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Biochemistry

Transcription of adenovirus cores *in vitro*: Major RNA products differ from those made from a DNA template

(chromatin protein/RNA polymerase/DNA–protein complex)

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ABSTRACT **Intact cores of adenovirus type 5 were transcribed *in vitro* in extracts derived from uninfected HeLa cells, and the products were analyzed by hybridization to restricted viral DNA. Predominant transcripts from cores were distinctly different from transcripts generated from deproteinized DNA. The extracts contained RNA polymerases II and III. Transcription from cores was completely inhibited by low levels of α-amanitin, and there was little or no hybridization of products to the region of the genome encoding the small polymerase III-dependent virus-associated RNA that was a major transcript from the DNA template. The major transcripts from cores hybridized to the rightward-transcribed strand of the region between 75 and 89 map units, early region 3 of the adenovirus genome. There was several-fold more transcription of this region in cores than of the region downstream from the major late promoter, which predominates among polymerase II transcripts from DNA.**

The transcription of group C human adenoviruses types 2 and 5 (Ad2 and Ad5) has been studied in detail. There are five major early transcription units that function primarily in the early phase of infection (before the onset of DNA replication) and are activated sequentially within that phase. Each has a separate promoter, and one or more mRNAs are spliced from primary transcripts (for review, see ref. 1). Early region 1A (E1A), located in the leftmost 4.5% of the genome, seems to control expression of regions E1B, E2, E3, and E4 (2–4) and has been designated "pre-early." Additional early transcripts have been identified including some "immediate early" products that seem independent of E1A control (5, 6). Late in infection, one major transcription unit extending rightward from a promoter at 16.75 map units nearly to the end of the genome is active. At least 16 different mature messages arranged in five 3′-coterminal families are produced from this transcription unit by polyadenylation and splicing of a large precursor (1). The relative amounts of different late messages are determined by these processing events, rather than at the level of initiation of transcription.

Control of the switch from the early to the late phase of viral gene expression is not well understood. The shift to expression of late genes is coupled to DNA replication and, in an elegant series of superinfection experiments, it has been shown that replication of a given DNA molecule is necessary for late gene expression from that molecule (7). Considerable evidence has been presented that shows that the major late (ML) promoter is active early in infection, with mRNA production being controlled by RNA processing and attenuation of transcription. The activity of the late promoter early in infection is similar to that of the other early promoters, but early transcripts from this region terminate at position 60–70 instead of at 99 map units and one 3′-coterminal family of late messages is expressed in the cytoplasm. The transcription rate in the late transcription unit increases relative to early transcription units as infection proceeds (8–13).

When full-length adenoviral DNA is transcribed in a cell-free system, the predominant polymerase II-dependent transcription is from the late promoter (14, 15); a number of the early promoters also function (15, 16). The relationship between efficiencies of promoters *in vitro* and control of RNA synthesis *in vivo* is not clear, since deproteinized DNA may well lack important regulatory signals. It is therefore of interest to see whether use of viral DNA–protein complex as template has an effect on transcription.

Infecting viral DNA is closely associated with virally coded core proteins (17–19). The major core protein, polypeptide VII, is a small arginine-rich protein with an NH_2-terminal basic domain similar to that of histones (20, 21). The total mass of this protein and two other minor core proteins in each virion is about equal to the mass of viral DNA. A portion of infecting viral DNA assumes a nucleosomal structure similar to that of cellular DNA shortly after infection (22–24), but it is not clear whether some components of the cores may be associated with these structures or whether they are templates for early transcription.

In this paper, we report the results of transcription of intact viral cores, showing that they are efficient templates and that the predominant transcripts are different from those resulting from transcription of deproteinized DNA.

MATERIALS AND METHODS

Preparation of Viral Cores and DNA. Ad5 cores were prepared by treatment of virus particles with pyridine as described (18). Virus particles were extracted from infected cells and purified by cesium chloride centrifugation as described (25). Gradient buffers contained no EDTA. Virions were dialyzed against 0.25 M sucrose/0.02 M Tris base/1 mM $MgCl_2$, pH 7.4/0.5% butanol at 4°C (18) and stored in this buffer. Before core preparation, they were dialyzed extensively against 5 mM Tris base (pH 7.5). About 400 μl of virions (500–700 μg of DNA; estimated by A_{260} in 0.1% $NaDodSO_4$) and 3×10^5 cpm of [^{3}H]thymidine-labeled virion purified in the same way were treated with 10% pyridine for 10–20 min at room temperature until the opacity of the solution decreased markedly. Virions in pyridine were layered onto 10–25% sucrose gradients (in 5 mM Tris base, pH 7.5/0.2 mM phenylmethylsulfonyl fluoride) and centrifuged for 1 hr at 29,500 rpm in an SW 41 rotor at 4°C. Fractions were collected through the bottom of the tube, and aliquots were assayed for radioactivity. The concentration of the peak fraction was measured as described for virus particles, and

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Abbreviations: Ad2 and Ad5, adenoviruses types 2 and 5; E1A, E2, etc., early regions 1A, 2, etc.; ML, major late (promoter); VA, virus-associated (RNA).

cores were used as transcription templates within 2 or 3 hr unless otherwise specified. Cores thus prepared contain virion proteins VII and V bound to intact viral DNA as determined by $NaDodSO_4$/polyacrylamide gel electrophoresis. These two species comprised >90% of the protein in these preparations and were present in the same molar amounts as in whole virions. Small amounts of capsid proteins including hexon were detected as described (18, 19). The level of these minor contaminants was minimized by taking only the peak fraction of the sucrose gradients; higher fractions contained more noncore proteins. DNA was prepared from virus particles or from cores as described (25).

Cloned DNA, Hybridization Assays, and Enzymes. Cloned fragments of Ad5 DNA (Ad5 *Hin*dIII b; Ad5 *Sma* F; Ad2 *Hin*dIII c) were the gift of Arnold Berk. Restriction enzymes *Xho* I and *Hin*dIII were purchased from Bethesda Research Laboratories, and *Eco*RI was the gift of David Gelfand. Restricted viral DNA or cloned fragments were subjected to electrophoresis through agarose and transferred to nitrocellulose by standard procedures (26). Hybridization of RNA to filters was carried out in 50% formamide/0.90 M NaCl/0.09 M Na citrate/0.2% bovine serum albumin/0.2% polyvinylpyrrolidine/0.2% Ficoll at 42°C for 16 hr. For the experiment determining polarity of separated strands of a restriction fragment, the large fragment of DNA polymerase I (27) was purchased from New England Nuclear and used according to supplier's specifications with [α-^{32}P]dATP and [α-^{32}P]dGTP.

Transcription Extracts and Reactions. Extracts of uninfected HeLa cells were provided to us by David Dignam and Robert Roeder (extract 1) and by Phillip Stuart (extract 2). Extract 1 is a high-salt extract of isolated nuclei developed by D. Dignam (personal communication); the behavior of this extract with respect to transcription of a variety of cloned adenovirus promoters is the same as that of previously described extracts (14, 16). Extract 2 was prepared by the method of Manley *et al.* (15). Both extracts were stored in small portions at −70°C and quick frozen in dry ice/ethanol after each use. Reaction mixtures were 50% extract/7.5 mM $MgCl_2$/cores or DNA/600 μM unlabeled ATP/CTP/UTP/50 μM [^{32}P]GTP (ICN; 20–25 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), which was dried down and suspended in H_2O with the unlabeled triphosphates. Extract was added last, and reactions carried out at 32°C for 1–1.5 hr. Reactions were stopped and RNA was extracted as described (14) followed by three or four rounds of ethanol precipitation, one of which was for 16 hr at −20°C.

RESULTS

Transcription of Cores Yields Predominantly E3 Transcripts. To analyze and compare the products of transcription from a variety of templates conveniently, we hybridized labeled RNA synthesized *in vitro* to restriction fragments of adenovirus DNA. Intact viral cores are crucial to the design of the experiment and must be compared with full-length viral DNA in their template function. Therefore we could not use the techniques of run off transcription from restricted DNA used by others to map promoter sites (14–16).

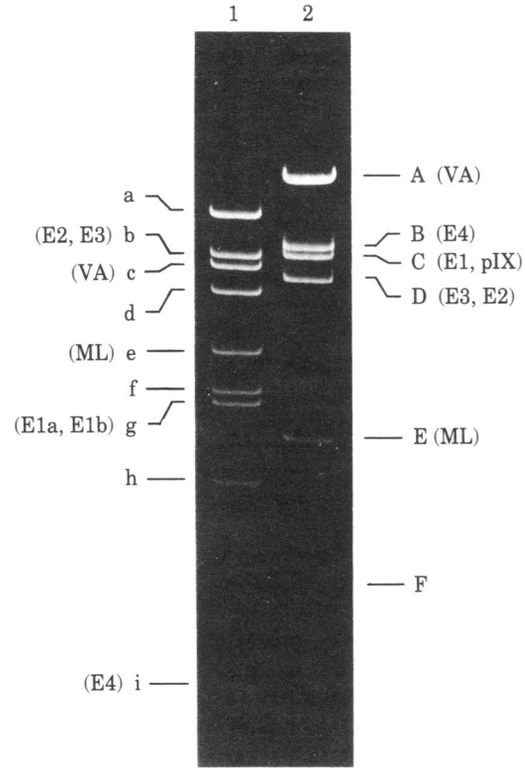

FIG. 1. Ad5 DNA was cleaved with *Hin*dIII (lane 1) or *Xho* I (lane 2) and 0.5 μg of each sample was subjected to electrophoresis through 10-cm 1.2% agarose gels at 20 V for 15 hr. Such gels, and gels that were subjected to electrophoresis for more volt-hours to better separate the higher molecular weight bands, were transferred to nitrocellulose filters for the hybridizations shown in Fig. 3. Designations in parentheses indicate genomic regions whose promoters would be expected to yield transcripts hybridizing to each fragment.

Cores freshly prepared by pyridine treatment and sucrose gradient centrifugation were added to reaction mixtures in ≈18% sucrose. Appropriate amounts of sucrose in gradient buffer were added to DNA-primed reaction mixtures to ensure that only the template differed between two reactions. The total amount of GTP incorporated in a reaction mixture was 2–10 pmol for reactions with either DNA or core template.

Extracted precipitated RNA was hybridized to *Xho* I and *Hin*dIII digests of Ad5 DNA immobilized on nitrocellulose filters by Southern blotting. The results are shown in Figs. 1 and 2, which indicate the promoters present on key fragments; the

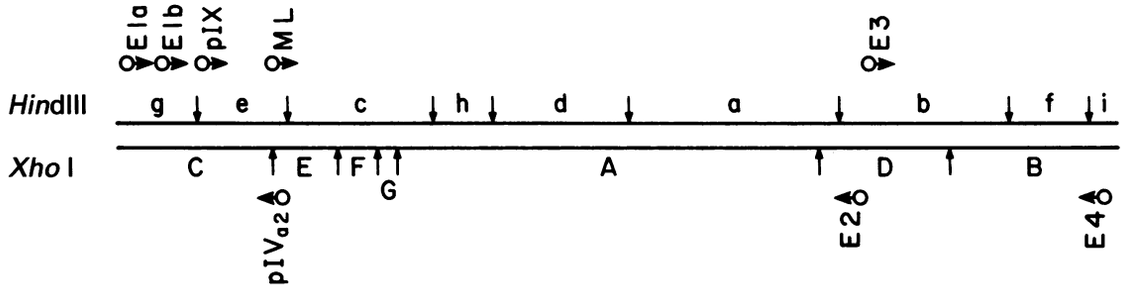

FIG. 2. Map of the Ad5 genome. ↑, Restriction site; O►, promoter.

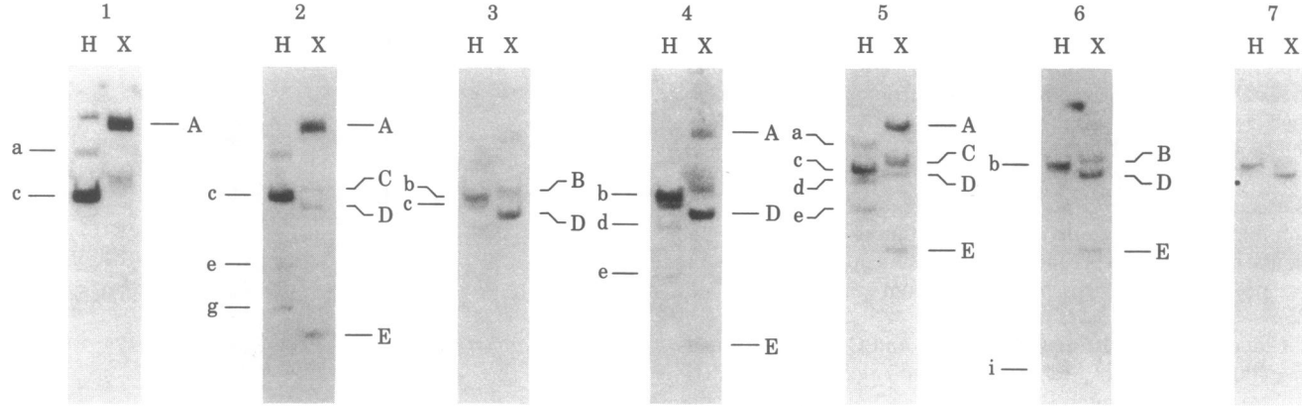

FIG. 3. Hybridization of *in vitro*-synthesized RNAs to blots of *Hin*dIII (H) and *Xho* I (X) digests of Ad5 DNA. The probe was transcribed with extract 1 (*1–4*) or extract 2 (*5–7*). (*1*) DNA at 80 μg/ml. (*2*) DNA at 40 μg/ml. (*3*) Cores at 40 μg/ml. (*4*) Cores at 40 μg/ml heated to 70°C for 10 min before addition to the transcription reaction mixture. (*5*) DNA at 60 μg/ml. (*6*) Cores at 60 μg/ml. (*7*) Cores at 30 μg/ml.

results for several representative transcription reactions are shown in Fig. 3. Restriction fragments were subjected to electrophoresis for different amounts of time to better resolve large fragments (Fig. 3-*1* to 3-*4*) or to include small ones on the gel (Fig. 3-5 to 3-*7*), so each set of filters is labeled to indicate the position of key fragments.

The first observation is that transcripts of cores (Fig. 3-*3*, -*6*, and -*7*) hybridize very little to the *Hin*dIII c and *Xho* I A fragments, which encode the small virus-associated (VA) RNAs and are hybridized strongly by DNA-derived RNA (Fig. 3-*1*, -*2*, and -*5*). Furthermore, hybridization of core transcripts is largely restricted to the *Hin*dIII b and *Xho* I D fragments, which are not among the dominant ones hybridized by DNA-derived transcripts.

Transcription of DNA and of cores was carried out in the presence of α-amanitin at 100 μg/ml, a concentration that inhibits polymerase II but not polymerase III. No hybridizing RNA was synthesized from core template in such a reaction. RNA made from DNA template in the presence of α-amanitin hybridizes only to the *Hin*dIII c and *Xho* I A fragments, confirming that most of the signal in those fragments is due to VA RNA (data not shown).

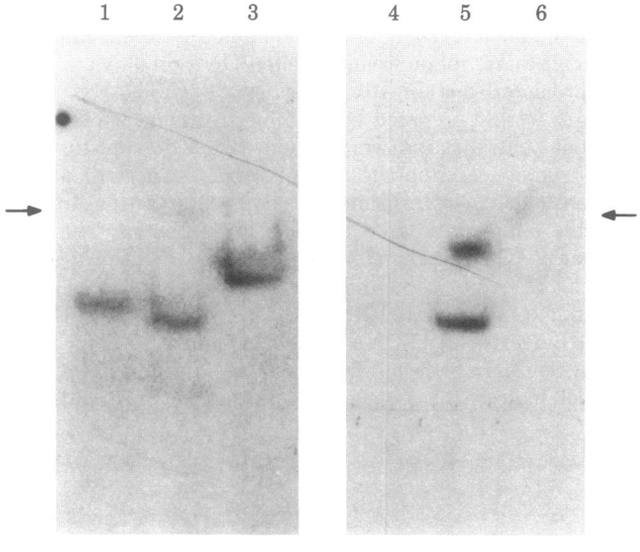

FIG. 4. Hybridization of *in vitro* RNAs to cloned fragments of Ad5. Lanes: 1 and 4, Ad5 *Sma* F (map position 2.8–11.1); 2 and 5, Ad5 *Hin*dIII B cleaved with *Eco*RI, generating three fragments [position 72.8–75.9, attached to plasmid DNA (→), position 75.9–84 (center band, prominent in lanes 2 and 5), and position 84–89.1]; 3 and 6, Ad2 *Hin*dIII c, position 7.9–17.0, corresponding exactly with the position of Ad5 *Hin*dIII e. The probes are RNA transcribed from DNA template (lanes 1–3) or from core template (lanes 4–6). In lane 5, the prominent but shorter upper "band" is due to an inadvertant concentration of triphosphates. There is no DNA at that position.

DNA-primed reactions vary more with concentration of template than do core-primed reactions. At concentrations ≤15 μg/ml (OD_{260}), cores did not yield any transcripts detectable in our assays, but the map position of the transcripts did not vary in reactions from 25–80 μg/ml. Lee and Roeder (16) demonstrate different concentration optima for different transcripts when restricted whole viral DNA is used as template. Consistent with their results, we note that the amount of ML promoter hybridization (*Xho* I E) relative to VA RNA is greater for DNA at 40 μg/ml than for DNA at 80 μg/ml (Fig. 3-*1* and -*2*).

The *Hin*dIII b and *Xho* I D fragments contain promoters for E2, transcribed leftward from 74.9 map units, and E3, transcribed rightward from 76.5 map units. To distinguish whether the transcripts detected corresponded to only one or to both of these regions, the *Hin*dIII b fragment was treated with *Eco*RI, which cleaves Ad5 DNA at position 75.9, between the E2 and the E3 promoters. Fig. 4 shows that core-derived RNA hybridizes only to the fragment at position 75.9–84, the fragment clearly hybridized in lanes 2 and 5. The absence of hybridization to a larger fragment, which contains sequences at position 72.8–75.9 attached to plasmid DNA, indicated that there is little transcription from the E2 promoter.

To determine the direction of transcription, RNA from cores was hybridized to separated strands of the *Hin*dIII b fragment. Fig. 5 shows that RNA hybridizes only to the rightward coding strand of this fragment. [Fig. 5*c* illustrates the method of assignment of the fast strand as rightward coding. This agrees with assignments by Flint *et al.* (28) for other fragments in this region of the genome.] Thus the transcripts are complementary to the same region and strand as the normal E3 transcripts.

Other Regions Transcribed From Core Template. Low-level hybridization of core-derived RNA to other regions is observed after extended exposure of films. *Xho* I B and *Hin*dIII i, representing the right-hand end of the genome, give the signal next in strength to the *Xho* I D hybridization. There is no hybridization to *Hin*dIII f, which can be explained if transcription from *Hin*dIII i is leftward from the E4 promoter, terminating before position 97, which is the junction of the *Hin*dIII f and *Hin*dIII i fragments. The *Xho* I B signal is probably due to both hybridization of the RNA to *Hin*dIII i and E3 transcripts

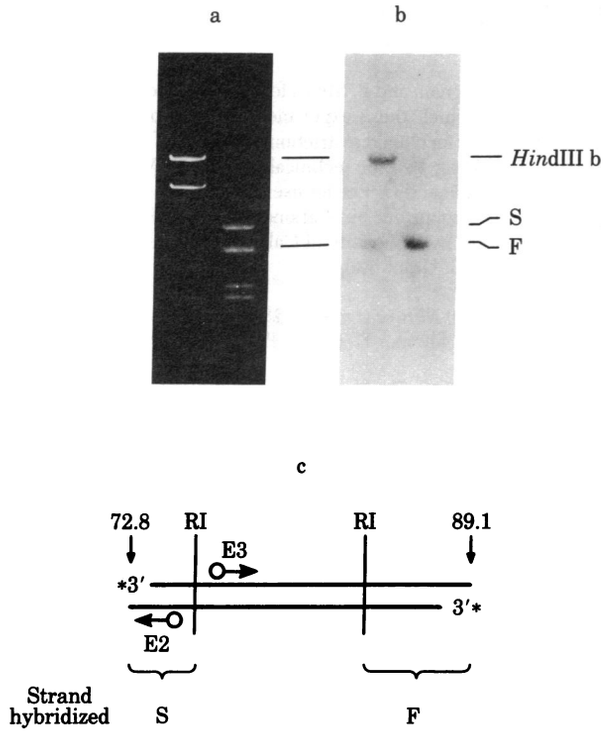

FIG. 5. Hybridization of *in vitro* RNA from core template to separated strands of the Ad5 *Hin*dIII B fragment. (*a*) Ad5 *Hin*dIII B cloned in plasmid pBR322 was cleaved with *Hin*dIII and subjected to electrophoresis as native DNA (*Left*) or after denaturation (*Right*). Restricted DNA was extracted with phenol/chloroform, precipitated with ethanol, and suspended in 1 mM EDTA; 0.5 μg of DNA was denatured with 0.1 M NaOH for 5 min at room temperature; dye-sucrose was added; and the sample was immediately loaded onto a 1% agarose gel and subjected to electrophoresis for 10 min at 100 V and then for 4 hr at 40 V. S and F, slow and fast strands, respectively, of the *Hin*dIII B fragment. (*b*) The DNA bands, shown in *a* were transferred to nitrocellulose and hybridized with total RNA derived from core template *in vitro*, and the filter was autoradiographed. (*c*) Schematic drawing of the experiment, showing the assignment of F and S strands as rightward and leftward coding. The *Hin*dIII B fragment was labeled at the 3' end of both strands by repair of the staggered ends of the *Hin*dIII cleavage sites with DNA polymerase (large fragment). The end-labeled fragment was cleaved with *Eco*RI, yielding two labeled fragments and one unlabeled fragment. The larger (righthand) labeled fragment will hybridize to the rightward coding strand, as the 3'-end label at position 89.1 is in the leftward coding strand. Likewise, the smaller fragment, labeled at position 72.8 in the rightward coding strand, will hybridize only to the leftward coding strand. The results confirm that the F strand is rightward coding.

that extend past position 86 (*Xho* I D/B junction).

The fragments containing ML promoter are *Xho* I E and *Hin*dIII e. These are hybridized strongly by DNA-primed transcripts and perceptibly but less strongly by transcripts from cores. *Hin*dIII e also contains the pIX promoter, which is about one-third as active *in vitro* as the ML promoter when DNA is transcribed (16).

While it is difficult to quantitate the data from the type of analysis presented here, estimates of the relative efficiencies of transcription of two different regions of the viral genome can be made. To compare the amount of transcription in E3 with that in the region of the ML promoter, numerous autoradiographs of the type shown in Fig. 2 were traced on a Joyce–Loebl densitometer, and intensities of bands at *Xho* I D and *Xho* I E were measured. Of fragments used in our analysis, *Xho* I E is the most likely to hybridize only transcripts originating at the ML region. This fragment begins 4.4 map units downstream from the pIX poly(A) addition site (5.8 units from the pIX promoter), so only extensive runoff transcription from pIX would yield hybridizing material. The promoter for IV_{a2} also lies within *Xho* I E, but this promoter is probably not active in these extracts (ref. 16; R. Roeder, personal communication). As shown here, *Xho* I D hybridization represents only E3 in core-derived RNA and predominantly E3 in DNA-derived RNA. If hybridization to *Xho* I E and D represented only ML and E3 transcription, respectively, 1,925 base pairs of *Xho* I E would be available for hybridization to ML transcripts and 2,450 base pairs of *Xho* I D would be available for hybridization to E3 transcripts. The results of gel traces, with values corrected for length of hybridizable sequence, are presented in Table 1. If some of the *Xho* I E hybridization is runoff from the pIX promoter, the calculated amount of ML transcription will be overestimated. Although this is a rough quantitation involving several assumptions, it is nonetheless clear that E3 is several-fold more actively transcribed in cores than is the region downstream from the ML promoter, while the latter region predominates in DNA transcription. The relative efficiency in DNA of the E3 and presumed ML promoter arrived at by these procedures (0.55) is in good agreement with that measured by more precise methods (16).

Table 1. Relative hybridization (*Xho* I D vs. *Xho* I E)

Template	D/E	(D*/E*)†
Cores	6	4.7
DNA	0.7	0.55

Results are in arbitrary units determined by measuring areas under peaks in densitometer tracings. Results for cores are the mean of tracing 10 autoradiograms from five different transcriptions; those for DNA are the mean of six autoradiograms from four different transcriptions.

† Values corrected for numbers of base pairs between the presumed active promoter and the downstream end of the fragment. D*, 2,450 base pairs; E*, 1,925 base pairs.

Hybridization to cloned restriction fragments of DNA provides a more sensitive blot assay for certain regions of interest because greater quantities of a given fragment can be subjected to electrophoresis and blotted. In Fig. 4, hybridization to *Sma* F (2.8–11.1 map units), *Eco*RI-cleaved *Hin*dIII b (72.8–75.9, 75.9–84, 84–89.1), and Ad2 *Hin*dIII c (7.9–17, corresponding to Ad5 *Hin*dIII e) is shown. The *Sma* F fragment, which contains both the E1b and the pIX promoters, is at least as well represented as E3 in transcripts from DNA (compare lanes 1 and 2) but is not hybridized to a detectable level by transcripts from cores (lane 4). A strong signal is likewise seen for DNA-derived transcripts hybridizing to the fragment containing the ML and pIX promoters (lane 3), and a faint signal is seen for core RNAs (lane 6).

Cores disrupted by heating (70°C for 10 min in sucrose) before transcription yield additional transcripts. Consistently, transcripts corresponding to the VA regions appear, while E3 remains a predominant transcript (Fig. 3-4). Hybridization to *Hin*dIII i seemed to be enhanced relative to the ML region in transcriptions of some preparations of disrupted cores. Cores are stable in their behavior as transcription templates for at least 2 days (stored at 4°C in sucrose).

DISCUSSION

We have shown that intact adenovirus cores serve as templates for transcription *in vitro* by uninfected cell extracts and that the selection of regions most actively transcribed is different than that observed in transcription of DNA. The absence of tran-

scription of the VA genes by polymerase III is striking. These genes fail to be transcribed in various early viral mutants and in nonpermissive cells in which DNA replication is blocked (ref. 4; unpublished results), and a role in splicing of late messages has been proposed for the VA RNA (29, 30). Because polymerase III is so active *in vitro*, VA RNA transcripts predominate in transcription of deproteinized adenoviral DNA. Clearly, the VA genes are not available for transcription in cores. Either the DNA is folded in such a way that these genes are sequestered or the particular conformation of core proteins blocks initiation by polymerase III in this region. It is possible that the core proteins may be phased with respect to DNA sequence. When cores are subjected to restriction endonuclease digestion, not all sites are equally susceptible to cleavage (ref. 31; unpublished results). This is consistent with either phasing or a specific folding of the DNA.

The block does result from core structure, not simply from the presence of core proteins in the mixture. After disruption of cores by heating, transcripts hybridizing to this region begin to appear (Fig. 3-4) and, when DNA is mixed with cores in a single reaction, the VA genes are transcribed (data not shown).

The relative amount of transcription in regions transcribed by polymerase II is also altered in cores. The predominance of strand-specific transcription in E3 of cores is striking, but its relationship to control of adenoviral gene expression *in vivo* is unclear. E1A is the first of the major early regions to be transcribed (in the first hour after infection), and transcription of E1B, E2, E3, and E4 are dependent on E1A expression (5–7, 32). Immediate early products, independent of E1A control, are a 13.5-kilodalton protein encoded between 17 and 21.5 map units and a 52–55-kilodalton protein encoded in the region of the ML transcription unit, which is transcribed early (3). The low-level transcription of the ML promoter region in cores *in vitro* is similar to immediate early expression *in vivo*, but the greater transcription of E3 than of any other region is not. The strand specificity of transcription in E3 suggests that it is accurately initiated. However, the E3 promoter may simply be the most exposed in core structure, allowing it to be transcribed *in vitro*, although it is dependent on E1A expression *in vivo*. The lack of transcription from the adjacent (leftward) E2 promoter may not be relevant to the question of exposure of this region, as E2 is not transcribed in deproteinized DNA either, presumably because it lacks a Goldberg–Hogness box (16). It has been shown that the left end of viral DNA is essential for packaging (33, 34). The predominance of E3, perhaps E4, transcription may reflect an "outside" position of the right end of the genome in core structure.

Clearly, precise mapping of transcripts from cores is required. It is also important to establish how the length of transcripts generated compares with those generated from DNA. Manley *et al.* (15) have detected elongation of transcripts >4,400 bases long. Our preliminary data on E4 suggest that, if there is specific initiation in that region, it is truncated before map position 97 (the *Hin*dIII i/f boundary), which would make it <700 base pairs.

The most exciting aspect of this work is that it provides an example in which chromatin-like proteins of a transcription template alter the products formed. This is an important complement to the characterization of factors that promote accurate initiation by polymerase II (35, 36). In studies of 5S genes transcribed by polymerase III, transcription factors have been shown to bind to specific DNA control regions (37, 38); the interaction of these with chromatin templates is of great interest. Further analysis of transcripts, and manipulation of the adenovirus cores that serve as templates, will be useful in studying polymerase–template interactions, and shedding light on early events in adenovirus infection.

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