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Presence of Two Deoxyribonucleic Acid Polymerases in Bull Spermatozoa

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A DNA polymerase–endogenous template complex was isolated from nuclear heads of bull spermatozoa. The buoyant density of the complex was 1.15 g/cm^3. The sedimentation coefficient of the nuclear DNA polymerase isolated from the complex was higher at low ionic strength, but approached 3.4S when centrifuged in a medium containing 2M-KCl. Activated exogenous DNA increased polymerase activity. Only very low activities were detected with synthetic templates such as poly(A)·$(dT)_{12-18}$ and poly(dT)·poly(A). The nuclear reaction was stimulated by 150mM-KCl and was slightly inhibited by *N*-ethylmaleimide; it was resistant to actinomycin D, netropsin and ethidium bromide. Another DNA polymerase, highly sensitive to ethidium bromide, was extracted from the mitochondria-rich middle-piece fraction. Its sedimentation coefficient was close to 9S, but fell to approx. 4S in high-ionic-strength medium.

The presence of DNA polymerase in spermatozoa has now been reported by several authors. Hecht (1974) extracted a DNA polymerase from intact bull spermatozoa whose properties were characteristic of a mitochondrial enzyme. A ribonuclease-sensitive DNA-synthesizing complex was isolated from human spermatozoa heads (Witkin *et al.*, 1975, 1977; Bendich *et al.*, 1976; Witkin & Bendich, 1977). By using a cytoenzymological method, we first localized a nuclear DNA polymerase in mouse spermatozoa heads (Chevaillier & Philippe, 1976*a*,*b*) and subsequently reported a more thorough characterization (Philippe & Chevaillier, 1976). The presence of this type of enzymic activity has also been demonstrated in spermatozoa heads of other animal species (Chevaillier & Philippe, 1977). Using biochemical methods, we have recently shown the presence of a nuclear DNA polymerase in isolated bull spermatozoa heads; the conditions of extraction and some characteristics of the enzyme activity have been defined (Philippe & Chevaillier, 1978).

We present here further details of the characterization of the nuclear enzyme as well as the description of another DNA polymerase of mitochondrial origin.

Materials and Methods

Bull spermatozoa heads were isolated as described in the preceding paper (Philippe & Chevaillier, 1978). The supernatant obtained after pelleting spermatozoa heads was diluted 10-fold and was centrifuged for 30min at 3500*g* to yield a pellet of middle pieces.

Preparation of nuclear and mitochondrial DNA polymerases

Spermatozoa heads (1.5×10^8/ml) were incubated for 1h at 37°C or for 20h at room temperature in 50mM-borate/NaOH buffer, pH9, containing 15mM-dithiothreitol, 4% Tween 80 and 1mg of soya-bean trypsin inhibitor/ml. A gelatinous chromatin solution was obtained and centrifuged at 40000*g* for 1h. In most cases supernatants were pooled and concentrated with an Amicon ultrafiltration cell by using a Diaflo UM2 filter or against aq. 15% (w/w) poly(ethylene glycol) (mol.wt. 4000; Serva, Heidelberg, Germany).

Mitochondrial extracts were similarly prepared from the middle pieces.

Buoyant-density sucrose-gradient centrifugation

The buoyant density of the nuclear DNA-synthesizing complex was determined by layering 0.3ml of nuclear extract on to 4.7ml of a 20–65% (w/w) linear sucrose gradient in 50mM-borate buffer, pH9, containing 1mM-dithiothreitol and 100mM-KCl. Centrifugation was performed in a swinging-bucket rotor (SW 50.1) at 4°C for 16 or 48h at 192000*g* in a Beckman L 3-50 ultracentrifuge. Fractions were collected from below in tubes containing 90μg of bovine serum albumin. Buoyant densities were determined by refractive index, and portions of each fraction were assayed for DNA polymerase activity with or without exogenous activated DNA.

Sedimentation analysis of nuclear and mitochondrial DNA polymerases

Sedimentation coefficients were determined by centrifugation through isokinetic sucrose gradients (Martin & Ames, 1961). A fraction (0.3ml) of the biological extract was loaded on to 4.7ml of 5–20% (w/w) linear sucrose gradient in 50mM-borate buffer, pH 9, containing 1mM-dithiothreitol and 0.3M- or 2M-KCl. Centrifugation was performed for 15.5h at 4°C in a SW 50.1 rotor at 192000*g*. Fractions were collected from the bottom into tubes containing 90μg of bovine serum albumin. Portions of each fraction were assayed for DNA polymerase activity as described below. Alcohol dehydrogenase (7.4S), bovine serum albumin (4.4S) and ovalbumin (3.6S) were used as markers and were centrifuged in parallel.

DNA polymerase assay

Poly(dT) homopolymer was obtained from P–L Biochemicals, Milwaukee, WI, U.S.A., and poly(A) from Miles laboratories, Elkhart, IN, U.S.A. Poly(dT)·poly(A) (ratio 2:1) was prepared by heating a mixture of poly(dT) and poly(A) at the same ratio at 80°C followed by slow cooling. Poly(A)·$(dT)_{12-18}$, dATP, dCTP, dGTP, dTTP and calf thymus DNA were purchased from Boehringer Mannheim Corp., West Germany. [*methyl*-^{3}H]-Thymidine 5′-triphosphate (ammonium salt) (40–60 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Fractions were incubated at 37°C in 50mM-borate buffer, pH 9.0, containing 100μg of soya-bean trypsin inhibitor/ml, 150mM-KCl, 15mM-$MgCl_2$, 100μM each of dATP, dGTP, dCTP, 10μM-dTTP and [*methyl*-^{3}H]dTTP (30μCi/ml; 1500c.p.m./pmol). Incubations were performed with or without native, heat-denatured or activated calf thymus DNA. DNA with different extents of activation was prepared as described by Fansler & Loeb (1974).

DNA polymerase activity was determined as the amount of radioactivity incorporated into the acid-insoluble fraction (de Recondo & Fichot, 1969).

Activity was also measured with different synthetic templates in order to distinguish polymerase-α, -β and -γ activities (Bollum, 1975; Weissbach, 1977).

RNA-primed DNA synthesis was assayed with 50μM-poly(dT)·poly(A) in 50mM-Tris/HCl, pH 7.6, containing 0.1mM-$MnCl_2$, 50mM-KCl, 10μM-dATP and [^{3}H]dATP (22Ci/mmol, 2500c.p.m./pmol).

DNA polymerase-γ activity was assayed with 40μM-poly(A)·$d(T)_{12-18}$ in 50mM-Tris/HCl, pH 7.5, or 20mM-potassium phosphate buffer, pH 7.2, containing 0.5mM-$MnCl_2$, 140mM-KCl, 10μM-dTTP and [*methyl*-^{3}H]dTTP (2500c.p.m./pmol). RNA-dependent DNA synthesis in this system is also detected with DNA polymerase-β (Chang, 1974; Knopf *et al.*, 1976). In this case, incubation was at 29°C because of the low 'melting' point of the template–primer hybrid (Méchali *et al.*, 1977).

Electron microscopy

Biological material was deposited on freshly glow-discharged carbon grids and negatively stained with 2% phosphotungstic acid. Observations were with an RCA electron microscope operating at 100kV.

Results

Buoyant-density sucrose-gradient centrifugation (Fig. 1)

After centrifugation on a 20–65% (w/w) sucrose gradient for 16h, nuclear DNA polymerase activity was found in a single band of density 1.15g/cm^3. The attainment of equilibrium was verified by

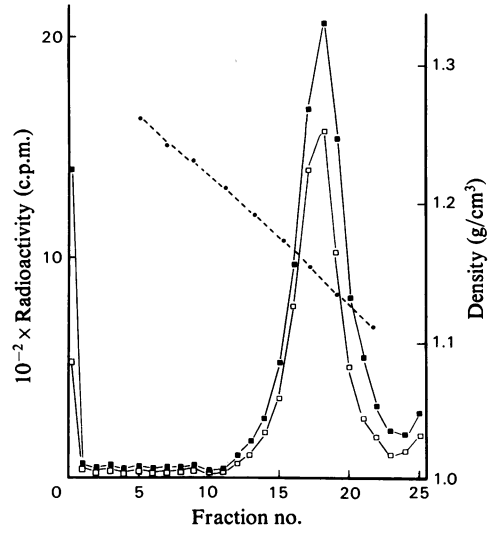
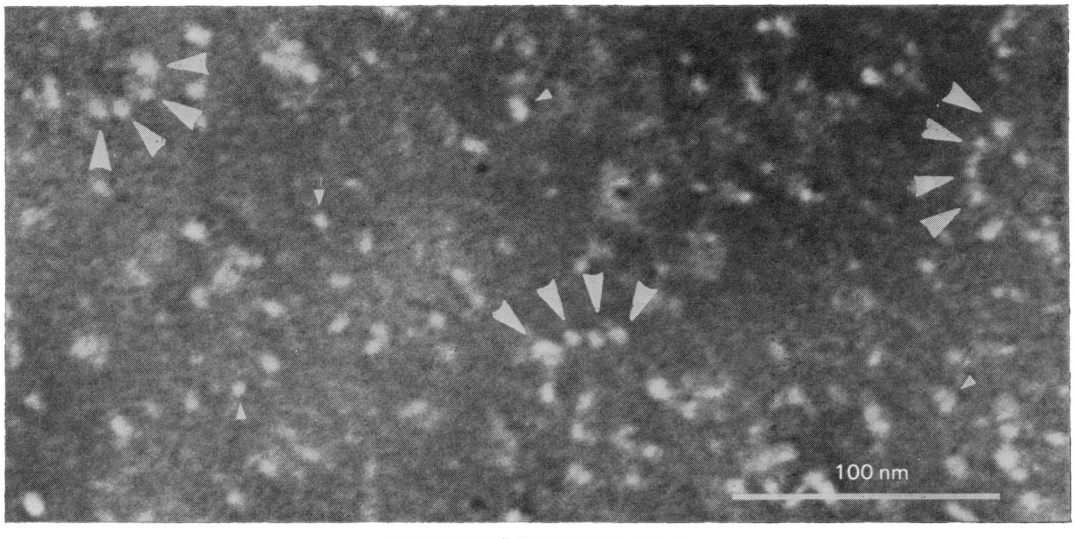


Fig. 1. *Buoyant-density sucrose-gradient centrifugation* Nuclear DNA polymerase extract was centrifuged on a linear 20–65% (w/w) sucrose gradient for 16h at 192000*g* (SW 50.1 rotor). Density (●) was determined by refractive index, and DNA polymerase activity was measured in the absence (□) or in the presence (■) of 400μg of activated DNA/ml. The DNA polymerase activity was expressed as c.p.m. of [^{3}H]dTTP incorporated into an acid-insoluble form under the same standard conditions as described in the Materials and Methods section.



EXPLANATION OF PLATE 1

Structural aspects of the nuclear DNA polymerase–endogenous template complex (density 1.15 *g/cm*^{3}*) shown by electron microscopy after negative staining*

Some particles appear free (small arrows), others seem to be aggregated (large arrows).

observing that the density of the enzyme was unchanged when centrifugation was conducted for 48h.

Electron-microscopic observation of the 1.15g/cm^3-density DNA polymerase complex (Plate 1) showed the presence of roughly spherical or slightly elongated small particles, which appeared either dispersed or associated in small groups by a thin fibre. The particles had a mean diameter of 5nm, thus well below the diameter of nucleosomes.

Sedimentation analysis of nuclear DNA polymerase

Nuclear extracts centrifuged on isokinetic 5–20% sucrose gradients showed that the sedimentation position of DNA polymerase was a function of KCl concentration. In the presence of 0.3M-KCl in the gradient (Fig. 2a), DNA polymerase activity was recovered in a wide zone of the gradient at a sedimentation coefficient greater than 4.4S (bovine serum albumin). The shape of the curve was wide and irregular. Moreover, variations were noted from one experiment to another, rendering difficult the precise determination of a sedimentation coefficient. When the gradients were prepared in 2M-KCl (Fig. 2*b*), however, nuclear DNA polymerase activity was recovered in a sharp peak that sedimented near 3.4S. This result was quite reproducible. An endogenous DNA polymerase activity was detected in both gradients, but activity was consistently found to be higher when exogenous activated DNA was added to the incubation medium.

Nuclear DNA polymerase activity in the presence of different exogenous templates (*Table* 1)

The DNA polymerase peaks at 3.4S were pooled and incubated in the presence of different exogenous templates. Single-stranded DNA had no effect on DNA polymerase activity. Activity was increased when native or activated calf thymus DNA was added to the incubation medium. This increase, which was a function of the extent of activation, reached a maximum with 8.8% activated DNA and decreased when more activated DNA (27.6%) was used.

Activity was slight when incubations were performed in the presence of synthetic templates such as poly(A)·$(dT)_{12-18}$ or poly(dT)·poly(A).

Other properties of the nuclear DNA polymerase

The 3.4S nuclear DNA polymerase was partially characterized, and the results were identical with those obtained with swollen spermatozoa heads (Philippe & Chevaillier, 1978).

The enzyme is Mg^{2+}-dependent with optimal activity at 20mM. It is also pH-dependent, with optimal activity at pH 8.6 and 9.0 with 50mM-Tris/HCl and 50mM-borate buffer respectively. Phosphate buffer had an inhibitory effect. Optimal activity was obtained with 150mM-K^+. The reaction was partially inhibited by *N*-ethylmaleimide (1mM-

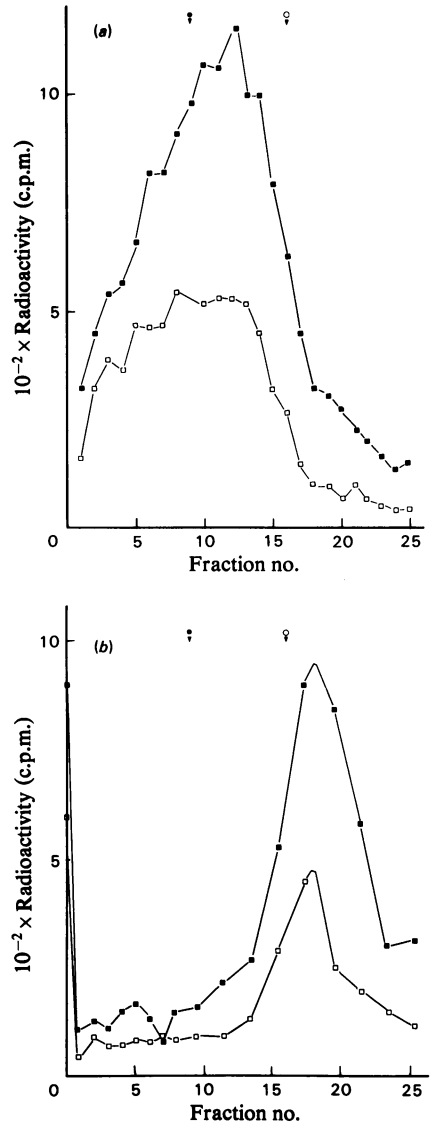


Fig. 2. *Sedimentation analysis of nuclear DNA polymerase*
A nuclear extract was centrifuged on a linear 5–20% (w/w) sucrose gradient for 15.5h at 192000*g* (SW 50.1 rotor). Two KCl concentrations were used, 0.3M (*a*) and 2M (*b*). DNA polymerase activity was measured in the absence (□) or in the presence (■) of 400μg of activated DNA/ml. Alcohol dehydrogenase (7.4S: ●) and bovine serum albumin (4.4S: ○) were used as markers.

Table 1. *Nuclear DNA polymerase activity measured in the presence of different templates*

All these experiments were with the same extract; the results in each case are given for the same quantities of extract and expressed by reference to a control incubated without any addition of exogenous template. Experimental conditions are described in the Materials and Methods section.

				+'Activated' DNA			+Poly(A)·$(dT)_{12-18}$		
	Endogenous	+Native DNA	+Heat-denatured DNA	1.4% activation	8.8% activation	27.6% activation	In 50mM-Tris/HCl, pH7.5	In 20mM-phosphate buffer	+ Poly(dT)·poly(A)
Activity (pmol of ^{3}H-labelled nucleotide incorporated)	0.647	0.710	0.639	0.762	0.934	0.751	0.079	0.062	0.059
(%)	100	109.7	98.7	117.8	144.3	116.1	12.2	9.6	9.2

N-ethylmaleimide led to 76% of control activity), but was found to be resistant to 50μg of actinomycin D/ml, 50μg of netropsin/ml and 25μM-ethidium bromide.

Sedimentation of mitochondrial DNA polymerase

The mitochondrial extract was centrifuged in 5–20% sucrose gradients under the same conditions as the nuclear extract. As in the latter case the position of mitochondrial DNA polymerase depended on the KCl concentration. In the presence of 0.3M-KCl (Fig. 3*a*) mitochondrial DNA polymerase sedimented at about 9S, but when centrifuged in a gradient containing 2M-KCl (Fig. 3*b*) the sedimentation coefficient was near 4S.

In addition, mitochondrial DNA polymerase activity was highly sensitive to ethidium bromide. The presence of 10μM-ethidium bromide led to the recovery of 36% of control activity.

Discussion

When a nuclear extract of bull spermatozoa heads is centrifuged to equilibrium through a 20–65% linear sucrose gradient, a single peak of material is isolated that can synthesize DNA *in vitro* in the absence of an exogenous template. The observation of thin fibres between some small particles seen with electron microscopy could be correlated with endogenous activity. These fibres might be DNA fragments obtained by the partial deproteinization produced by the solvents used during the extraction procedure and for the spreading of the material contained in the gradient fractions on the grids. However, they might also represent chromatin fibres with nuclear proteins remaining associated with the DNA, as it seems that nucleosomes do not persist in nuclei containing protamines (Honda *et al.*, 1974). Whatever the exact interpretation of these structures, the fractions contained both a template and an enzyme which are needed for the expression of an endogenous activity. The density of this active fraction is 1.15g/cm^3, which is in good agreement with the most recently reported density value (Witkin & Bendich, 1977) for the nuclear DNA-synthesizing complex isolated from human spermatozoa heads. They also found that the density of the complex depended on the conditions used to prepare the extract. When the complex was prepared by incubating isolated human spermatozoa heads in 20mM-dithiothreitol for 10min, its density was between 1.21 and 1.25g/cm^3 (Witkin *et al.*, 1975) and decreased to 1.16g/cm^3 when the incubation lasted for over 80min (Bendich *et al.*, 1976); density was found to be 1.15g/cm^3 when the extract was subsequently incubated with trypsin and pancreatic deoxyribonuclease (Witkin & Bendich, 1977).

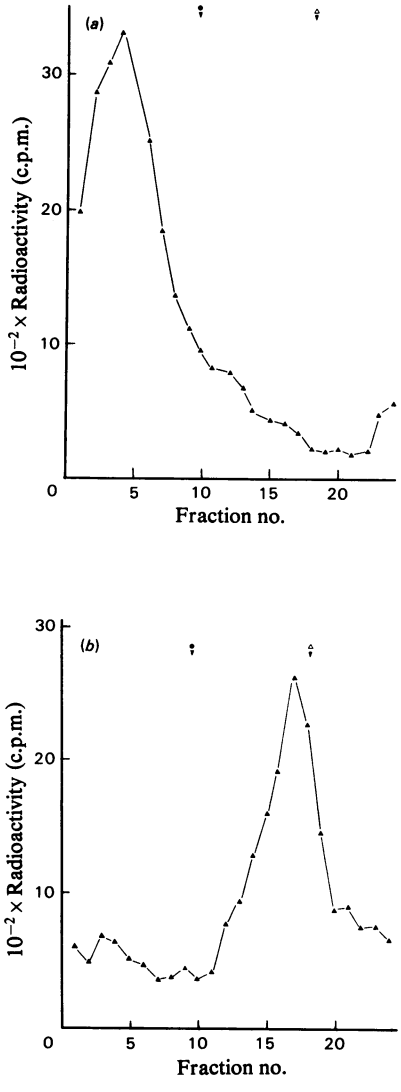


Fig. 3. *Sedimentation analysis of mitochondrial DNA polymerase*

A mitochondrial extract was centrifuged on a linear 5–20% (w/w) sucrose gradient for 15.5h at 192000*g* (SW 50.1 rotor). Two KCl concentrations were used, 0.3M (*a*) and 2M (*b*). DNA polymerase activity was measured in the presence of 400μg of activated DNA/ml. Alcohol dehydrogenase (7.4S: ●) and ovalbumin (3.6S: △) were used as markers.

Determinations of sedimentation coefficient in sucrose gradients revealed that the position of the nuclear enzyme is dependent on ionic strength. In the presence of 2M-KCl, the sedimentation coefficient is close to 3.4S, which is characteristic of DNA polymerase-β (Craig & Keir, 1974; Fansler, 1974; Bollum, 1975; Weissbach, 1977). When centrifuged in the presence of a lower KCl concentration (0.3M), DNA polymerase is recovered in a zone with a higher sedimentation coefficient. The profile of this zone is irregular, and variations are noted from one experiment to another. At very low ionic strength, DNA polymerase-β is known to self-aggregate (Bollum, 1975; Weissbach, 1975) or to form complexes with membrane components or with other proteins (de Recondo & Abadiedebat, 1976). We believe that this interpretation is not justified in the present case because of the relatively high concentration of KCl used (0.3M). It is more probable that the enzyme in 0.3M-KCl still remains bound to very small fragments of chromatin. If the size of fragments is variable in the same gradient, this could explain the shape of the sedimentation curve as well as the lack of reproducibility from one experiment to another.

When centrifugation is performed in a very-high-ionic-strength medium (2M-KCl), however, DNA polymerase is free, and always sediments at the same position (3.4S). Nevertheless, an endogenous DNA polymerase activity is also detected, which may be due to co-sedimentation of very small chromatin fragments. After re-association, they could be used as template by the enzyme. We use the term 'chromatin' because protamines were electrophoretically detected in the 3.4S peak (results not shown).

Nuclear DNA polymerase was stimulated by exogenous native or activated DNA. Optimal activity was obtained when the DNA was 8.8% activated. A similar result was reported by Stalker *et al.* (1976), who studied DNA polymerase-β extracted from Novikoff hepatoma. On the contrary, DNA polymerase-α is known to prefer more highly activated DNA (Fansler & Loeb, 1974; Baril *et al.*, 1977). When synthetic templates such as poly(dT)·poly(A) and poly(A)·$(dT)_{12-18}$ were used, practically no activity was detected compared with endogenous activity. These results differ from those of Witkin *et al.* (1975), who obtained dTTP incorporation when poly(A)·$(dT)_{12-18}$ was used as template. Other differences were noted, especially a very strong inhibition of human spermatozoa DNA polymerase by KCl and *N*-ethylmaleimide, and a stimulatory effect of ethidium bromide (Witkin *et al.*, 1977). The present results for bull spermatozoa nuclear DNA polymerase, as well as those previously found on swollen spermatozoa heads (Philippe & Chevaillier 1978) lead to the conclusion that this enzyme is a DNA polymerase-β.

An ethidium bromide-sensitive DNA polymerase was extracted from the mitochondria-rich middle-piece fraction, whose sedimentation coefficient was close to 9S. This corresponds to properties generally

found for mitochondrial DNA polymerases (Probst & Meyer, 1973; Hecht, 1975; Fujisawa *et al.*, 1977; Tanaka & Koike, 1977), and is in good agreement with the results obtained with intact bull spermatozoa by Hecht (1974). Moreover, a dissociation of mitochondrial bull spermatozoa DNA polymerase (4S) was noted at high ionic strength (2M-KCl). This dissociation was also described by Bolden *et al.* (1977) for rat liver mitochondrial DNA polymerase.

Several authors have reported the presence of at least two distinct DNA polymerase activities in fully differentiated spermatozoa, nuclear (Witkin *et al.*, 1975, 1977; Bendich *et al.*, 1976; Chevaillier & Philippe, 1976*a*,*b*, 1977; Philippe & Chevaillier, 1976, 1978; Witkin & Bendich, 1977) and mitochondrial (Hecht, 1974). A problem posed by these results is the biological role of these two polymerases in spermatozoa, since no DNA replication occurs during spermatozoal differentiation and maturation. The role of these enzymes may be to protect and repair DNA, thus maintaining its integrity. This function is of course essential, at least for nuclear DNA. These enzymes may also be involved in the DNA replication which occurs after fertilization.

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References

Baril, E., Mitchener, J., Lee, L. & Baril, B. (1977) *Nucleic Acid Res.* **4**, 2641–2653

Bendich, A., Borenfreund, E., Witkin, S. S., Beju, D. & Higgins, P. J. (1976) *Prog. Nucleic Acid Res. Mol. Biol.* **17**, 43–75

Bolden, A., Noy, G. P. & Weissbach, A. (1977) *J. Biol. Chem.* **252**, 3351–3356

Bollum, F. J. (1975) *Prog. Nucleic Acid Res. Mol. Biol.* **15**, 109–144

Chang, L. M. S. (1974) *J. Biol. Chem.* **249**, 7441–7446

Chevaillier, Ph. & Philippe, M. (1976*a*) *Chromasoma* **54**, 33–37

Chevaillier, Ph. & Philippe, M. (1976*b*) *Exp. Cell Res.* **99**, 237–244

Chevaillier, Ph. & Philippe, M. (1977) *Chromosoma* **63**, 385–399

Craig, R. K. & Keir, H. M. (1974) in *The Cell Nucleus* (Busch, H., ed.), vol. 3, pp. 35–66, Academic Press, London and New York

de Recondo, A.-M. & Abadiedebat, J. (1976) *Nucleic Acid Res.* **3**, 1823–1837

de Recondo, A.-M. & Fichot, O. (1969) *Biochim. Biophys. Acta* **186**, 390–392

Fansler, B. S. (1974) *Int. Rev. Cytol.* **S4**, 363–415

Fansler, B. S. & Loeb, L. A. (1974) *Methods Enzymol.* **29**, 53–70

Fujisawa, T., Tanaka, S., Kobayashi, M. & Koike, K. (1977) *Biochim. Biophys. Acta* **475**, 611–622

Hecht, N. B. (1974) *J. Reprod. Fertil.* **41**, 345–354

Hecht, N. B. (1975) *Biochim. Biophys. Acta* **414**, 59–70

Honda, B. M., Baillie, D. L. & Candido, E. P. M. (1974) *FEBS Lett.* **48**, 156–159

Knopf, K. W., Yamada, M. & Weissbach, A. (1976) *Biochemistry* **15**, 4540–4548

Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **237**, 519–525

Méchali, M., Girard, M. & de Recondo, A.-M. (1977) *J. Virol.* **23**, 117–125

Philippe, M. & Chevaillier, Ph. (1976) *Biochim. Biophys. Acta* **447**, 188–202

Philippe, M. & Chevaillier, Ph. (1978) *Biochem. J.* **175**, 585–594

Probst, G. S. & Meyer, R. R. (1973) *Biochem. Biophys. Res. Commun.* **50**, 111–117

Stalker, D. M., Mosbaugh, D. W. & Meyer, R. R. (1976) *Biochemistry* **15**, 3114–3121

Tanaka, S. & Koike, E. (1977) *Biochim. Biophys. Acta* **479**, 290–299

Weissbach, A. (1975) *Cell* **5**, 101–108

Weissbach, A. (1977) *Annu. Rev. Biochem.* **46**, 25–47

Witkin, S. S. & Bendich, A. (1977) *Exp. Cell Res.* **106**, 47–54

Witkin, S. S., Korngold, G. C. & Bendich, A. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3295–3299

Witkin, S. S., Traganos, F. & Bendich, A. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1404–1410