissive temperature will be required to determine if any of these mutants are affected in histone acetylation.

The amounts of different forms of H3 and H4 histones have been determined by examining scans such as those of Fig. 3 on a Dupont 310 Curve Resolver. Table 2 shows the results on the gels discussed here. All hr-t mutants show a reduced level of acetylation relative to wild-type polyoma virus. Both wild-type and mutant viruses show a range of

Table 2. Quantitation of acetylated forms of H3 and H4 histones in different strains of polyoma virus and normal BMK cells

Virus strain	Percent of histones in various states of acetylation*							
	H3				H4			
	0	1	2	3	0	1	2	3
Wild type								
	31	29	27	13	40	29	20	11
	30	35	25	10	33	33	22	12
	29	29	26	16	26	30	28	16
†	—	—	—	—	44	29	19	8
Mutants								
Hr-t:								
NG-18	41	30	22	7	55	30	12	3
NG-23	42	33	16	9	51	34	11	4
30'b	46	32	17	5	52	34	10	4
B-2	46	32	16	6	51	33	12	4
Ts:								
616	34	29	23	14	44	33	15	8
3	33	32	22	13	46	32	14	8
1260	32	29	23	16	39	36	16	9
BMK	48	37	15	—	63	27	6	4

* Distributions of various acetylated forms determined as described in *Materials and Methods*. 0, 1, 2, and 3 refer to the number of internal acetylations of each form. Results of four separate wild-type experiments are given.
† Wild-type stock grown at 31°.

acetylation patterns in different preparations, although the two ranges do not appear to overlap.

DISCUSSION

The viral chromatins of wild-type polyoma and SV40 show a high degree of acetylation of their H3 and H4 species compared to the histones in the chromatins of their host cells (5). The extent of acetylation seen in these viral chromatins makes them among the most highly acetylated of any chromatins reported thus far.

When various classes of conditional lethal virus mutants are each grown under their respective permissive conditions, the temperature-sensitive mutants of all complementation groups show wild-type levels of histone acetylation, while mutants of the hr-t class show markedly reduced levels. As noted earlier, the normal (high) levels of histone acetylation in temperature-sensitive mutants is readily understood as arising from a restoration to wild-type action of the altered viral gene product at the permissive temperature. The analogous situation apparently does not hold for hr-t mutants which are complemented by cellular factors (12, 13); in a permissive host, the constitutive expression of these factors bypasses the need for an active hr-t viral gene product.

The absence of a high degree of acetylation has been noted in seven out of seven hr-t mutants. The chromatins of these mutants show a degree of acetylation in their H3 and H4 components which is close to but usually slightly higher than that seen in the chromatin of uninfected host cells. This increment may be due to a residual amount of normal function by the mutants; it may also be a passive reflection of increased host metabolism after infection, particularly of histone synthesis and modification occurring during viral-induced cellular DNA synthesis.

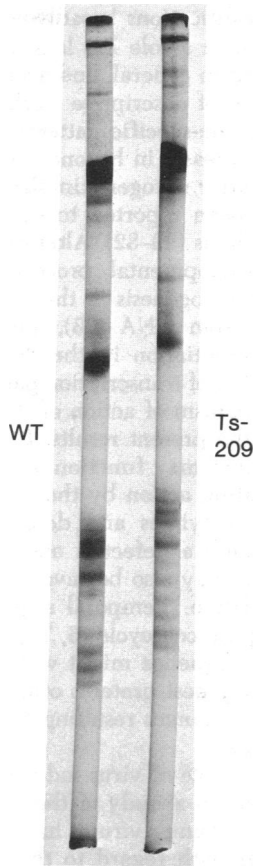

FIG. 6. Acetic acid–urea gels of wild-type SV40 (WT) and a *ts-A* mutant (*ts-209*).

A role of the hr-t viral gene in bringing about histone acetylation is consistent with biological properties of the mutants: (*i*) Failure to alter the cell membrane. Transformed cells generally express a permanent surface alteration measured by lectin agglutinability, a change that is regulated in normal cells to occur only during mitosis (26). Wild-type polyoma virus induces this membrane change during productive infection of 3T3, while hr-t mutants do not (10). For wild-type virus to induce this change, it must be able to induce cellular DNA synthesis (10, 25). Although hr-t mutants retain this ability, the stimulation of cellular DNA synthesis is not followed by the onset of agglutinability. These results suggest that the induction of cellular DNA synthesis, while necessary, is not sufficient for the subsequent membrane change, and that some event linked to cellular DNA synthesis is also essential but lacking in hr-t infected cells. Histone synthesis and modification, known to be closely associated with DNA synthesis, may be implicated in the process by which the virus induces this cell surface change. (*ii*) Restrictions in virus growth and transformation. All hr-t mutants show effectively the same limitations of host range (13), and all are defective in transformation (8). Analysis of the growth of the mutants in a variety of cell types has lead to the hypothesis that the primary function of the hr-t gene is to bring about the expression of cellular genes required for productive infection (13). The perfect concordance of "non-transforming behavior" and "reduced host range" suggests further that cellular factors in transformation as well as cellular permissive factors for virus growth are elicited from the cell by action of the hr-t viral gene. The hr-t function therefore appears to act pleiotropically in altering the expression of cellular genes.

Could histone modifications broadly alter the expression of cellular genes? Such a role has long been suggested for histone modifications in general, and acetylation in particular (27). A great deal of descriptive work has been done on the temporal and tissue-specific patterns of histone modifications (6, 7, 28). Increases in histone acetylation after hormonal stimulation, after mitogen stimulation, or during liver regeneration have been reported to occur at times of increase in RNA synthesis (29–32). Alterations have also been noticed during developmental processes: acetylation increases during spermatogenesis in the trout prior to the removal of histones from DNA (33), and decreases during erythroid cell differentiation in the duck, paralleling the progressive narrowing of transcription patterns (34).

No specific mechanism of action of the hr-t function can be deduced from the present results. It is conceivable that the hr-t gene product may function as a histone acetylase, although a modulating action by the virus on the levels or activities of host acetylases and de-acetylases is perhaps more likely. Although a defect is observed in acetylation, other modifications may also be involved such as phosphorylation or methylation. Temporal sequences of modifications occur during the cell cycle (6, 7, 28, 35–37); an effect on one step in the sequence might well affect others down the line. Finally, key host proteins other than histones may undergo functional changes resulting from similar viral induced modifications.

Evaluation of the role of virus induced histone modifications must be based on a study of the effects on histones in host cell chromatin. Tumor viruses have not been extensively studied thus far with regard to their effects on histone metabolism. However, SV40 transformed (WI38) human cells (38) and adenovirus 2- and 12-infected human embryonic-kidney cells (39) have been reported to show increased histone acetylation. The results reported here call attention to the need for further study of the role of virus induced changes in histone metabolism in processes of virus growth and cell transformation.

The authors wish to acknowledge the expert technical assistance of Ingrid Lane and Anne Caesar. The work of this laboratory has been supported by Grant no. DRG-13-F from the Damon Runyon-Walter Winchell Cancer Fund, and Contract no. N01-43299 from the National Cancer Institute. B.S.S. is a Post-Doctoral Fellow of the National Institutes of Health. T.L.B. is a Scholar of the Leukemia Society of America, Inc.

1. Murakami, W. T. & Schaffhausen, B. (1972) in "Molecular studies in viral neoplasia," *MD Anderson Symposium* (Williams and Wilkins, Baltimore), pp. 43–62.
2. Schaffhausen, B. & Murakami, W. T. (1972) *Fed. Proc.* **31**, 806.
3. Frearson, P. & Crawford, L. (1972) *J. Gen. Virol.* **14**, 141–155.
4. Fey, G. & Hirt, B. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 235–241.
5. Schaffhausen, B. & Murakami, W. T. (1976) *Virology* **69**, in press.
6. Hnilica, L. S. (1972) *The Structure and Biological Functions of Histones* (CRC Press, Cleveland, Ohio).
7. Phillips, D. M. P. (1971) *Histones and Nucleohistones* (Plenum Press, New York).
8. Benjamin, T. L. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 394–399.
9. Benjamin, T. L. & Norkin, L. (1972) in "Molecular studies in viral neoplasia," *MD Anderson Symposium* (Williams and Wilkins, Baltimore, Md.), pp. 158–168.
10. Benjamin, T. L. & Burger, M. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 929–934.
11. Siegler, R. & Benjamin, T. (1975) "Oncogenicity of wild type and mutant strains of polyoma virus," abstract: *Proc. Am. Assoc. Cancer Res.* **16**, 99.
12. Benjamin, T. L. & Goldman, E. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 41–44.
13. Goldman, E. & Benjamin, T. L. (1975) *Virology* **66**, 372–384.
14. Eckhart, W. (1969) *Virology* **38**, 120–125.
15. Dulbecco, R. & Eckhart, W. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1775–1781.
16. Chou, J. Y. & Martin, R. G. (1974) *J. Virol.* **13**, 1101–1109.
17. Murakami, W. T., Fine, R., Harrington, M. R. & Ben Sassan, Z. (1968) *J. Mol. Biol.* **36**, 153–166.
18. Yoshiike, K. (1968) *Virology* **34**, 391–401.
19. Gelb, L. D., Kohne, D. E. & Martin M. A. (1971) *J. Mol. Biol.* **57**, 129–145.
20. Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346.
21. Wangh, L., Ruiz-Carrillo, A. & Allfrey, V. G. (1972) *Arch. Biochem. Biophys.* **150**, 44–56.
22. Francke, B. & Eckhart, W. (1973) *Virology* **55**, 127–135.
23. Oxman, M., Takemoto, K. & Eckhart, W. (1972) *Virology* **49**, 675–682.
24. Eckhart, W. & Dulbecco, R. (1974) *Virology* **60**, 359–369.
25. Eckhart, W., Dulbecco, R. & Burger, M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 283–286.
26. Fox, T., Sheppard, J. & Burger, M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 244–247.
27. Allfrey, V. G., Faulkner, R. & Mirsky, A. E. (1964) *Proc. Nat. Acad. Sci. USA* **51**, 786–794.
28. "The structure and function of chromatin" (1975) *The CIBA Foundation Symposium* (Associated Scientific Publishers, Amsterdam), No. 28.
29. Pogo, B. G. T., Allfrey, V. G. & Mirsky, A. E. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 805–812.
30. Pogo, B. G. T., Pogo, A. O., Allfrey, V. G. & Mirsky, A. E. (1968) *Proc. Nat. Acad. Sci. USA* **59**, 1337–1344.
31. Libby, P. R. (1972) *Biochem. J.* **130**, 663–669.
32. Libby, P. R. (1973) *Biochem. J.* **134**, 907–912.
33. Candido, E. P. M. & Dixon, G. H. (1972) *J. Biol. Chem.* **247**, 5506–5510.
34. Ruiz-Carrillo, A., Wangh, L. J., Littau, V. C. & Allfrey, V. G. (1974) *J. Biol. Chem.* **249**, 7358–7368.
35. Shepherd, G. R., Hardin, J. M. & Noland, B. J. (1971) *Arch. Biochem. Biophys.* **145**, 1–5.
36. Shepherd, G. R., Noland, J. B. & Hardin, J. M. (1971) *Arch. Biochem. Biophys.* **142**, 299–302.
37. Shepherd, G. R., Noland, B. J. & Hardin, J. M. (1971) *Biochim. Biophys. Acta* **228**, 544–549.
38. Krause, M. V. & Stein, G. S. (1975) *Exp. Cell Res.* **92**, 175–190.
39. Ledinko, N. (1970) *J. Virol.* **6**, 58–68.