and 20 by 60 μm to 25 by 80 μm (elongate) on days 11 to 16. In differentiating oocysts, we observed small refractile droplets similar to those seen in vivo and internal compartmentalization. Sporozoites, either free (Fig. 2B) or still connected to the residual body with a flowerlike appearance (Fig. 2D), were also observed in 12 different flasks from five different experiments, beginning on days 10 to 12. The number of sporozoites peaked on days 14 to 16, but some oocysts continued to produce sporozoites until day 22. These sporozoites expressed circumsporozoite protein antigenicity [Fig. 2C (*18*)].

In experiments in which ookinetes were seeded onto Matrigel but *D. melanogaster* cells were omitted from the culture system, none of the attached oocysts grew beyond 7 to 8 μm and clear signs of deterioration were seen within 4 to 7 days. However, when both L2 cells and Matrigel were excluded from the culture system, virtually all the parasites became round and many adhered to each other, forming large aggregates consisting of hundreds of parasites. About 20% of the clumped parasites grew to a size of 7 to 10 μm and ceased to develop. Nevertheless, in three different experiments rare oocysts continued to grow and produced sporozoites beginning on day 10.

In conclusion, sporozoites of *P. gallinaceum* were observed in cultures maintained under two different sets of in vitro conditions. Of the two, the combination of a Matrigel substrate and L2 cells in suspension probably approximated more closely the natural conditions for development and thus promoted the completion of sporogonic development in a larger proportion of the parasites. In the absence of Matrigel, mutual adhesion of ookinetes may have provided the necessary cue for development, albeit in a very small proportion of the parasites.

One approach for the control of malaria is the introduction into mosquitoes of genes that render them refractory to plasmodial infections. This strategy requires the identification of extrinsic factors involved in the critical developmental stages of the malaria parasite in the mosquito vector (*19*). This paper describes the development of *Plasmodium* in vitro from the blood form to the sporozoite under special conditions that may mimic the signals for development in the mosquito. The factors required for this development can now be more readily identified. Furthermore, the ability to study the development of sporozoites in vitro may help identify necessary components for a still elusive sporozoite vaccine.

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14. L2 cells were grown in Schneider's *Drosophila* medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (50 μg/ml), pH 6.8.
15. Oocyst-culture medium: RPMI 1640 (Gibco, catalog no. 320-1875) supplemented with 15% (v/v) heat-inactivated FBS, trehalose (2 g/liter), hypoxanthine (2 mg/liter), 10 mM Hepes, 1 to 2% (v/v) lipoprotein-cholesterol solution (ICN Biomedicals, Costa Mesa, CA; final concentration of cholesterol, 0.2 to 0.8 mg/ml), and gentamicin (50 μg/ml). The pH was adjusted to 7.0; 0.7 ml of medium was used per well (in 24-well cluster) or 3.0 ml per flask (25 cm^2).
16. Matrigel used in these experiments was produced by H. Kleinman (National Institute of Dental Research/NIH). An equivalent product is commercially available through Collaborative Research (Bedford, MA). Matrigel at 4°C was pipetted onto the bottom of the culture vessel [0.25 to 0.3 ml per well (24-well cluster), 0.8 to 1.0 ml per 25-cm^2 cell-culture flask—spread out over approximately 10 to 12.5 cm^2] and allowed to gel for 30 min at room temperature.
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Polymerase II Promoter Activation: Closed Complex Formation and ATP-Driven Start Site Opening

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Studies on bacterial RNA polymerases have divided the initiation pathway into three steps, namely (i) promoter binding to form the closed complex; (ii) DNA melting to form an open complex, and (iii) messenger RNA initiation. Potassium permanganate was used to detect DNA melting by mammalian RNA polymerase II in vitro. Closed complexes formed in a rate-limiting step that was stimulated by the activator GAL4-VP16. Adenosine triphosphate was then hydrolyzed to rapidly melt the DNA within the closed complex to form an open complex. Addition of nucleoside triphosphates resulted in the melted bubble moving away from the start site, completing initiation.

FOR INITIATION OF TRANSCRIPTION to occur, the duplex DNA that composes the start site must open (melt) to expose the nucleotides on the template strand. Melting of DNA requires energy and is a key event in regulation of transcription by bacterial polymerases (*1*), where the open complex containing melted DNA has become a centerpiece for regulatory studies. However, the open complex formed by RNA polymerase II (pol II), which synthesizes mRNA in eukaryotes, has not been unequivocally demonstrated.

Biochemical studies have detected intermediates in the pol II initiation pathway (*2, 3*), including a rapid start complex (*4*) and a phenanthroline-copper–sensitive complex (*5*). Melted DNA within the start site has not been detected in either complex. Both form in the absence of adenosine triphosphate (ATP) hydrolysis, which is required for initiation (*6*). The single-strand selective reagent potas-

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sium permanganate ($KMnO_4$) has been used to detect open complexes formed by bacterial polymerases (*7*), eukaryotic RNA polymerase III (*8*), and the mammalian vaccinia viral polymerase (*9*). Here we use this reagent to identify the pol II open complex.

We used templates composed of a truncated adenovirus E4 promoter that contained its TATA sequence and a variable number of synthetic upstream binding sites for the yeast transcriptional activator GAL4 (*10*). The transcriptional activator protein that we used contained the DNA binding domain of GAL4 fused to the potent transcription activation domain of the Herpes simplex virus protein VP16 (GAL4-VP16). The E4 promoter contains six consecutive thymines and an adenosine in the initiation region that can all be used as start sites (*11*) (Fig. 1). Because single-stranded thymines are the strongest DNA targets of $KMnO_4$, these six nucleotides should be highly reactive when the start site is opened.

We tested the effect of GAL4-VP16 on the permanganate attack pattern of the E4 promoter in the presence of HeLa cell extract and ATP (Fig. 1) (*12*). In the absence of GAL4-VP16, thymines in the region of the transcription start site reacted poorly

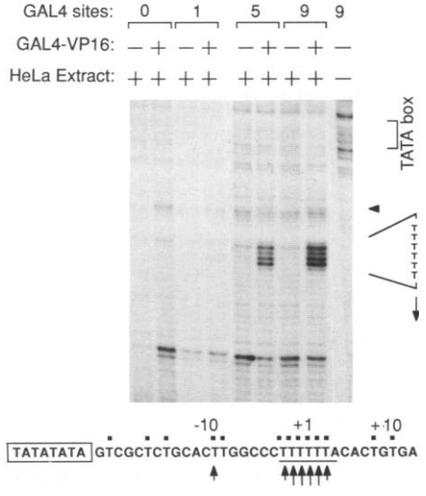
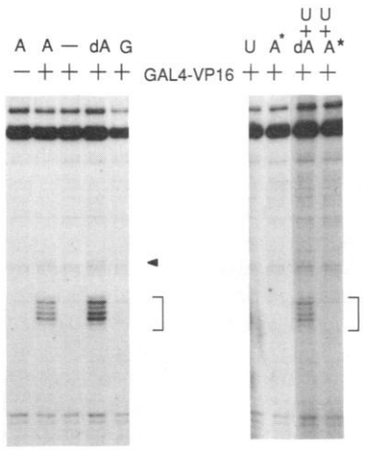


Fig. 1. Sensitivity to $KMnO_4$ of E4 promoters that contain various numbers of GAL4 binding sites. Templates bearing the indicated number of GAL4 binding sites were incubated in a HeLa cell extract with ATP and template-saturating amounts of GAL4-VP16 where indicated (+). The $KMnO_4$ modification sites near the start site and at position −10 (arrowhead) are indicated. The arrow shows the direction of transcription. Below, the DNA sequence of the sense strand of the E4 promoter is shown. The open box indicates the position of the TATA box. The underlined sequence is the region of the multiple start sites. The squares indicate the thymines near the start site region that could be potential targets of $KMnO_4$. The arrows indicate the residues that are hyperreactive to $KMnO_4$, with longer arrows representing greater reactivity.

Fig. 2. Requirement of ATP hydrolysis for open complex formation. The presence (+) or absence (−) of GAL4-VP16 is indicated on the top of each lane. The nucleotides added are indicated; abbreviations are A, ATP; dA, dATP; G, GTP; U, UTP; A*, AMP-PNP; −, no addition. The multiple start site region is marked with a bracket. The arrowhead indicates the T at −10.



with $KMnO_4$. These patterns are similar to those generated by attack on the template DNA alone or the template DNA with GAL4-VP16 and ATP but without extract (*13*). When GAL4-VP16 was added, the start site region of templates bearing multiple GAL4 binding sites became sensitive to $KMnO_4$ attack. The $KMnO_4$ sensitivity was evident with five GAL4 binding sites and increased with nine sites, but was undetectable with a single site. The increase in sensitivity to $KMnO_4$ with increased numbers of GAL4 binding sites paralleled the effect of the number of sites on the amount of transcriptional activation (*10*). The $KMnO_4$-sensitive residues corresponded to thymines located between −10 and +3 within the start site region of the E4 promoter, with the most sensitive residues centered between −2 and +2. We conclude that binding of GAL4-VP16 facilitates a process leading ultimately to DNA melting at the start site. By analogy with studies of prokaryotic polymerases (*7*), we refer to this as formation of an open complex.

ATP is required for transcription initiation by pol II (*6*), but the mechanistic step at which it acts is unclear. In order to determine whether ATP is required for open complex formation we measured $KMnO_4$ sensitivity in the absence of ATP or in the presence of other nucleotides (Fig. 2). Opening of the start site region, as evidenced by $KMnO_4$ hypersensitive sites, was observed in the presence but not in the absence of ATP; thus, ATP was required for open complex formation. Deoxyadenosine triphosphate (dATP), which can substitute for ATP in the transcription initiation reaction (*14*), also substituted for ATP in the formation of the open complex (*15*). Guanosine triphosphate (GTP) could not substitute for ATP in open complex formation. The formation of the first phosphodiester bond of the mRNA was prevented by omitting both uridine triphosphate (UTP) and cytidine triphosphate (CTP) in these experiments. Because the open complex can still be observed, phosphodiester bond formation is not required for DNA melting by pol II.

The nonhydrolyzable ATP analog 5′ adenylyl imidodiphosphate (AMP-PNP) cannot substitute for ATP during open complex formation (Fig. 2). The requirement for ATP hydrolysis was further emphasized by the lack of open complexes even under conditions (*16*) where the substrates for making a short transcript are present. We conclude that ATP hydrolysis is required to open the DNA strands prior to formation of the first phosphodiester bond.

To confirm that the open complex was functional, we added all four nucleoside triphosphates (NTPs) so that initiation and elongation could occur. Most of the pol II open complexes disappeared within 1 min after addition of NTPs (Fig. 3). We also

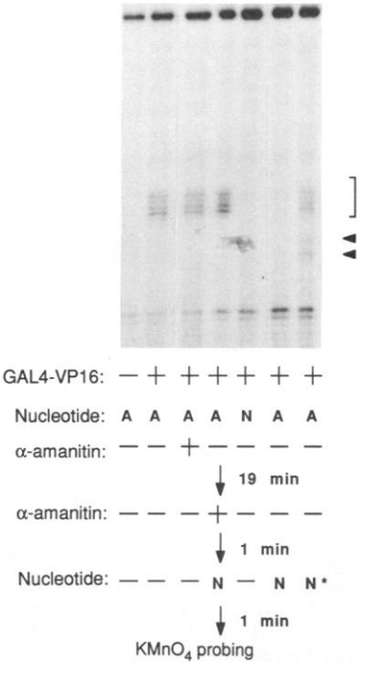


Fig. 3. Properties of the pol II open complex during initiation. The order of addition of various components and the presence (+) or absence (−) of the activator or α-amanitin is indicated below each lane. The abbreviations for nucleotides are A, ATP; N, all four NTPs; N*, 3′-*O*-methyl-GTP plus ATP, CTP, and UTP; −, no addition. The multiple start site region is marked with a bracket. The arrows indicate the residues at +8 and +10, which are hyperreactive to $KMnO_4$.

replaced the normal elongation substrate GTP with the chain terminator 3′-*O*-methyl-GTP, which should block elongation at +9. This caused two thymines at +8 and +10 to become hyperreactive to $KMnO_4$, while at the same time the start site thymines became less reactive. The results are consistent with the transcription bubble moving downstream with the polymerase during elongation, which is analogous to the properties of bacterial RNA polymerase (*7, 17*). This movement was blocked by the elongation inhibitor α-amanitin at a low concentration that was sufficient to inactivate only pol II. The open complex is typically short-lived (*7*) and cannot be detected unless initiation is blocked.

The assembly of general transcription factors on the template is believed to be slow, whereas the steps that require NTPs are fast (*4, 18*). In these experiments it takes about 20 min for the open complex to form fully. To learn where GAL4-VP16 and ATP participate in the initiation pathway, we performed kinetic experiments (Fig. 4). When both activator and ATP were added at the beginning of the 22-min incubation, $KMnO_4$ hyperreactive sites were observed within the start site region. We next added GAL4-VP16 at the beginning of the reaction, incubated the mixture for 20 min, and then added ATP for 2 min before probing with $KMnO_4$. The presence of hypersensitive sites indicated that ATP need not be present throughout the incubation, but, rather, functions late in the assembly pathway in the rapid opening of the DNA strands in the complex. When ATP was added for the first 20 min and GAL4-VP16 for the final 2 min, sensitivity to $KMnO_4$ was not observed. This indicates that GAL4-VP16 must be present at the beginning of the incubation and, therefore, participates in the slow 20-min step, which we infer to be assembly of a closed complex. No $KMnO_4$ sensitivity was observed when the template and HeLa extract were incubated for the first 20 min in the absence of activator and ATP, and then both GAL4-VP16 and ATP were added for the last 2 min. The binding of GAL4-VP16 to DNA is not rate-limiting, as it occurred within 2 min (*13*).

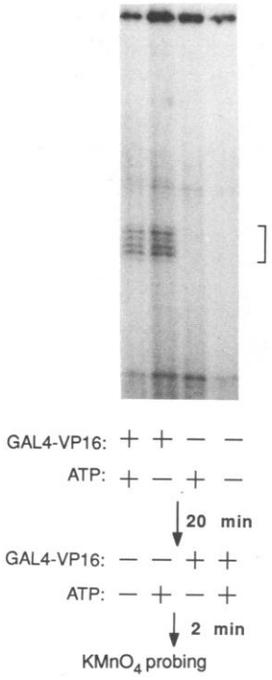


Fig. 4. Kinetics of open complex formation. The protocol and the presence (+) or absence (−) of GAL4-VP16 and ATP in the reaction mixture is indicated below each lane. The multiple start site region is marked with a bracket.

We propose the following pathway for activation of transcription at the E4 promoter by GAL4-VP16. First, the activator binds to the DNA and promotes formation of a closed transcription complex. This is the slow rate-limiting step in the assembly pathway. In a subsequent rapid step, the β-γ phosphoanhydride bond of ATP is hydrolyzed to melt the start site DNA. Next, the polymerase uses nucleotides complementary to the template strand to initiate rapidly and copy the melted DNA. During elongation the melted bubble accompanies pol II down the DNA. This is followed by the closing of the DNA around the start site, requiring reopening in subsequent rounds of transcription. This description is consistent with studies of pol II activation (*19*), where activators function prior to the step shown here to require hydrolysis of ATP.

Our results imply that transcription complexes generated by binding of TFIID, IIA, IIB, pol II, and IIE/F are all closed complexes, as they form in the absence of ATP (*3, 4, 20*). ATP changes the electrophoretic mobility of complexes that contain a complete complement of these factors (*3*). We suggest that these factors are bound to the DNA in a closed complex that is opened by ATP. This closed complex may be analogous to the phenanthroline-copper–sensitive complex that forms at the adenovirus major late promoter in the absence of ATP (*5*). We suspect that the promoter DNA upstream from the start site undergoes a conformational change prior to opening within this closed complex because phenanthroline detects a broad range of conformational changes (*21*) and because the start site is not hyperreactive.

The pathway described here for pol II is reminiscent of those of bacterial promoters that use the factor σ^{54} (*22, 23*) and promoters that respond to the T4 replication enhancer (*24*). In all three systems transcription can be stimulated by activators bound at close or distant positions, and all require ATP hydrolysis to form open complexes (*6, 22–24*). In contrast, promoters that utilize pol III or the factor σ^{70} cannot be activated at a distance and do not require ATP hydrolysis to melt the DNA (*1, 7, 23*), suggesting that there may be two distinct classes of mechanisms for transcription initiation that cross the eukaryotic-prokaryotic boundary (*23*).

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DNA Binding Activity of Recombinant SRY from Normal Males and XY Females

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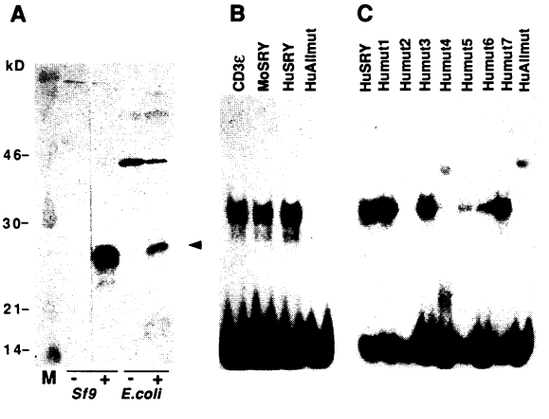
The protein encoded by the human testis determining gene, *SRY*, contains a high mobility group (HMG) box related to that present in the T cell–specific, DNA-binding protein TCF-1. Recombinant SRY protein was able to bind to the same core sequence AACAAAG recognized by TCF-1 in a sequence dependent manner. In five XY females point mutations were found in the region encoding the HMG box. In four cases DNA binding activity of mutant SRY protein was negligible; in the fifth case DNA binding was reduced. These results imply that the DNA binding activity of SRY is required for sex determination.

THE Y CHROMOSOME IS A DOMINANT inducer of male sex determination in mammals (*1*). In the absence of the Y chromosome, fetal genital ridges develop as ovaries; in the presence of the Y chromosome, the genital ridges develop as testes (*2*). The position of the Y-located gene responsible for inducing testis formation was deduced from the study of the genomes of individuals with two X chromosomes but with testicular development (*3*). In four cases, the sex-reversed individuals had inherited less than 40 kb of Y chromosomal DNA, and in this region we identified and cloned the gene *SRY* (sex-determining region Y gene) (*4*). *SRY* and the mouse homolog *Sry* (*5*) have many of the predicted properties of the testis-determining gene (*6–9*).

By analogy with the genetic control of other developmental processes, it would be predicted that *SRY/Sry* is part of a pathway of genes that control sex determination. The existence of this pathway can also be deduced directly: (i) the induction of *Sry* transcription at day 10.5 postcoitum implies the existence of "upstream" regulatory genes (*9*); and (ii) the testicular differentiation found in some XX males, in the absence of any Y-derived sequences, can best be explained by gain-of-function mutations in "downstream" genes (*4*). Similarly, many other sex-reversing mutations in both humans and mice must be affecting genes other than *SRY/Sry* in the sex determination pathway (*10*).

Regulatory genes can exert their control by different mechanisms. In *Drosophila*, although the sex determination pathway is set by transcriptional regulation, the mechanism for transferring information between genes in the pathway is posttranscriptional; in nematode sex determination, transcriptional regulation is the main method used (*11*). The sequence of *SRY/Sry* suggests that it may encode a DNA binding protein, and this would be consistent with a role as a transcription factor. The protein product of *SRY/Sry* contains an 80–amino acid sequence motif, which is found in several proteins with known or suspected DNA



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Element	Probe sequence	DNA binding
CD3ε	ACACTGAG AACAAAG CGCTCTCACAC	+++
MoSRY	GTGCTTGT AACAAAG ATTTTAT	+++
HuSRY	GTTAACGT AACAAAG AATCTGGTAGA	+++
Humut1	..... C.....	+++
Humut3	....G....	+++
Humut7	....T	+++
Humut2	..... .C.....	+/-
Humut4	....C...	+/-
Humut5	....G..	+/-
Humut6	....G.	+/-
HuAllmut	..... CCGCGGT	-

Fig. 1. Identification of SRY as a sequence-specific DNA binding protein. (**A**) Western blot analysis of human SRY expressed in *E. coli* and in insect cells (*19*). Soluble extracts from Sf9 insect cells infected with baculovirus expressing β-galactosidase (−) or human SRY (+) and from induced *E. coli* cultures transformed with plasmid pJLA503 (−) and pJLA503-huSRY (+) were analyzed by Western blotting after SDS–polyacrylamide gel electrophoresis and probed with an anti-SRY rabbit serum. M: Prestained markers with molecular weights in kilodaltons as indicated. The arrow indicates the position of SRY, which migrates as a 24/25-kD doublet (predicted molecular weight, 23.9) from baculovirus-infected Sf9 cells and as a single band at 25 kD from bacteria. The doublet appears to be caused by an insect cell modification of the SRY protein. (**B**) DNA binding of SRY to the sequence AACAAAG as measured by electrophoretic mobility shift assay (*20*). *Escherichia coli* extracts containing SRY were incubated with 29-bp ^{32}P-labeled oligonucleotides probes containing the sequence AACAAAG or with a negative control, HuAllmut, where the AACAAAG in the human *SRY* probe has been replaced with CCGCGGT. (**C**) Electrophoretic mobility shift assay of SRY and probes with single point mutations across the AACAAAG sequence. (**D**) Summary of DNA-binding specificity of SRY to the DNA elements used as probes for DNA binding assays shown in (B) and (C). Dots indicate sequence homology to the human *SRY* probe. Binding of at least 30% of the DNA added to the reaction was judged as strongly positive (+++); weak, but detectable binding was recorded as +/−.

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