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Interrelationship Between Poly(ADP-Rib) Synthesis, Intracellular NAD Levels, and Muscle or Cartilage Differentiation from Mesodermal Cells of Embryonic Chick Limb

(developmental gradient/intracellular pools/chromatin/nicotinamide/3-acetylpyridine)

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ABSTRACT **Mesodermal cells of embryonic chick limbs have the capacity to differentiate into either muscle or cartilage. Previous reports from this laboratory show a correlation between pyridine nucleotide levels and this differentiation, and thus suggest that fluctuations in the cellular NAD levels play a role in the control of muscle versus cartilage development. Poly(ADP-Rib) is chromatin-associated and forms from the polymerization of NAD with the excision of nicotinamide. The studies reported here show that: (*A*) the rate of net synthesis of poly(ADP-Rib) is correlated with the differentiation of chondrogenic cells from stage 24 limb mesodermal cells grown *in vitro;* (*B*) inhibition of chondrogenic expression caused by exposure to nicotinamide or BrdUrd is correlated with maintenance of basal levels of poly(ADP-Rib) synthesis, and this inhibition is dependent on the concentration of nicotinamide or BrdUrd exogenously supplied; (*C*) potentiation of chondrogenic expression caused by exposure of limb mesodermal cells *in vitro* to 3-acetylpyridine is correlated with stimulation of the rate of poly(ADP-Rib) synthesis if corrected for the specific activity of the ATP pool or compared to untreated cultures on a per cell basis; (*D*) isolated chromatin from mesodermal cells has the enzymatic machinery for synthesizing poly(ADP-Rib); (*E*) this machinery is inhibited by nicotinamide, thymidine, and 3-acetylpyridine; and (*F*) newly synthesized poly(ADP-Rib) is either associated with a discrete fraction of chromatin or is completely extracted from chromatin by the high column salts, which result in an aggregation with eventual elution at the exclusion volume position of the agarose column. Taken together, these observations provide a possible explanation for how fluctuations in cellular NAD levels can communicate with or be "sensed" by genomic related machinery and eventually result in differential phenotypic expression.**

Previous reports from this laboratory have shown a correlation between the intracellular levels of NAD and whether an undifferentiated mesodermal cell of embryonic chick limbs will express myogenic or chondrogenic properties (1–9). Our studies show that high levels of NAD are correlated with myogenic expression and with inhibition of chondrogenic expression; conversely, low levels of NAD are correlated with chondrogenic expression while inhibiting myogenic expression. An anatomical basis for the unequal distribution of NAD levels radially from core to periphery across the limb has been described (6). The developing vascular network establishes an avascular area at the pre-chondrogenic core region and a dense capillary system at the premyogenic peripheral region. Such an unequal distribution of nutrient flow radially across the limb is established long before observable signs of cytodifferentiation. We have hypothesized that limb mesodermal cells are capable of *sensing* such regional differences and respond by differentiating as dictated by such differences. NAD has been implicated in the process(es) governing such differential gene activity since high versus low intracellular levels of NAD have been correlated with differential phenotypic expression of limb mesodermal cells.

If NAD has a causal relationship with the events observed as differential gene activity then, as a minimum requirement, NAD must communicate with the genome. Others have described an NAD-related enzyme–product system which is strongly associated with chromatin (for review see ref. 10). This enzyme system catalyzes the synthesis and degradation of the homopolymer, poly(ADP-Rib). Poly(ADP-Rib) forms via the polymerization of the ADP-Rib moiety of NAD with the excision of nicotinamide. Poly(ADP-Rib) has been shown to be associated with or possibly bound to histones (11, 12), and thus this polymer can be implicated in mechanisms governing chromosomal structures and function. The studies reported here were designed to ascertain if a relationship exists between poly(ADP-Rib) synthesis and muscle versus cartilage differentiation since we have shown a close correlation between changes in NAD levels and limb mesodermal cell commitment to myogenic or chondrogenic phenotypes. The results of these studies can be taken to indicate that changes in intracellular NAD levels are "sensed" by the chromatin-associated poly(ADP-Rib) synthesizing machinery and result in differential rates of synthesis of this polymer. Such differential rates of synthesis of poly(ADP-Rib) are correlated with the differentiation of limb mesodermal cells into muscle and cartilage phenotypes.

MATERIALS AND METHODS

Materials. [*2*-^{14}C]Thymidine, diphosphopyridine[*adenine-U*-^{14}C]nucleotide 253 mCi/mmol, and [*carbonyl*-^{14}C]nicotinamide adenine dinucleotide 62 mCi/mmol, were purchased from Amersham/Searle Corp. [*2*-^{3}H]Adenine, 15 Ci/mmol; [*2*-^{3}H]adenosine, 16 Ci/mmol, and diphosphopyridine nucleotide [*G*-^{3}H-adenosine], 5 Ci/mmol, were purchased from New England Nuclear Corp. [^{3}H]Poly(A) was purchased from Miles Laboratory. RNases, DNase, firefly lantern extract, thymidine, nicotinamide, and 3-acetylpyridine were purchased from Sigma. NAD was purchased from P.L. Laboratories. Powdered medium and sera were purchased from Grand Island Biological Co. Embryo extract was made no more than one month before use (1). All other materials were reagent grade.

Cell Cultures. Stage 24 limb mesodermal cells were obtained as described previously (1, 2, 8). For pulse experiments in-

volving intact cells (see below), cells were plated at 1.0 or 1.25 × 10^7 cells in 3 ml of complete medium into 60 mm plastic petri dishes. For samples of chromatin, 5.0 × 10^7 cells in 8 ml of complete medium were plated into a 100 mm plastic petri dish.

Poly(ADP-Rib) Analysis. A number of protocols were used for assays of newly synthesized poly(ADP-Rib) that involved RNase, DNase, and KOH treatment in various combinations; most of the data reported here were obtained by the assay of Colyer *et al.* (13), with the primary modification that 70 μg/ml of cell culture medium of cytosine arabinoside to inhibit DNA synthesis was added with radioactively labeled nucleoside or nucleotide. The protocol used by Colyer *et al.* (13) gave 1–3% thymidine contamination and gave the most reproducible results.

Radioactively labeled adenine and adenosine were used interchangeably although all of the data reported here are for labeled adenine. On various culture days, the specific radioactivity of the soluble ATP was measured by the luciferase assay of Emmerson and Humphreys (14). Except where noted, the specific radioactivity of the ATP pool did not vary by more than 11%; thus, it may be argued that uptake and incorporation of adenine into poly(ADP-Rib) was not limiting and that variations in rate of synthesis of this polymer represented variation in the amount of synthesizing machinery. Also, these measurements show that 93% of the ATP pool equilibrates with radioactively-labeled adenine in 15 min.

Chromatin Isolation and In Vitro Synthesis of Poly(ADP-Rib). Cells were washed with two changes of Tyrodes balanced salt solution and then scraped from the petri dish with a rubber policeman in buffer A (0.075 M NaCl; 0.025 EDTA at pH 8.0) into a Teflon-glass homogenizer attached to a model RZR1-64 motor (Infromo, Wayne, N.J.) set at 80% maximum speed. After two up-and-down strokes, the nonionic detergent, Nonidet-40 (Shell Oil Co.), was added to a final concentration of 1% (v/v), followed by seven more up-and-down strokes. Nuclei were isolated at 1000 × *g* for 15 min and the resulting pellet was resuspended in buffer A and centrifuged again at 1000 × *g* for 15 min. The pellet was again suspended in buffer A and then mixed into the upper third of a 2.0 M sucrose solution made up in 0.05 M Tris at pH 8.0 in a 30 ml polyallomer tube for an SW 25.1 rotor (Beckman). The sample was spun at 25,000 rpm for 90 min. The liquid was poured off and the gelatinous pellet was resuspended with a loose fitting Dounce homogenizer in 0.001 M Tris at pH 8.0, allowed to swell on ice for 15 min, and spun at 12,000 × *g* for 15 min. The very gelatinous pellet was resuspended in 0.001 M Tris and recentrifuged; this was repeated one more time. The final resuspended pellet was sheared at 6000 lb./inch² (41.37 kPa) in a French pressure cell at ice temperature followed by a centrifugation at 12,000 × *g* for 15 min. A small white pellet was discarded.

The resulting solubilized chromatin was then brought to a single concentration in the agarose column buffer by adding a 10 times concentrate of buffer B. Buffer B was exactly as described by Janowski *et al.* (15) and contained 0.1 M $MgCl_2$, 0.1 M KCl, 0.001 M mercaptoethanol, and 0.01 M Tris, all at pH 8.0. Reagents like nicotinamide, 3-acetylpyridine, and thymidine were made up in buffer B and then added to the incubation medium. Nonradioactive NAD made up in buffer B was subsequently added to a final concentration of 0.0025 M followed by 2.5 μCi of [2-^{3}H]adenine-labeled NAD. At the end of the incubation period, the sample was precipitated with 10% trichloroacetic acid or loaded directly on a column (2.5 × 65 cm) of agarose Bio-Gel A-50 (100–200 mesh) with the flow rate of buffer B at 0.3–0.4 ml/min. Then fractions of 4.1 ml were collected, optical density at 260 nm measured, and then each fraction precipitated with cold 10% trichloroacetic acid. Various duplicate samples were exposed to DNase (2 μg/ml) for 30 min at 37° followed by 2 M KOH for 2 hr at 37° before being precipitated by trichloroacetic acid. Since duplicate or split samples were within 8% of one another, the trichloroacetic acid precipitated material appeared not to be DNA or RNA and is probably authentic poly(ADP-Rib).

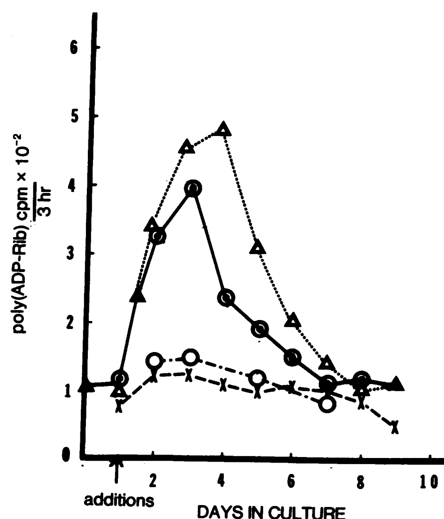

FIG. 1. Rate of [^{3}H]adenine incorporation into poly(ADP-Rib) as a function of the day in culture in untreated cultures (⊙) and those exposed to nicotinamide (×), 3-acetylpyridine (Δ), or BrdUrd (O). Stage 24 limb mesodermal cells which had been seeded at 1.25 × 10^7 were exposed to [^{3}H]adenine for 3 hr and then analyzed for poly(ADP-Rib) (13). Since the incorporation of [^{3}H]adenine is linear, the cpm/3 hr time period is represented as the rate of poly(ADP-Rib) synthesis. Cultures were first exposed to 10 mg of nicotinamide, 4 mg of 3-acetylpyridine, or 10^{-5} M BrdUrd on day 1 and continuously maintained at that level thereafter. Cytosine arabinoside was added to each sample simultaneously with labeled adenine.

RESULTS

In Vivo Labeling Studies: Limb Mesodermal Cells in Culture. All of the determinations for poly(ADP-Rib) utilize radioactive adenine or adenosine and thus exclusively represent *newly synthesized* polymer. These determinations do not give information on the absolute amounts of poly(ADP-Rib) nor do they relate to the turnover or degradation of poly(ADP-Rib). Thus, in all cases stated here, "poly(ADP-Rib)" refers to net amounts of newly synthesized polymer with no consideration to absolute amounts or degradation. The *in vivo* rates of incorporation of radioactive adenine or adenosine into poly-(ADP-Rib) are linear with time on all of the days cell cultures were pulsed. Since intracellular pool sizes of ATP were essentially unchanged during these times, uptake of radioactive label over a 3 or 4 hr time exposure reflects the *in vivo* net rate of poly(ADP-Rib) synthesis.

Fig. 1 shows that when untreated cell cultures were assayed for newly synthesized poly(ADP-Rib) on various days, a

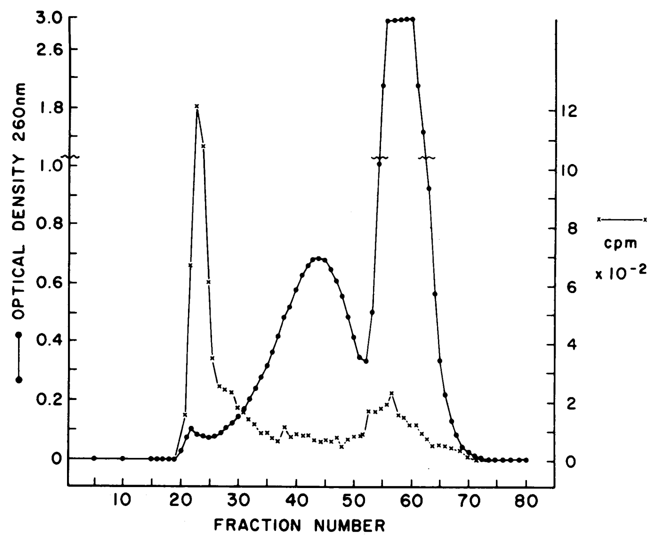

FIG. 2. Optical density and radioactivity elution profiles of sheared chromatin from limb mesodermal cells after incubation with radioactively labeled NAD. Chromatin from ten 100 mm petri dishes initially inoculated with 5×10^7 cells was isolated on day 3 and incubated in buffer B with 0.0025 M NAD and 2.5 μCi of NAD-labeled with [^{14}C]adenine. About 50 OD_{260} units of sheared chromatin with a 260 nm: 280 nm ratio of never less than 1.50 was incubated for 20 min at 25°, cooled to ice temperature, and loaded on a reverse gravity-flow agarose (Bio-Gel A-50) column with a pumping rate of 0.3–0.4 ml/min of buffer B. Fractions of 4.1 ml were collected, exposed to 5 μg of DNase for 1 hr at 37°, exposed to 2 M KOH at 37° for 6 hr, and then precipitated with 10% trichloroacetic acid with 200 μg of bovine serum albumin as carrier. The precipitate was collected by filtration on Millipore filters and the radioactivity quantitated.

rise is observed in the net rate of poly(ADP-Rib) synthesis with a maximum on day 3. Thereafter, poly(ADP-Rib) synthesis falls off to initial levels. Variation in the maximum was observed in that the highest rate of poly(ADP-Rib) synthesis was either on day 3 or day 4, but never before or after that 24 hr period. This variation was independent of the method used to assay for poly(ADP-Rib) but was correlated with the batch variation of limb mesodermal cells. In some cases, such as depicted in Fig. 1, the earliest visible sign of differentiation of cartilage nodules occurred on late day 2–early day 3. Such correlations were not subjective since the visual observation was made before obtaining the results of quantitative determinations for poly(ADP-Rib). Thus, a strong correlation seems to exist between the cellular commitment for chondrogenic expression and the increase or maximum of poly(ADP-Rib) synthesis.

We have reported that exogenously added nicotinamide inhibits chondrogenic expression in cultures of limb mesodermal cells (1, 2, 9). Fig. 1 also shows that under conditions of nicotinamide- or BrdUrd-suppressed chondrogenic expression, poly(ADP-Rib) synthesis does not rise but remains at basal levels. In this case a correlation seems to exist between the lack of increased poly(ADP-Rib) synthesis and inhibition of chondrogenic expression. This lack of increase of poly(ADP-Rib) synthesis is dependent on nicotinamide or BrdUrd concentration (data not shown) just as was the previously reported nicotinamide- or BrdUrd-caused inhibition of chondrogenic expression shown to be concentration dependent.

TABLE 1. *Poly(ADP-Rib) synthesis as a function of exposure to 3-acetylpyridine and of the specific radioactivity of the ATP pool*

	Untreated	3-acetylpyridine
cpm poly(ADP-Rib)	1040	1452
Specific activity ATP dpm/0.1 nmol	178	118
cpm corrected for specific activity of ATP	1040	"2190"

Cultures were initially seeded with 10^7 cells in 3 ml of medium into a 60 mm plastic petri dish. Cells were pulsed with [^{3}H]-adenine for 3 hr on day 3 before extraction for poly(ADP-Rib) and determination of the specific activity of the total cellular ATP pool. The "corrected cpm" for the cells exposed to 3-acetylpyridine is calculated by multiplying the observed cpm (1452) by the ratio of the ATP specific activity of untreated and 3-acetylpyridine treated cells (178:118).

We have also reported that 3-acetylpyridine causes a marked potentiation of chondrogenic expression (1–3, 7, 9). Under these conditions which maximize the potentiation of chondrogenic expression, poly(ADP-Rib) synthesis is seen to increase until a maximum is reached on day 4 or 5 with subsequent decreasing synthesis of poly(ADP-Rib) until basal levels are reached. This shift in the day of maximum synthesis of poly(ADP-Rib) from day 3 or 4 in untreated cultures to day 4 or 5 in 3-acetylpyridine treated cultures is again correlated with visibility of chondrogenic expression. Previous reports have shown that continuous exposure to 3-acetylpyridine starting on day 2 shifts observed chondrogenic expression by 24–48 hr; that is, the first visible signs of chondrogenic expression occur on late day 3 or day 4 as opposed to day 2 or 3 in untreated cultures (1, 2).

Synthesis of poly(ADP-Rib) on a "per plate" basis is 19% higher in 3-acetylpyridine cultures when compared with untreated cultures. We have previously shown that exposure to 3-acetylpyridine results in a loss of 30–40% of the cells; presumably only myogenic cells are lost (7). Thus, if the values pictured in Fig. 1 were corrrected to reflect poly(ADP-Rib) synthesis on a "per cell" basis, it would appear that exogenously added 3-acetylpyridine greatly stimulates poly(ADP-Rib) synthesis since the maximum rate would be 30–40% higher than already apparent. Such stimulation may be viewed as being quantitatively correlated with 3-acetylpyridine's observed potentiation or selection of chondrogenic expression.

Others have suggested that the exposure of embryos *in situ* to nicotinamide-antagonized teratogens like 3-acetylpyridine results in the decrease of total ATP pool in the chick embryo (16, 17). It might be argued that decreased ATP pools would result in an increased specific radioactivity of ATP when exogenous [^{3}H]adenine is added. Since ATP is the immediate precursor to NAD, it is possible that the observed increased rates of poly(ADP-Rib) synthesis may be due to the artifactual consequence of the proposed changes in ATP pool size. Table 1 shows that the observed specific activity of total cellular ATP is *lower* in cells exposed to 3-acetylpyridine. In fact, if the data are corrected for this lowered ATP specific radioactivity, 3-acetylpyridine stimulates poly(ADP-Rib) synthesis by over 100% as opposed to the 43% calculated

from uncorrected values. Although such a correction is probably not justified since we have not measured the effects on the specific radioactivity of the cellular NAD pool, we tentatively conclude that exposure to exogenously added 3-acetylpyridine results in a stimulation of poly(ADP-Rib) synthesis which is correlated with the observed potentiation of chondrogenic expression and that this observed stimulation is not the result of an increased specific radioactivity of the soluble ATP pool.

In Vitro Synthesis of Poly(ADP-Rib): Isolated Chromatin. It is known that poly(ADP-Rib) is exclusively associated with nuclei and purifies with chromatin as opposed to nuclear sap (see ref. 10 for review). Two questions can be asked relative to poly(ADP-Rib) and limb mesodermal cells. First, does the poly(ADP-Rib) synthetic machinery cofractionate with chromatin from stage 24 limb mesodermal cells grown *in vitro*? Second, is this machinery sensitive to 3-acetylpyridine or nicotinamide in a manner comparable to that seen with *in vivo* labeling? Agarose-column separation, as described by Janowsky *et al.* (15) seems appropriate for both answering these questions and obtaining additional information related to the relationship of poly(ADP-Rib) and chromatin.

After shearing, the chromatin is incubated at 25° with radioactively labeled NAD and then passed through an agarose (Bio-gel A-50) column. Three separate peaks of optical density at 260 nm can be seen (Fig. 2). The first relatively small peak is seen at the exclusion volume of the column as separately identified by dextran blue exclusion. The middle peak represents the majority of the sheared chromatin, whereas, the final large peak is identified as the NAD included in the incubation for synthesis of poly(ADP-Rib). Also shown in Fig. 2 is the trichloracetic acid precipitable, DNase and KOH insensitive radioactive material associated with newly synthesized poly(ADP-Rib). The majority of the radioactivity is associated with the void volume although some is clearly associated with the middle optical density peak of nonexclusion-volume chromatin. The radioactivity associated with the NAD is variable from experiment to experiment and is essentially an artifact of the precipitation and filter technique as seen in Fig. 3A–D although it is possible that some small proportion of radioactivity is free poly(ADP-Rib).

Fig. 3 shows radioactivity profiles from agarose gel columns of various control and experimental situations.

(*A*) *[^{14}C]Adenine plus cold NAD:* The only radioactivity seen was at the small molecular position of the column effluent with no radioactivity associated with chromatin. Thus, NAD breakdown with fortuitous labeling by adenine does not seem to occur as evidenced by the lack of radioactivity at the exclusion volume.

(*B*) *No incubation: 2°:* Instead of incubating at 25°, the chromatin was incubated at 2° for an equivalent amount of time. A relatively small amount of radioactivity was associated with the exclusion volume and main peak chromatin. At the most, this radioactivity represents 15% of that obtained after a 25° incubation. Thus, a temperature sensitive system is responsible for radioactivity associated with the exclusion volume material.

(*C*) *5 mM nicotinamide, thymidine, or 3-acetylpyridine:* It has been shown that the enzyme responsible for the synthesis of poly(ADP-Rib) is inhibited by nicotinamide and thymidine (18). We confirm these observations and extend them to include inhibition of synthesis of poly(ADP-Rib) by 3-acetylpyridine. In these situations, no radioactivity marking newly synthesized poly(ADP-Rib) is associated with chromatin after passage through the agarose column.

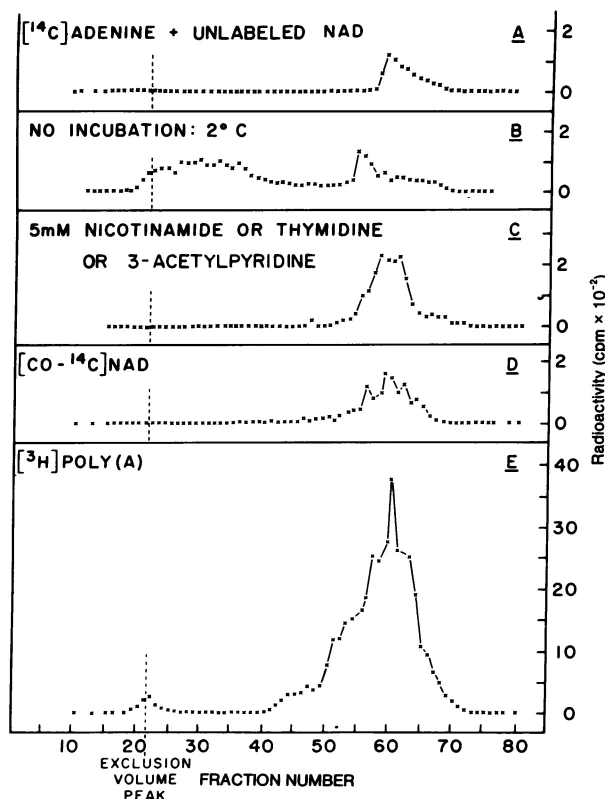

FIG. 3. Radioactivity profiles after agarose A-50 separation of sheared chromatin incubated with a variety of reagents as indicated in the figure and *text*. The optical density profile at 260 nm for A through E was essentially as in Fig. 2. (*A*) [^{14}C]Adenine (5 μCi) and nonradioactive NAD were added to the incubation medium in place of radioactively labeled NAD, incubated for 20 min at 25°, cooled to ice temperature, and loaded onto the column; (*B*) "no incubation," complete reaction mixture as in Fig. 2 except left on ice (2°) for 20 min before loading onto the agarose column; (*C*) five millimolar nicotinamide, thymidine, or 3-acetylpyridine made up in buffer B was added just prior to adding NAD. All other details are the same as in Fig. 2; (*D*) [*CO*-^{14}C]NAD (5 μCi) was added in place of [^{14}C]adenine-labeled NAD; (*E*) [^{3}H]poly(A) was added to the reaction mixture in place of radioactive NAD.

(*D*) *[CO-^{14}C]NAD:* It is possible that NAD itself binds to chromatin and this association is not DNase or KOH sensitive. To test for this possibility, we used [^{14}C]carbonyl-labeled NAD (label in the carbonyl-group of nicotinamide) instead of [^{14}C]adenine-labeled NAD. In the formation of poly(ADP-Rib), nicotinamide is excised and would not be expected to be associated with newly synthesized polymer. The observed absence of radioactivity associated with chromatin suggests that poly(ADP-Rib) has formed, with the elimination of nicotinamide. A double label experiment mixing [*CO*-^{14}C]NAD and [*adenine*-^{3}H]NAD, not reported here, clearly shows ^{3}H associated with the exclusion peak and main peak as in Fig. 2, whereas, ^{14}C is distributed as in Fig. 3*D*. These observations exclude the possibility that NAD labeled in the adenine moiety binds to chromatin without excision of nicotinamide in the process.

(*E*) [^{3}*H*]*Poly*(*A*): It is possible that poly(ADP-Rib) or similar polymers might aggregate in the high salt buffer and fortuitously elute at the exclusion volume of the column. [^{3}H]Poly(A) was mixed with chromatin and 98% of the radioactivity associated with poly(A) elutes well away from the exclusion volume (Fig. 3*E*). Although authentic poly-(ADP-Rib) would better serve in this experiment, it is technically unfeasible to obtain sufficient quantities due to the small amount of mesodermal tissue available although more adequate procedures have been described for other tissues (10). Because of the salt concentrations used here, it is unlikely that poly(A) or possibly polymers like poly(ADP-Rib) would aggregate and elute at the exclusion volume of the column. Other possibilities are discussed below.

DISCUSSION

The studies reported here clearly show that: (*A*) the rate of net synthesis of poly(ADP-Rib) is correlated with differentiation of chondrogenic cells from stage 24 limb mesodermal cells grown *in vitro;* (*B*) inhibition of chondrogenic expression caused by exposure to nicotinamide or BrdUrd is correlated with maintenance of basal levels of poly(ADP-Rib) synthesis; this inhibition is dependent on the concentration of exogenously supplied nicotinamide, (*C*) potentiation of chondrogenic expression caused by exposure of limb mesodermal cells *in vitro* to 3-acetylpyridine is *correlated* with stimulation of the rate of poly(ADP-Rib) synthesis if correlated with specific activity of ATP pool or compared to untreated cultures on a per cell basis, (*D*) isolated chromatin from mesodermal cells has the enzymatic machinery for synthesizing poly(ADP-Rib), (*E*) this machinery is inhibited *in vitro* by nicotinamide, thymidine, and 3-acetylpyridine; and (*F*) newly synthesized poly(ADP-Rib) is either associated with a discrete fraction of chromatin or is completely extracted from chromatin by the high salt concentration of the columns and results in an aggregation with eventual elution at exclusion volume positions of the agarose column. Taken together, these observations provide a possible explanation for how fluctuations in cellular NAD levels can communicate or be "sensed" by genomic-related machinery eventually resulting in differential phenotypic expression. Data reported here cannot be used to decide whether the observed differences in poly(ADP-Rib) synthesis affect replicative or transcriptional events; also not eliminated is the possibility that poly(ADP-Rib) is a unique storage form of NAD.

Various studies support the view that a single limb mesodermal cell has the capacity to differentiate either into a chondroblast or into a myoblast (19). Once committed to one choice, the cell seems to be unable to revert back to an embryonic cell which eventually differentiates into the other phenotype. The choice process or time of phenotypic commitment takes place over a very narrow time period *in ovo*, a matter of a few hours between late stage 24 and 25 (19, 20); see ref. 22 for a contrasting view. Stage 24 limb mesodermal cells may be arranged in cell culture to essentially mimic these *in situ* differentiation events where the proper proportion of cells develop into myogenic and into chondrogenic phenotypes (1, 2, 21). Nicotinamide or pyridine nucleotide-related reagents have a dramatic effect on these commitment events, and studies employing these reagents have led to the general hypothesis that local levels of pyridine nucleotides play a central or controlling role in the phenotypic expression of limb mesodermal cells (1–9).

If cellular NAD levels play a role in initiating or directing differentiation events, such levels or changes in such levels must communicate with the genome. The poly(ADP-Rib) synthesizing and degradation machinery might provide such a genome-associated NAD "sensing" system. Data presented in Fig. 1 show a correlation between observed differentiation and changes in the rate of poly(ADP-Rib) synthesis. In the cases of nicotinamide- or BrdUrd-caused inhibition and 3-acetylpyridine-caused potentiation of chondrogenic expression, changes in poly(ADP-Rib) synthesis were also inhibited or stimulated, respectively. Previous reports (3, 8, 9) show that in cultures of stage 24 limb mesodermal cells such inhibition or stimulation reflects the changes in cellular NAD levels as opposed to a direct action of nicotinamide or 3-acetylpyridine on genomic events. When cultures are exposed to nicotinamide, cellular NAD levels increase by a factor of from 2 to 10, whereas, exposure to 3-acetylpyridine decreases cellular NAD levels by a factor of 5 to 10 (8, 9). Taken together, these observations show that by experimentally manipulating the cellular NAD levels, differential rates of poly(ADP-Rib) synthesis can be observed. These events are correlated with differential gene activity resulting in either myogenic or chondrogenic phenotypes.

Our previous hypothesis that high NAD levels trigger myogenic commitment while inhibiting chondrogenic events and that low NAD levels trigger chondrogenic while inhibiting myogenic commitment can now be expanded. We would propose that cellular NAD levels are sensed by the poly-(ADP-Rib) synthesizing apparatus: high cellular levels of NAD result in low or basal rates of poly(ADP-Rib) synthesis, whereas low levels result in high or increased rates of poly-(ADP-Rib) synthesis. Such an increase in the rate of polymer synthesis is important only during the time period of phenotypic commitment. After the cells are committed to be a specific phenotype, the rate of poly(ADP-Rib) synthesis decreases to basal levels. The questions remain as to what effect this newly synthesized poly(ADP-Rib) has on chromatin structure and on chromatin functions.

Our studies with isolated limb mesodermal chromatin have attempted to answer these last questions. The techniques used to analyze chromatin are exactly those described by Janowski *et al.* (15). These workers show that the chromatin eluting with the exclusion volume of similar agarose columns may be tentatively identified as transcriptively active chromatin. If this assertion is correct, it would link newly synthesized poly(ADP-Rib) with the transcriptively active chromatin fraction. Such a linkage would dictate that differential synthesis of poly(ADP-Rib) could be responsible for differential gene activity. However, experiments conducted in our laboratory (Caplan and Saffitz, unpublished observations) clearly show that the chromatin eluting with the exclusion volume is not exclusively chromatin that is transcriptively active. Thus, although the newly synthesized poly(ADP-Rib) seems to be associated with a discrete chromatin fraction, the functional identity of that fraction is subject to some uncertainty.

A possible explanation for the observation that the newly synthesized poly(ADP-Rib) elutes in the exclusion volume is that the high salt conditions used to operate the agarose column, strips poly(ADP-Rib) or more likely poly(ADP-Rib) that is attached to histone (most likely F_1) from the chromatin. This aggregated material then elutes at the exclusion

volume. The fact that newly synthesized poly(ADP-Rib) is not firmly associated with the bulk of the chromatin argues either that the chromatin at the exclusion volume is a special type of chromatin or that the sites of synthesis of poly(ADP-Rib) can be extracted and aggregate under these high salt conditions. Two facts argue against nonspecific aggregation. First, the fact that poly(A) does not aggregate itself under these conditions (Fig. 3*E*), suggests that the newly synthesized poly(ADP-Rib) is attached to either the chromatin of the exclusion volume or a salt-extractable moiety which eventually aggregates. Second, newly synthesized poly(ADP-Rib) seems to be associated with a discrete chromatin fraction under low salt conditions on an agarose-A50 column or a sucrose gradient (Caplan, unpublished observations).

Of pertinence to the above hypothesis is the observation that *both* 3-acetylpyridine and nicotinamide inhibit *in vitro* poly(ADP-Rib) synthesis (Fig. 3*C*). The inhibition by nicotinamide has been reported previously and correlates with the observations that exogenously added nicotinamide inhibits chondrogenic expression, and with the studies here showing that such exposure does not cause an increase in the net rate of poly(ADP-Rib) synthesis; yet, when added to the purified chromatin, 3-acetylpyridine inhibits poly(ADP-Rib) synthesis. One must conclude that exogenously added 3-acetylpyridine does not directly interact with chromosomal machinery but causes an effect which is then sensed by this machinery and results in the observed increased rate of net poly(ADP-Rib) synthesis in intact cells. As we have shown previously, this 3-acetylpyridine effect is the lowering of the cellular NAD pool by a factor of 5 to 10 (3, 8, 9). Such 3-acetylpyridine-caused lowering of the NAD pool is, thus, correlated with the potentiation of chondrogenic expression. These observations strengthen our view that the poly(ADP-Rib)-synthesizing machinery is capable of sensing cellular NAD levels or changes in these levels. The act of sensing such levels or changes is transposed into differential rates of poly-(ADP-Rib) synthesis that are correlated with differential phenotypic expression into either myogenic or chondrogenic cell types.

The advantage of using stage 24 limb mesodermal cells in culture over other systems used to study poly(ADP-Rib) synthesis is that these cells, both *in ovo* and *in vitro*, have been shown to be exquisitely sensitive to changes in nicotinamide or pyridine nucleotide levels as well as differentially sensitive to nicotinamide-antagonized teratogens some of which are analogs of nicotinamide (1–9, 16, 17, 21). In other systems used to study poly(ADP-Rib), no such physiological correlation has been established.

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